Development of new tools for the characterisation of transient enzyme species by EPR spectroscopy

A thesis submitted to The University of Manchester for the degree of Doctor of Philosophy (PhD) in the Faculty of Science and Engineering

2018
Emma Castiglioni
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### 5.2. Results and discussion

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#### 5.2.2. Extinction coefficient and reaction quantum yield determination

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#### 5.2.4. Laser photolysis experiments

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##### 5.2.5.4. EPR measurements at cryogenic temperatures of EAL in the presence of caged-2-AE

### 5.3. Summary and conclusion

### 6. Discussion and future directions

Final words count: 44,407 including captions
List of abbreviations and symbols

**A**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Symbol/Description</th>
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<tbody>
<tr>
<td>A, Abs</td>
<td>absorbance</td>
</tr>
<tr>
<td>AcOEt</td>
<td>ethylacetate</td>
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<tr>
<td>AdoCbl</td>
<td>5'-deoxyadenosylcobalamin</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>2-AE</td>
<td>2-aminoethanol, ethanolamine</td>
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<tr>
<td>$^2$H$_2$-2-AE</td>
<td>deuterated 2-aminoethanol, deuterated ethanolamine</td>
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<td>2-AP</td>
<td>(S) 2-aminopropanol</td>
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**B**

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<td>βME</td>
</tr>
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<td>B</td>
<td>magnetic field</td>
</tr>
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<td>B$_9$</td>
<td>folic acid</td>
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<td>BAPTA</td>
<td>1,2-<em>bis</em>(o-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid</td>
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<td>Boc</td>
<td><em>ter</em>-butyl dicarbonate</td>
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**C**

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<td>degrees Celsius</td>
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<td>c</td>
<td>concentration</td>
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<td>Ca$^{2+}$</td>
<td>calcium cation</td>
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<td>CBD</td>
<td>chitin binding domain</td>
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<td>Cbl or B$_{12}$</td>
<td>cobalamin</td>
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<td>CDCl$_3$</td>
<td>deuterated chloroform</td>
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<td>CIDEPE</td>
<td>chemically induced dynamic electron polarization</td>
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<tr>
<td>CIDNP</td>
<td>chemically induced dynamic nuclear polarization</td>
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<td>cm$^{-1}$</td>
<td>wavenumbers</td>
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<td>α-carboxy-ortho-nitrobenzyl</td>
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<td>Co-C</td>
<td>cobalt-carbon bond</td>
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<td>continuous wave</td>
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<td>Cyt c</td>
<td>cytochrome c</td>
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<td><strong>D</strong></td>
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<td>-------------</td>
<td>----------------------------------------------------------------------------------</td>
</tr>
<tr>
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<td>deuterium</td>
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<td>decibel</td>
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<td>DMNP-EDTA</td>
<td>$1\text{-}(4,5\text{-Dimethoxy-2-Nitrophenyl})\text{-}1,2\text{-Diaminoethane-}$ \</td>
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<td></td>
<td>$N,N,N',N'\text{-Tetraacetic Acid}$</td>
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<td>sodium dithionite</td>
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<td>$\varepsilon$</td>
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<td>ethylenediaminetetraacetic acid</td>
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<td>ethylene glycol-\text{bis}(2\text{-aminoethylether})\text{-}N,N,N',N'\text{-} \</td>
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<td>tetraacetic acid</td>
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<td>EM</td>
<td>electromagnetic</td>
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<td>expressed protein ligation</td>
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<td>$\Phi$</td>
<td>quantum yield</td>
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<td>Fe$^{2+}$/Fe$^{3+}$</td>
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<td>Fmoc</td>
<td>fluorenyllmethyl carbamate</td>
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<td>FR</td>
<td>flow-rate</td>
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<tr>
<td>Symbol</td>
<td>Definition</td>
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<td>G</td>
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<td>2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid</td>
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<td>H₂O</td>
<td>water</td>
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<td>HLADH</td>
<td>Horse liver alcohol dehydrogenase</td>
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<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<td>high spin</td>
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<td>Hz</td>
<td>Hertz</td>
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<td>I</td>
<td>intensity of incident light</td>
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<td>I₀</td>
<td>intensity of transmitted light</td>
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<td>IDH</td>
<td>isocitrate dehydrogenase</td>
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<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
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<td>ITC</td>
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<td>observed/apparent rate constant</td>
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</tr>
<tr>
<td>Laser</td>
<td>light amplification by stimulated emission of radiation</td>
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<td>LED</td>
<td>light emitting diode</td>
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<td>Ln</td>
<td>natural logarithm</td>
</tr>
<tr>
<td>LS</td>
<td>low spin</td>
</tr>
<tr>
<td>M</td>
<td>molar, moles per litre</td>
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<td>Mb</td>
<td>myoglobin</td>
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<td>MeCbl</td>
<td>methylcobalamin</td>
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<tr>
<td>MFE</td>
<td>magnetic field effect</td>
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<td>Mn^{2+}</td>
<td>manganese cation</td>
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<td>MS</td>
<td>mass spectroscopy</td>
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<td>MW</td>
<td>micro wave</td>
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<td>nitrogen</td>
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<td>Na₂CO₃</td>
<td>Sodium carbonate</td>
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<tr>
<td>NADH/NAD⁺</td>
<td>nicotinamide adenine dinucleotide (reduced/oxidised)</td>
</tr>
<tr>
<td>NADP</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NaN₃</td>
<td>sodium azide</td>
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<td>NaOH</td>
<td>sodium hydroxide</td>
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<td>Nd:YAG</td>
<td>neodymium-doped yttrium aluminium garnet</td>
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<td>NH₃</td>
<td>ammonia</td>
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<tr>
<td>NMN</td>
<td>nicotinamide mononucleotide</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
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<td>NPPOC-Cl</td>
<td>2-nitrophenylpropylchloroformate</td>
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<tr>
<td>O</td>
<td>optical density</td>
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<tr>
<td>O.D.</td>
<td>optical density</td>
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<tr>
<td>OPO</td>
<td>optical parametric oscillator</td>
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<tr>
<td>P</td>
<td>phosphoenolpyruvate</td>
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<tr>
<td>PEP</td>
<td>phosphoenolpyruvate</td>
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<tr>
<td>pET SEAL</td>
<td>pET vector containing EAL gene sequences</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
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<tr>
<td>PG</td>
<td>protecting group</td>
</tr>
<tr>
<td>pH</td>
<td>negative logarithm of the proton ((H^+)) concentration</td>
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<td>PMT</td>
<td>photomultiplier tube</td>
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<tr>
<td>POM</td>
<td>polyoxymethylene</td>
</tr>
<tr>
<td>R</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>rapid freeze-quench</td>
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<tr>
<td>Rpm</td>
<td>revolutions per minute</td>
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<td>r.t.</td>
<td>room temperature</td>
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<td>S</td>
<td>substrate</td>
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<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>SPPS</td>
<td>solid-phase peptide synthesis</td>
</tr>
<tr>
<td>T</td>
<td>transmittance</td>
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<tr>
<td>TD-DFT</td>
<td>time-dependent density functional theory</td>
</tr>
<tr>
<td>(t_f)</td>
<td>Freezing-time</td>
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<td>THF</td>
<td>tetrahydrofuran</td>
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<td>TLC</td>
<td>Thin layer chromatography</td>
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<td>Mixing-time</td>
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<td>(t_Q)</td>
<td>Quenching-time</td>
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<tr>
<td>(t_T)</td>
<td>Sample transport time</td>
</tr>
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<td>U</td>
<td>ultraviolet</td>
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<tr>
<td>UV-vis</td>
<td>ultraviolet-visible</td>
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<tr>
<td>V</td>
<td>reaction rate</td>
</tr>
<tr>
<td>(V_0)</td>
<td>maximum reaction rate</td>
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<tr>
<td>W</td>
<td>wild-type</td>
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X

Xe  xenon

Y

YADH  yeast alcohol dehydrogenase
### Lists of equations

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<tr>
<td>Equation 1.1</td>
<td>Schematic representation of a system where a substrate (S) binds reversibly to an enzyme (E) to form an enzyme-substrate complex (ES), which then reacts irreversibly to generate a product (P) and to regenerate the free enzyme (E).</td>
<td>29</td>
</tr>
<tr>
<td>Equation 1.2</td>
<td>Michaelis-Menten equation.</td>
<td>29</td>
</tr>
<tr>
<td>Equation 3.1</td>
<td>Quenching-time described by the sum of the mixing-time (t_M), the transport-time (t_T) and the freezing-time (t_F).</td>
<td>83</td>
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<th>Description</th>
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<td>30</td>
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<tr>
<td>Figure 1.2</td>
<td>Schematic representation of a rapid freeze-quench device with copper wheels [12 and 15].</td>
<td>33</td>
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<tr>
<td>Figure 1.3</td>
<td>Schematic representation of a rapid freeze-quench device with rotating plate [21].</td>
<td>34</td>
</tr>
<tr>
<td>Figure 1.4</td>
<td>Schematic representation of the modified cryopress which enables rapid freezing and laser photolysis. Samples were loaded on a freezing head that could be released to slam onto a metal plate cooled with liquid helium. An optical sensor activated a frequency-doubled ruby laser for photolysis of caged ATP at selected time points [22].</td>
<td>35</td>
</tr>
<tr>
<td>Figure 1.5</td>
<td>Zeeman splitting for a S = 1/2 system with one unpaired electron in an external magnetic field B_0.</td>
<td>36</td>
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<tr>
<td>Figure 1.6</td>
<td>A small additional oscillating magnetic field, B_m, is applied in the same direction as the main field B. B_m is commonly at 100 kHz. As B_m increases from the value B_m1 to B_m2, the crystal detector output increases from i_1 to i_2. The output of the 100 kHz phase-sensitive detector is the derivative of the absorption curve.</td>
<td>37</td>
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<tr>
<td>Figure 1.7</td>
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<td>Figure 1.8</td>
<td>Photocleavage of the caged group.</td>
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<tr>
<td>Figure 1.9</td>
<td>Multistep synthesis and direct caging, both followed by photocleavage.</td>
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<td>Synthesis of caged compounds by chemical modifications [37].</td>
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<tr>
<td>Figure 1.11</td>
<td>Solid- phase peptide synthesis [37].</td>
<td>46</td>
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<tr>
<td>Figure 1.12</td>
<td>Recombinant protein α-thioester and EPL [38].</td>
<td>47</td>
</tr>
<tr>
<td>Figure 1.13</td>
<td>Tandem ligation procedure. The protecting group (PG) can be a peptide. All the fragments can be produced by synthetic or recombinant methods [38].</td>
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<tr>
<td>Figure 1.14</td>
<td>Structure of cobalamin: A)Methylcobalamin, B)Adenosylcobalamin</td>
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<tr>
<td>Figure 1.15</td>
<td>EAL (holoenzyme) catalyses the conversion of 2-aminoethanol into acetaldehyde and ammonia.</td>
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<td>Figure</td>
<td>Description</td>
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<td>1.16</td>
<td>General mechanism of AdoCbl-dependent isomerases. Substrate binding initiates homolysis and the Ado radical promotes the formation of the substrate radical by H-abstraction followed by rearrangement to the product radical. The full mechanism of EAL-B_{12} in the presence of ethanolamine is illustrated in figure 4.7.</td>
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<td>1.17</td>
<td>Direct caging of 2-aminoethanol with 2-nitrophenylpropylchloroformate.</td>
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<td>1.18</td>
<td>Project goals summary</td>
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<tr>
<td>2.1</td>
<td>Alkaline hydrolysis of DNPA with NaOH. 1) Nucleophilic attack of OH- at the electrophilic C of the ester leading to a tetrahedral intermediate. 2) The intermediate collapses forming acetic acid and the alkoxide. 3) Acid/base reaction where the alkoxide works as a base deprotonating the acetic acid. DNP and the sodium salt of the acetic acid are formed.</td>
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<tr>
<td>3.1</td>
<td>RFQ device combining a stopped-flow driving unit (Hi-Tech HSU-63, now TgK Scientific) and a rotating plate set in a Dewar (Spearlab).</td>
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<tr>
<td>3.2</td>
<td>The ageing loops that have been used in the RFQ instrument. a) Nozzle; b) loop 1, volume 27 μL; c) loop 2, volume 42 μL; d) loop 3, volume 144 μL; e) loop 4, volume 238 μL; f) loop 5, volume 312 μL. All the loops are made of Tefzel tubing and the metal holders are made of stainless steel.</td>
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<td>3.3</td>
<td>Technical drawing of the initial rotating wheel that was used in the RFQ instrument. Adapted from TgK Scientific. The Teflon coated aluminium alloy plate and the original rotating wheel are shown.</td>
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<tr>
<td>3.4</td>
<td>The initial rotating system provided by TgK Scientific for the RFQ instrument: a) Rotating system based on magnetic coupling was designed using a low-cost lab stirrer (non-heating) from CAT M5 (M. Zipperer GmbH, 79282 Ballrechten-Dottingen, Germany); b) Rotating wheel inside the polyurethane Dewar in which the liquid nitrogen is poured.</td>
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<td>3.5</td>
<td>The new magnetic coupling rotating system used in the RFQ instrument: a) Rotating disc and stand set-up; b) Set-up inside the polyurethane Dewar; c) Upper part where the Teflon coated aluminium alloy plate is placed; d) Upper part where one of the magnets is placed; e) Stand; f) Stand where the second magnet is placed.</td>
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<tr>
<td>3.6</td>
<td>Technical drawing of the new magnetic coupling system. Adapted from Dr. Roger J. Kutta.</td>
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<tr>
<td>3.7</td>
<td>Summary of the calibration steps used to characterise the RFQ instrument.</td>
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<tr>
<td>3.8</td>
<td>Alkaline hydrolysis of DNPA with NaOH. 1) Nucleophilic attack of OH- at the electrophilic C of the ester leading to a tetrahedral intermediate. 2) The intermediate collapses forming acetic acid and the alkoxide. 3) Acid/base reaction.</td>
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where the alkoxide works as a base deprotonating the acetic acid. DNP and the sodium salt of the acetic acid are formed.

Figure 3.9 Absorbance spectra of DNPA solutions representing the unreacted sample t0 and the fully reacted sample tinf. About 10 mg of DNPA was dissolved in 1 mL of ethanol and then, the solution was made up to 50 mL in HCl (2 mM). The blanks were prepared manually as follow: tinf was prepared by mixing 1 mL of stock solution containing DNPA (0.88 μM) and 1 mL of NaOH (1 M) up to 10 mL of HCl (2 M). For t0 1 mL of solution containing DNPA (0.88 μM) was mixed with 5 mL of HCl (2 M) and 1 mL of NaOH (1 M) was added. At the end, the solution was made up to 10 mL of HCl (2 M). The spectra were measured between 240 and 420 nm by using a Varian Cary 50 UV-visible spectrophotometer.

Figure 3.10 Hydrolysis test of the DNPA stock solution obtaining as follows: 20 mg of DNPA were dissolved in 2 mL of ethanol and made up to 100 mL in HCl (2 mM). Then, the traces were recorded every 2 minutes for 90 minutes.

Figure 3.11 Example quench flow data obtained using different collection vessels in the RFQ instrument. a) UV absorbance spectra of DNPA (0.88 μM) sprayed against NaOH (1 M) via loop 1 and 2 at different flow-rates and collected in disposable plastic bijous containing HCl (2 M); b) Fractions of sample reacted obtained from the absorbance spectra shown in a) and calculated as follows: [(Aratio)t - (Aratio)0] / [(Aratio)inf - (Aratio)0], where (Aratio)t is the ratio A294/A260 as a function of time; (Aratio)0 and (Aratio)inf correspond to the ratio of absorbances at t=0 and at completion of the reaction respectively. 260 nm is the isosbestic point and 294 nm corresponds to the formation of dinitrophenol; c) UV absorbance spectra of DNPA (0.88 μM) sprayed against NaOH (1 M) via loop 1 and 2 at different flow-rates and collected in disposable plastic bijous containing HCl (2 M); d) Fractions of sample reacted obtained from the absorbance spectra shown in c) and calculated as described in b).

Figure 3.12 Values of sample reacted obtained with different loops and flow-rates (plastic beaker 25 mL). Fractions of sample reacted calculated as follows: [(Aratio)t - (Aratio)0] / [(Aratio)inf - (Aratio)0], where (Aratio)t is the ratio A294/A260 as a function of time; (Aratio)0 and (Aratio)inf correspond to the ratio of absorbances at t=0 and at completion of the reaction respectively; 260 nm is the isosbestic point and 294 nm corresponds to the formation of dinitrophenol.

Figure 3.13 Determination of the rate constant of DNPA hydrolysis. a) The fraction of reacted sample was measured with different loops and flow-rates (PYREX Griffin beaker, volume=10 mL) and plotted against time. Fractions of sample reacted calculated as
follows: \[(\text{Aratio})_t - (\text{Aratio})_0 \] / \[(\text{Aratio})_{\text{inf}} - (\text{Aratio})_0\], where \((\text{Aratio})_t\) is the ratio \(A_{294}/A_{260}\) as a function of time; \((\text{Aratio})_0\) and \((\text{Aratio})_{\text{inf}}\) correspond to the ratio of absorbances at \(t=0\) and at completion of the reaction respectively; 260 nm is the isosbestic point and 294 nm corresponds to the formation of DNP. 

b) Stopped-flow trace (TgK Scientific) of DNPA (1 mL of stock solution 0.88 μM up to 10 mL in HCl 2 mM) against NaOH (1 M). Absorbance changes were measured at 310 nm. The red lines show the fit to a single exponential function \(y = y_0 + A \times \exp(R_0 \times x)\) to obtain the pseudo-first-order rate constants.

Figure 3.14
Stopped-flow absorbance transients at 575 nm upon mixing of myoglobin (70 μM) with sodium azide at different concentrations: a) \([\text{NaN}_3] = 0.35 \text{ mM}\); b) \([\text{NaN}_3] = 0.70 \text{ mM}\); c) \([\text{NaN}_3] = 2 \text{ mM}\); d) \([\text{NaN}_3] = 5 \text{ mM}\). All data were fitted to a single exponential equation to obtain the \(k_{\text{obs}}\) values (red lines).

Figure 3.15
Stopped-flow absorbance transients at 575 nm upon mixing of myoglobin (70 μM) with sodium azide at different concentrations: a) \([\text{NaN}_3] = 0.35 \text{ mM}\); b) \([\text{NaN}_3] = 0.70 \text{ mM}\); c) \([\text{NaN}_3] = 2 \text{ mM}\); d) \([\text{NaN}_3] = 5 \text{ mM}\). All data were fitted to a single exponential equation to obtain the \(k_{\text{obs}}\) values (red lines).

Figure 3.16
CW-EPR spectra of free and azide-bound myoglobin. a) CW-EPR spectrum of myoglobin in high-spin conformation (Fe³⁺, \(S=2/5\), \(g_{xy}=6\), \(g_z=2\)) measured at 12 K; b) CW-EPR of the myoglobin-azide complex in low-spin conformation (Fe²⁺, \(S=1/2\), \(g_1=2.59\), \(g_2=2.18\), \(g_3=1.83\)) measured at 12 K.

Figure 3.17
Summary of the problems encountered during testing of the RFQ instrument. a) CW-EPR spectrum of Mb-azide complex at 12 K (loop 5, flow-rate 5) showing a strong manganese signal; b) CW-EPR spectra of Mb-azide complex at 12 K (loop 1, flow-rate 7) showing oxygen background noise and its reduction by blowing helium in the sample; c) CW-EPR spectrum of Mb-azide complex at 12 K (loop 2, flow-rate 2) showing an unknown peak.

Figure 3.18
CW-EPR of Mb-azide complex measured at 12 K (loop 2, flow-rate 6). Oxygen background noise covering entirely the signal of interest.

Figure 3.19
Schematic representations of the two packing systems used in the RFQ instrument.

Figure 3.20
Selected CW-EPR spectra of the Mb-azide complex measured at 12 K and sprayed at a distance of 7 mm between the spray nozzle and the rotating plate: a) loop 1, flow-rate 7, 3.9 ms; b) loop 2, flow-rate 4, 10.5 ms; c) loop 2, flow-rate 2, 21.0 ms; d) loop 3, flow-rate 5, 28.8 ms; e) loop 5, flow-rate 5, 62.4 ms; f) loop 5, flow-rate 4, 78.0 ms. Different signal intensities are due
| Figure 3.21 | Calculation of the freezing time from the freeze-quench test upon formation of the Mb-azide complex: a) Plot of \( \ln(f_{HS}/f_{HS_0}) \) against time for freeze-quench experiments carried out with the rotating plate at a distance of 2 cm from the nozzle; b) Plot of \( \ln(f_{HS}/f_{HS_0}) \) against time for freeze-quench experiments carried out with the rotating plate at a distance of 7 mm from the nozzle. The data were fitted to a straight line and the intercept on the x-axis was used to calculate the freezing times. The value of \( f_{HS_0} \) corresponds to HS at \( t=0 \). |

| Figure 3.22 | Summary of the problems in obtaining reproducible data from the RFQ instrument. Both CW-EPR spectra in a) and b) of the formation of the Mb-azide complex were collected under identical conditions (loop 1, flow-rate 7, 12 K). |

| Figure 3.23 | RFQ experimental set-up showing how the sample is illuminated in the mixer-cell. LEDs can be placed on either side of the instrument in order to illuminate the optical cell. |

| Figure 3.24 | Photo-induced reduction of Cyt c in the presence of NADH. Excitation of NADH produces NAD` radicals and solvated electrons and facilitates the reduction of ferric Cyt c to ferrocytochrome. |

| Figure 3.25 | EPR spectra of oxidised and reduced Cyt c. a) CW-EPR spectrum of Cyt c at different concentrations (500 \( \mu \)M, 1 mM, 2 mM) were measured at 12 K, centre field 2600 G, modulation amplitude 5 G, sweep width 5000, number of points 2048, microwave power 25 dB. Oxidised ferricytochrome c is low-spin ferric (S=1/2), but at a concentration of 2 mM a high-spin signal for haem proteins was detected (at 1130 G g=5.9); b) CW-EPR spectrum of reduced Cyt c at different concentrations (500 \( \mu \)M, 1 mM, 2 mM) measured under the same conditions as in a). As expected the reduced form is diamagnetic. |

| Figure 3.26 | CW-EPR spectra of Cyt c (1 mM final concentration) mixed against NADH/NMN (2 and 50 mM respectively) and illuminated with LED (ThorLabs) at \( \lambda_{exc}=365 \) nm measured at 12 K at different temperatures and ageing times: a) 4°C (loop 1, different flow-rates: 6 (11.7 ms), 5 (12.6 ms), 4 (14.0 ms), 2 (20.7 ms)); b) 15°C (loop 1 flow-rates: 6 (11.7 ms), 5 (12.6 ms), 4 (14.0 ms), 2 (20.7 ms), loop 2 flow-rate 2 (28.2 ms) and loop 3 flow-rates: 4 (43.2 ms), 3 (55.2 ms); c) 20°C (loop 1 flow-rates: 6 (11.7 ms), 5 (12.6 ms), 4 (14.0 ms), 2 (20.7 ms), loop 2 flow-rate 2 (28.2 ms) and loop 3 flow-rates: 4 (43.2 ms), 3 (55.2 ms)); d) Comparison of Cyt c (1 mM oxidised), Cyt c reduced with DT and Cyt c in the presence of NADH and NMN after 5 minutes of continuous illumination with an LED (365 nm) |
nm) at room temperature.

Figure 3.27  UV-vis absorbance spectra to monitor the photoreduction of Cyt c upon illumination of NADH. a) Spectra of oxidised Cyt c (1 mM), Cyt c reduced with sodium dithionite and of samples collected in black Eppendorf after rapid mixing with NADH/NMN and illumination through the RFQ device (loop 1, different flow-rates: 6 (4.5 ms), 5 (5.4 ms), 4 (6.8 ms), 2 (13.5 ms)). There are not significant changes in the samples illuminated via LED (365 nm); b) Difference traces of Cyt c collected as in a) using the non-illuminated sample as the blank; c) Spectra of Cyt c (500 μM) in the presence of NADH/NMN after progressive illumination inside the UV-vis Cary 50 spectrophotometer by an LED (365 nm). The characteristic peak of the reduced form is visible (550 nm), but only after prolonged illumination; d) Difference traces of Cyt c collected as in c) using the non-illuminated sample as the blank.

Figure 3.28  Photo-cleavage of DMNP-EDTA yielding free Mn$^{2+}$ and two iminodiacetic acid photoproducts.

Figure 3.29  CW-EPR spectra of caged, uncaged and enzyme bound Mn2+measured at 20 K, centre field 3000 G, modulation amplitude 5 G, sweep width 5000, number of points 2048, microwave power 30 dB: a) Samples containing MnCl$_2$ (200 μM) in HEPES (20 mM, pH=7) are shown; b) Samples containing MnCl2 (400 μM) in HEPES (20 mM, pH=7) are shown.

Figure 3.30  Structure of cobalamin. The structure consists of a corrin ring, containing four pyrrolic units, three methylene bridges and a central cobalt ion. In methylcobalamin the Co$^{3+}$ is bound to a methyl group.

Figure 3.31  CW-EPR spectra of MeCbl measured at 12 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000 G, number of points 2048, microwave power 25 dB: a) 400 μM MeCbl before and after irradiation with LED at 365 nm; b) 1 mM MeCbl before and after irradiation with LED at 365 nm; c) 2 mM MeCbl after irradiation with LED at 365 nm; d) Comparison of different concentrations (0.2, 0.4, 1 and 2 mM) of MeCbl before 1 minute irradiation with LED at 365 nm.

Figure 3.32  CW-EPR spectra of MeCbl measured at 12 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000 G, number of points 2048, microwave power 25 dB: a) 400 μM MeCbl before and after irradiation with LED at 530 nm; b) 1 mM MeCbl before and after irradiation with LED at 530 nm; c) 2 mM MeCbl after irradiation with LED at 530 nm.

Figure 3.33  CW-EPR spectra of MeCbl measured at 12 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000 G, number of points 2048, microwave power 25 dB: a) 2 mM MeCbl was
irradiated via LED at 365 nm in the quartz cell of the RFQ instrument and sprayed into black Eppendorf tubes; b) 2 mM MeCbl was irradiated via LED at 365 nm in the quartz cell of the RFQ instrument, frozen onto the rotating plates and packed into EPR tubes.

**Figure 3.34** UV-vis absorbance spectra of 320 μM MeCbl: a) MeCbl before (black trace) and after illumination inside the optical cell of the RFQ device (LED 365 nm, loop 1, ms, red and blue traces) and after 5 seconds of illumination inside the optical cell of the RFQ device (LED 365 nm, magenta trace); b) MeCbl before illumination (black trace) and difference spectrum of MeCbl after illumination inside the optical cell of the RFQ device (LED 365 nm, loop 1, ms, blue trace) and after 5 seconds of illumination inside the optical cell of the RFQ device (LED 365 nm, magenta trace).

**Figure 4.1** Structure of cobalamin. The structure consists of a corrin ring, containing four pyrrolic units, three methylene bridges and a central cobalt ion. In 5'-deoxyadenosylcobalamin the Co$^{3+}$ is bound to an adenosyl group.

**Figure 4.2** General mechanism of AdoCbl-dependent isomerases. Substrate binding initiates homolysis and Ado radical generates substrate radical by H-abstraction.

**Figure 4.3** SDS-PAGE gel of EAL purification fractions. S4 is the final pure sample containing both α and β subunits. In the current figure S stands for supernatant, P stands for pellet and M for marker.

**Figure 4.4** UV-vis spectrum of EAL after purification and binding to AdoCbl ($\varepsilon_{525}$ nm= 8 mM$^{-1}$cm$^{-1}$) [105].

**Figure 4.5** EAL catalyses the conversion of 2-aminoethanol to acetaldehyde which is the substrate for the reaction catalysed by YADH. The final products are ethanol and NAD$^+$. 

**Figure 4.6** Kinetic trace of EAL (100 nM) steady-state assay monitoring NADH (150 nM) consumption at 340 nm in the presence of ethanolamine (1 mM) and YADH (100 units).

**Figure 4.7** Schematic representation of the EAL reaction mechanism. Substrate binding initiates homolysis and the Ado radical promotes the formation of the substrate radical by H-abstraction, followed by rearrangement to the product radical, which then dissociates to acetaldehyde and ammonia.

**Figure 4.8** Stopped-flow transients of EAL holoenzyme (15 μM) mixed against 2-AE (2.5 mM) recorded at 525 nm at 5℃. Data are the average of three kinetic transients. The data were fitted in Origin with the following equation: $y=A_1*\exp(-x/t_1)+y_0$.

**Figure 4.9** Schematic representation of the EAL reaction mechanism for (S)-2-aminopropanol. Substrate binding initiates homolysis and the Ado radical promotes the formation of the substrate radical by H-abstraction followed by rearrangement to the product radical, which then dissociates to propionaldehyde.
and ammonia.

Figure 4.10  Stopped-flow transients of EAL holoenzyme (15 μM) mixed against 2AP a) 0.5 mM, b) 1 mM and c) 2.5 mM) recorded at 525 nm at 5°C. The data are the average of three kinetic transients. The data were fitted in Origin with the following equation: y = A1*exp(-x/t1)+A2*exp(-x/t2)+y0.

Figure 4.11  CW-EPR spectra of EAL-B12 (46 μM) measured at 20 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000 G, number of points 2048, microwave power 30 dB: a) EAL-B12 by itself (black trace), EAL-B12 against 2-aminoethanol (10 mM, red trace, loop 1, flow-rate 7, 11.1 ms), EAL-B12 against 2-aminoethanol (blue trace, loop 1, flow-rate 4, 14.0 ms), EAL-B12 against 2-aminoethanol (pink trace, loop 1, flow-rate 4, 14.0 ms), EAL-B12 against 2-aminoethanol (green trace, loop 1, flow-rate 2, 20.7 ms); b) EAL-B12 by itself (black trace), EAL-B12 against 2-aminopropanol (10 mM, red trace, loop 1, flow-rate 7, 11.1 ms), EAL-B12 against 2-aminopropanol (red trace, loop 1, flow-rate 2, 20.7 ms).

Figure 4.12  CW-EPR spectra of EAL-B12 (46 μM) measured at 20 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000 G, number of points 2048, microwave power 30 dB: a) EAL-B12 against 2-aminopropanol (10 mM, loop 2, flow-rate 2, 21 ms). Black and green traces show the reported doublet due to Co2+ and substrate radical spin-spin interaction, whereas the red trace shows a multiplet structure.

Figure 4.13  CW-EPR spectra of EAL-B12 (46 μM) measured at 20 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000 G, number of points 2048, microwave power 30 dB: a) EAL-B12 against 2-aminoethanol multiplet structure (10 mM, black trace, loop 1, flow-rate 7, 3.9 ms), EAL-B12 against 2-aminoethanol doublet structure (black trace, loop 1, flow-rate 7, 3.9 ms); b) EAL-B12 against 2-aminoethanol multiplet structure (10 mM, black trace, loop 1, flow-rate 7, 3.9 ms), EAL-B12 against 2-aminoethanol doublet structure (black trace, loop 1, flow-rate 7, 3.9 ms). The double headed arrows show the decay and conversion from one paramagnetic species to Co2+ and substrate radical spin-spin interaction; c) EAL-B12 against 2-aminopropanol multiplet structure (10 mM, red trace, loop 1, flow-rate 2, 13.5 ms), EAL-B12 against 2-aminopropanol doublet structure (black trace, loop 1, flow-rate 2, 13.5 ms); d) EAL-B12 against 2-aminopropanol multiplet structure (10 mM, black trace, loop 1, flow-rate 2, 13.5 ms), EAL-B12 against 2-aminopropanol doublet structure (black trace, loop 1, flow-rate 2, 13.5 ms). The double headed arrows show the decay and conversion from one paramagnetic species to Co2+ and substrate radical spin-spin interaction.
Figure 4.14 CW-EPR spectra of EAL-B_{12} (46 μM) measured at 20 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000 G, number of points 2048, microwave power 30 dB: a) EAL-B_{12} against 2-aminoethanol (10 mM, loop 1, flow-rate 4, 6.8 ms); b) EAL-B_{12} against 2-aminoethanol (10 mM, loop 1, flow-rate 4, 6.8 ms), same data sets as in a); c) EAL-B_{12} against 2-aminopropanol (10 mM, loop 1, flow-rate 4, 6.8 ms); d) EAL-B_{12} against 2-aminopropanol (10 mM, loop 1, flow-rate 4, 6.8 ms), same data sets as in c). In both cases, residual signals from the formation of adenosyl radical and Co^{2+} are present in all the traces.

Figure 4.15 CW-EPR spectra of EAL-B_{12} (46 μM) measured at 20 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000 G, number of points 2048, microwave power 30 dB: EAL-B_{12} against 2-aminopropanol (loop 3, flow-rate 3, 48 ms).

Figure 5.1 Organic synthesis of caged 2-aminoethanol: 1) Nucleophilic addition to the carbonyl group; 2) Proton transfer between nitrogen and oxygen; 3) Chloride ion elimination; 4) Proton loss from oxygen and final product formation.

Figure 5.2 $^1$H-NMR spectrum of NPPOC-caged-2-AE in deuterated chloroform processed with Mestrenova. The product analysed was obtained after purification via a silica column using ethyl acetate and hexane (1:1).

Figure 5.3 $^1$H-NMR spectrum of NPPOC-caged-2H4-AE in deuterated chloroform processed with Mestrenova. The product analysed was obtained after purification via a silica column using ethyl acetate and hexane (1:1).

Figure 5.4 Kinetic trace of EAL steady-state assay monitoring NADH consumption at 340 nm. The amount of reagents involved in the assay was determined spectrophotometrically in order to have 100 units of YADH, NADH 150 nM, EAL-B12 100 nM (HEPES 20 mM pH 7.5).

Figure 5.5 UV-vis spectra of caged-2-AE (stock solutions in water 5% EtOH (v/v) and then diluted in HEPES 20 mM pH 7.5) recorded at λ_{exc}= 365 nm: a) caged-2-AE before illumination at different concentrations: 273 μM (black trace), 173 μM (red trace) and 68μM (blue trace); b) extinction coefficient determination by plotting concentration (M) against absorbance at 258 nm for the three samples (68 μM, 137 μM, 273 μM). After linear fitting: ε= 3x103 M$^{-1}$ cm$^{-1}$.

Figure 5.6 UV-vis spectra of caged-2-AE (stock solution in water 5% EtOH (v/v) and then diluted in HEPES 20 mM pH 7.5) illuminated with LEDs at 365 nm (a), 280 nm (b) and 265 nm (c). The illumination steps were regulated via oscilloscope as follows: ten pulses of 100 ms each, twenty-one pulses of 500 ms each and 17 pulses of 1 s each.

Figure 5.7 Quantum yield determination from the slop of the caged
compound loss expressed in pmoles against pmoles of photons for 365 nm LED. The data were fitted in Origin with linear fitting (red line).

Figure 5.8 Changes in absorbance of caged-2-AE (20-23 μM) over time upon illumination with different LEDs (365, 280, 265 nm) at 258 nm (a) and at 325 nm (b).

Figure 5.9 Schematic representation of a laser flash-photolysis set-up. The monochromatic light beam is generated by a xenon arc lamp and the transmitted photons counted at a photomultiplier (PMT). The sample is contained into a sample cuvette and excited with a Nd:YAG laser at a selected wavelength.

Figure 5.10 UV-vis spectra of caged-2-AE (a) and caged-2-^H_4-AE (b) (stock solutions in water 5% EtOH (v/v) and then diluted in HEPES 20 mM pH 7.5 to a final concentration of 90 μM) illuminated with LED 365 nm. The illumination steps were regulated via oscilloscope as follows: ten pulses of 100 ms each, thirty pulses of 500 ms each and twenty-one pulses of 1 s each.

Figure 5.11 Schematic representation of a laser flash-photolysis set-up. The monochromatic light beam is generated by a xenon arc lamp and the transmitted photons counted at a photomultiplier (PMT). The sample is contained into a sample cuvette and excited with a Nd:YAG laser at a selected wavelength.

Figure 5.12 Flow-cell set-up showing the quartz cuvette connected to the pump. The sample was irradiated with laser (Quantel Brilliant B) at both 266 and 355 nm excitation wavelengths laser over a range of wavelengths from 600 to 270 nm with 5 nm steps.

Figure 5.13 Flow-cell experiments on caged-2-AE (6 mM in HEPES 20 mM pH 7.5) irradiated at 266 nm (Quantel brilliant B, 30 mJ) and measured at multiple wavelengths (600 to 270 nm). a) Kinetic traces recorded at different wavelengths; b) Absorbance profiles derived from the kinetic measurements shown in a); c) 3D plot of the flow-cell experiments on caged-2-AE measured at multiple wavelengths (600 to 270).

Figure 5.14 Flow-cell experiments on caged-2-AE (6 mM in HEPES 20 mM pH 7.5) irradiated at 355 nm (Quantel brilliant B) and measured at multiple wavelengths (600 to 270 nm). a) Kinetic traces recorded at different wavelengths with a laser power of 30 mJ; b) Absorbance profiles derived from the kinetic measurements shown in a); c) 3D plot of the flow-cell experiments on caged-2-AE measured at multiple wavelengths (600 to 270).

Figure 5.15 Flow-cell experiments on caged-2-AE (6 mM in HEPES 20 mM pH 7.5) irradiated at 355 nm (Quantel brilliant B) and measured at multiple wavelengths (600 to 270 nm). a) Kinetic
traces recorded at different wavelengths with a laser power of 100 mJ; b) Absorbance profiles derived from the kinetic measurements shown in a); c) 3D plot of the flow-cell experiments on caged-2-AE measured at multiple wavelengths (600 to 270).

Figure 5.16 Kinetic transients after 1 pulse (black), 5 pulses (blue), 10 pulses (red), 20 pulses (green), 30 pulses (magenta) of: a) caged-2-AE (1 mM in HEPES 20 mM pH 7.5) with laser (355 nm, 100 mJ) monitored at 420 nm; b) deuterated caged-2-AE (1 mM in HEPES 20 mM pH 7.5) monitored at 420 nm. All the transients are an average of three data sets.

Figure 5.17 Kinetic transients after 1 pulse (black), 5 pulses (blue), 10 pulses (red), 20 pulses (green), 30 pulses (magenta) of: a) caged-2-AE (1 mM in HEPES 20 mM pH 7.5) in the presence of EAL-B12 (15 μM) with laser (355 nm, 100 mJ) monitored at 420 nm; b) deuterated caged-2-AE (1 mM in HEPES 20 mM pH 7.5) in the presence of EAL-B12 (15 μM) with laser (355 nm, 100 mJ) monitored at 420 nm. All the transients are an average of three data sets.

Figure 5.18 Kinetic transients after 1 pulse (black), 5 pulses (blue), 10 pulses (red), 20 pulses (green), 30 pulses (magenta) of: a) caged-2-AE (1 mM in HEPES 20 mM pH 7.5) in the presence of EAL-B12 (15 μM) with laser (355 nm, 100 mJ) monitored at 525 nm; b) deuterated caged-2-AE (1 mM in HEPES 20 mM pH 7.5) in the presence of EAL-B12 (15 μM) with laser (355 nm, 100 mJ) monitored at 525 nm. All the transients are an average of three data sets.

Figure 5.19 Cryo-chamber (OptistatDN Oxford Instrument liquid nitrogen cryostat) with a valve-controlled liquid nitrogen reservoir. The device is connected to a vacuum pump (Pfeiffer vacuum) for insulation purposes.

Figure 5.20 77 K absorbance spectra showing the uncaging of caged-2-AE (about 500 μM in HEPES 20 mM pH 7.5) after illumination at 77 K (a) and 100 K (b). Spectra were recorded in sucrose (100% w/v in HEPES 20 mM pH 7.5) as cryo-protectant.

Figure 5.21 a) 77 K absorbance spectra showing the uncaging of caged-2-AE (1 mM in HEPES 20 mM pH 7.5) in the presence of EAL-B12 (15 μM) after illumination at 100 K. Spectra were recorded in sucrose (100% w/v in HEPES 20 mM pH 7.5) as cryo-protectant; b) Difference spectra of caged-2-AE in the presence of EAL-B12 after illumination at 100 K. The black trace corresponds to the sample prior to illumination; c) Changes in absorbance at 325 nm at different times (0, 10, 20, 25 and 30 s).

Figure 5.22 77 K absorbance spectra showing the uncaging of caged-2-AE (1 mM in HEPES 20 mM pH 7.5) in the presence of EAL-B12 (15 μM) after illumination at 100 K. Spectra were recorded in
sucrose (100% w/v in HEPES 20 mM pH 7.5) as cryoprotectant. a) blue trace recorded at 77 K after dark incubation at 150 K; b) blue trace recorded at 77 K after dark incubation at 160 K; c) blue trace recorded at 77 K after dark incubation at 170 K; d) blue trace recorded at 77 K after dark incubation at 180 K; e) blue trace recorded at 77 K after dark incubation at 190 K; f) blue trace recorded at 77 K after dark incubation at 200 K.

Figure 5.23 a) Difference spectra of caged-2-AE (1 mM in HEPES 20 mM pH 7.5) in the presence of EAL-B$_{12}$ (15 μM) after dark incubation at the stated temperatures. The black trace corresponds to the sample prior to illumination; b) Changes in absorbance at 325 nm at different temperatures (150, 160, 170, 180, 190 and 200 K).

Figure 5.24 CW-EPR spectra of EAL-B$_{12}$ (60 μM) were measured at 20 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000, number of points 2048, microwave power 30 dB before (black trace) and after illumination (red trace).

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Abstract

By using light as trigger it is possible to study many biological processes, such as the activity of genes, proteins and other molecules, with precise spatiotemporal control. Caged compounds, where biologically active molecules are generated from an inert precursor upon photolysis, offer the potential to initiate such biological reactions with high temporal resolution [1 to 3]. As light acts as the trigger for cleaving the protecting group the ‘caging’ technique provides a number of advantages as it can be intracellular, rapid and controlled in a quantitative manner [1 to 5]. In general, to design suitable caged compounds two important requirements are: 1) the uncaging step needs to be faster than the process under study; 2) the efficiency of uncaging should be high to remove easily the caging groups. Another main point to consider is that caged compounds must be biologically inert before photolysis [2, 3 and 5]. A caging strategy was applied to study the catalytic cycle of ethanolamine ammonia lyase (EAL). EAL is a coenzyme B_{12}–dependent enzyme and it has become an important model system to understand the catalytic mechanism of cobalamin B_{12}-dependent enzymes in general. In particular, it has been used to study the relationship between catalysis and enzyme–substrate complex geometry, and the properties of the active site [6].

Moreover, in conjunction with TgK Scientific, a novel rapid freeze-quench (RFQ) instrument, which combines fast mixing and flashing capabilities, was characterised. The purpose of this device consists in trapping reaction intermediates at low temperatures and analysing them by electron paramagnetic resonance (EPR) spectroscopy to identify the involvement of any radical species during catalysis [7 and 8].
Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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1. Introduction

The introduction provides an overview of rapid mixing techniques and freeze-quench devices, and design and application of caged compounds. Short descriptions of photochemistry and EPR are covered. In the latter part, the enzyme, ethanolamine ammonia lyase (EAL), which has been studied with these methodologies, is described.

1.1. Enzyme kinetics: basics and techniques

Enzymes are biological catalysts responsible for supporting chemical reactions that are essential for life processes. One of their significant roles in living systems is to catalyse the making and breaking of chemical bonds and almost every relevant life process is regulated by enzyme activity. Enzymes are present in all tissues and fluids of the body and they catalyse the reactions of metabolic pathways. Therefore, enzyme kinetics is important in providing essential information that is necessary to understand their physiological effects, investigate chemical mechanism of catalysis and design inhibitors for specific enzymes [9]. Enzyme reactions differ from standard chemical reactions as they are highly specific catalysts and increase the reaction rate by lowering the energy of the transition state much faster than non-biological catalysts. The region of an enzyme where catalysis occurs, which is called the active site, includes the residues directly involved in the chemical steps. These residues are known as catalytic groups. The interaction of the enzyme and substrate to form the enzyme-substrate (ES) complex is followed by the formation of a transition state in the active site, from which the reaction products are formed. The kinetic characterisation of these catalytic events was elucidated by the Michaelis-Menten model in 1913. This approach, also known as the rapid equilibrium assumption, investigates the reaction rate by following the increase of the reaction product as a function of time. The first step is the binding of a substrate to an enzyme with the formation of the ES complex. This complex has two possible fates. It can dissociate to enzyme (E) and substrate (S) or release the product (P) [9].
Equation 1.1 Schematic representation of a system where a substrate (S) binds reversibly to an enzyme (E) to form an enzyme-substrate complex (ES), which then reacts irreversibly to generate a product (P) and to regenerate the free enzyme (E).

$k_1$, $k_{-1}$ and $k_2$ are rate constants. The Michaelis-Menten equation is a quantitative description of the relationship among the rates of an enzyme-catalysed reaction.

$$V_0 = V_{\text{max}} \frac{[S]}{[S] + K_M}$$

Equation 1.2 Michaelis-Menten equation.

The symbols used in the Michaelis-Menten equation refer to the reaction rate ($V_0$), maximum reaction rate ($V_{\text{max}}$), substrate concentration [$S$] and the Michaelis-Menten constant ($K_M$). When [$S$] = $K_M$, $V_0 = V_{\text{max}}/2$. As a consequence, $K_M$ is equal to the substrate concentration at which the reaction rate is half of the maximal value. $K_M$ is an essential feature of an enzyme-catalysed reaction (figure 1.1) [9].
The Michaelis-Menten equation is used to describe the steady-state behaviour of an enzyme and is dependent upon the rate-limiting step of the reaction. However, enzyme catalysis often involves multiple steps and many of these enzyme-catalysed processes occur on sub-second or sub-millisecond timescales. To study these individual reaction steps different techniques have been developed that aim to quickly mix the reactants together or perturb the system in a controlled manner, and to monitor the formation and disappearance of reaction intermediates over time. The most common technique that is used to study enzyme mechanisms is a rapid mixing technique, known as stopped-flow spectroscopy and described in the next paragraph. These rapid mixing techniques are generally user-friendly but are normally limited to a time-resolution of ~ 1-2 ms. To overcome this, equilibrium perturbation techniques, such as temperature jump or pressure jump, have been used, which are generally based on the perturbation of a system by rapidly varying an external parameter, such as temperature or pressure. The most established relaxation technique is the temperature jump, where the sample solution is rapidly heated, either electrically or by using a laser, and changes in reaction equilibrium are then followed optically. Perturbations induced by pressure can also be measured using a pressure jump instrument, although the changes are often smaller than

**Figure 1.1** Plot of the Michaelis-Menten equation’s predicted reaction velocity as a function of substrate concentration.
those induced by temperature. Although most of these techniques are well-established and used routinely to study enzyme catalysis, the use of kinetic techniques to study short-lived enzyme species by electron paramagnetic resonance (EPR) or nuclear magnetic resonance (NMR) spectroscopy is less common [9].

1.2. Techniques

1.2.1. Flow-techniques and freeze-quench

To understand enzymatic reaction mechanisms in detail it is necessary to identify catalytic intermediates and any potential structural transformations during the catalytic cycle. As many of these processes occur on very fast timescales enzyme reactions are often studied using rapid mixing techniques [7, 8, 10 and 11]. All flow techniques drive two solutions at high speed into a mixing chamber and the reaction is then normally detected by using a variety of spectroscopic methods. In most cases the kinetics of enzymatic reactions are studied by using the stopped–flow technique, which involves the mixing of two reactants together into a measurement cell. The flow is stopped, and the reaction can typically be detected by absorbance, fluorescence or circular dichroism over time [7 and 8]. However, several enzyme systems do not give any optical signal and, if they do it can often be difficult to attribute the changes to a specific intermediate. In some cases, it is possible to solve these problems by using quenched-flow methods, either in continuous or time delayed mode. As in the stopped-flow technique, two solutions are mixed and pushed through an ageing tube at a constant speed, and then the reaction is stopped by a quenching agent, usually acid. The quenching, in order to work optimally, should be rapid, complete and irreversible [8 and 10]. This method is employed to follow reactions on the millisecond to seconds time range. The age time is dependent on the volume of the age loop and on the flow-rate of the reactants. It can be varied by changing the loop volume and/or the flow-rate.

A variation of this approach is the rapid freeze-quench (RFQ) technique, where the mixed solution is sprayed into a cryogenic medium and the reaction is stopped after a certain time [10 and 11]. The frozen samples can then be packed into glass tubes for detection by low–temperature EPR or another suitable analytical technique. Freezing of the sample stops the reaction from proceeding any further and prevents any large-scale protein
motions. The freeze-quench instrument is basically composed of a mixing / ageing system and a quenching system. The earliest RFQ instrument consisted of a hydraulic ram unit as a drive unit with direct spraying of the sample into a cryogenic liquid (nitrogen, isopentane or petroleum) [11]. Improvements to the RFQ technique have arisen from the design of new mixers in order to reduce both the volume and the mixing time [12 to 15]. New packing systems have also been invented to reduce sample loss [16 and 17]. In addition, the cryogenic isopentane bath has been coupled to a filtering system [18 and 19] or replaced by spraying the solution onto a surface that is frozen via thermal conductivity with a cryogenic liquid [12, 15, 20 and 21]. One of these new freeze-quenching devices consists of two oxygen-free copper wheels arranged in a side-by-side fashion and temperature-equilibrated in liquid nitrogen at 77 K (Figure 1.2). The driving wheel is attached to a motor and drives the other one through frictional contact. As a result, the two wheels rotate in opposite directions but at the same speed. The mixed solution instantaneously freezes on the surface of the wheels and is crushed into a powder by the friction of the rotating wheels. The frozen powder is then collected into an EPR tube through a collecting funnel that is placed in the liquid nitrogen directly below the interface of the wheels [12 and 15].
Figure 1.2 Schematic representation of a rapid freeze-quench device with copper wheels [12 and 15].

In another design, the rotating wheels were replaced by a rotating plate that is in contact with liquid nitrogen (Figure 1.3). The plate, made of aluminium for its high thermal conductivity (150 W/(mK)) and characterised by a conical shape, was cooled to 77 K by immersing the bottom into a liquid nitrogen bath. In this apparatus, in order to reduce the freezing time, the plate rotates around its centre at a speed correlated to the flow speed of the solution in the microfluidic channel (about 0.05 rad/s for a 500 mm/s flow) [21].
Figure 1.3 Schematic representation of a rapid freeze-quench device with rotating plate [21].

Although the RFQ method is now a proven technique to analyse short-lived species by EPR spectroscopy, and commercial RFQ devices are available, its application to study enzymatic reaction intermediates has been limited. This is due to the challenges in obtaining an efficient packing system that often requires a large amount of sample. The irreproducibility in packing affects the magnitude of the EPR signals and entails oxygen background noise. In the current work we describe a novel RFQ instrument, which combines the freeze-quench technology with flashing capabilities [22] to enable the studies of both thermally-activated and light-activated biological reactions. This unique instrument also uses a new rotating plate design based on magnetic couplings and removes the need for mechanical motorised rotation, which can otherwise be problematic at cryogenic temperatures.

1.2.2. Rapid flash-freeze apparatus

Reaction mechanisms are characterised by two main aspects: kinetic and structural. The kinetic behaviour is usually analysed by stopped-flow experiments, while the structural aspects, such as formation of intermediates and their structural changes during the reaction, require other techniques. However, the development of instrumentation and suitable techniques to simultaneously follow reaction kinetics and structural changes during light-induced reactions has been limited. In 1993 the transient structural state of cross-bridges during activation of rabbit skinned muscle fibres by photolysis of adenosine
triprophosphate (ATP) in the presence of calcium (Ca$^{2+}$) was studied with a novel device [22]. The fibres were rapidly frozen at selected times after illumination in order to trap the structural states in the transient condition. A new device was created by modifying a cryo-press to enable laser photolysis and tension measurements (Figure 1.4). Samples were loaded on a freezing head that could be released to slam onto a metal plate cooled with liquid helium. An optical sensor activated a frequency-doubled ruby laser for photolysis of caged ATP at selected time points. By optimising the position of the optical sensor and the mirror it was possible to modify the time interval between the laser pulse and freezing.

![Figure 1.4 Schematic representation of the modified cryopress which enables rapid freezing and laser photolysis.](image)

However, as yet, there are no reported instruments that combine RFQ with flashing capabilities for the analysis of samples by EPR spectroscopy. Hence, this is one of the primary goals of the current work.
1.3. Electron paramagnetic resonance (EPR)

EPR (Electron Paramagnetic Resonance), also known as ESR (Electron Spin Resonance) and EMR (Electron Magnetic Resonance), is a spectroscopic technique that uses magnetic fields and microwaves to study species that contain unpaired electrons. Unpaired electrons play crucial roles in several biological processes such as catalysis, oxidation, polymerisation and photosynthesis. They behave as small magnets and can change their spin state by absorbing microwave energy in the presence of a magnetic field. Spin is an intrinsic property of a particle and can have only two orientations in space. Only a paramagnetic system can show EPR signals, for example certain transition metals (V, Mn, Co, Ni, Cu etc.) and organic radicals \[23\]. When a paramagnetic sample is placed in a uniform magnetic field, the energy level of the ground state splits by an amount $\Delta E$. In order to determine $\Delta E$, which is the energy between the two spin levels, the sample is irradiated by microwaves with a set of frequencies. $\Delta E = h \nu = g \beta_e B$ where $h$ is the Planck’s constant \(6.62607004 \times 10^{-34} \text{ m}^2 \text{ kg} / \text{s}\), $\nu$ is the frequency (GHz), $g$ is the gyroscopic factor and the fingerprint of a molecule, $\beta_e$ is the Bohr’s magneton and $B$ is the magnetic field (Gauss). The $g$ factor is the parameter that is used for the identification of the paramagnetic species. $\beta_e$, $h$ are constant, frequency is known and $B$ can be measured. It is important to point out that without a magnetic field there is no energy difference to measure and that this difference depends linearly on the magnetic field (figure 1.5) \[23\].

![Figure 1.5 Zeeman splitting for a S = 1/2 system with one unpaired electron in an external magnetic field B₀.](image)

**Figure 1.5** Zeeman splitting for a $S = 1/2$ system with one unpaired electron in an external magnetic field $B₀$. 
The EPR signal is transformed into a sine wave with an amplitude proportional to the slope of the signal. As a result the first derivative of the signal is measured (figure 1.6). EPR spectra can be recorded in different frequency regions and the most important are: S-band (2-4 GHz), X-band (8-10 GHz), Q-band (~35 GHz) and W-band (~90 GHz). Continuous wave (CW) EPR spectra are recorded by putting a sample into a microwave (MW) irradiation field of constant frequency and sweeping the external magnetic field until the resonance condition is fulfilled. In the experimental set-up, the MW field is built up in a resonator (typically a rectangular cavity), into which the sample tube is introduced. Time-resolved electron paramagnetic resonance (TR-EPR) combines high spectral resolution and time resolution in the 10-100 ns domain. This technique allows EPR spectra to be recorded for any transiently formed species. In addition, the combination of high-field studies with light excitation is promising for determination of very short-lived species. In general, this method is employed to study photo-induced electron transfer and energy transfer processes [24].

![Figure 1.6](image.png)

**Figure 1.6** A small additional oscillating magnetic field, Bm, is applied in the same direction as the main field B. Bm is commonly at 100 kHz. As Bm increases from the value Bm₁ to Bm₂, the crystal detector output increases from i₁ to i₂. The output of the 100 kHz phase-sensitive detector is the derivative of the absorption curve.

EPR spectra can be recorded at room temperature for a large number of spin systems, including radicals and transition metal ions, due to its high sensitivity. However, many EPR spectra of biological transition metals and radicals can only be detected on frozen
solutions. There are various reasons to measure biological samples at low temperatures rather than room temperature. The act of freezing fixes molecules in all possible orientations, each with slightly different 3D structures and g-values. In general, at lower temperatures the EPR signals will sharpen up, while at higher temperatures they tend to broaden. Another important aspect to consider is the solvent. Biological samples are commonly dissolved in aqueous buffers but water will absorb the microwaves, causing the sample to heat up. It is also important to take into consideration a property of the sample known as spin-lattice relaxation. The electrons are distributed between the two energy levels (S= -1/2 and S=1/2) involved in the transition with a small excess in the lower energy level. All the electrons in both levels will absorb energy quanta provoking a complete reversal of the electron distribution. However, it is possible to keep observing a signal only if the lower energy level is more populated. Therefore, some electrons have to move back to the S=-1/2 level using relaxation. When this process does not occur the signal will not be detected because the electron distribution between the two levels will be equal. Even if the signal intensity is higher at lower temperatures, it is necessary to find the optimal temperature region to detect the EPR signal of a specific sample. This region needs to be defined for each new species under study [25]. In addition, the way the sample is collected plays a crucial role. For the study of enzyme intermediates it is necessary to trap states at different timepoints. The RFQ technology coupled with EPR offers an ideal approach to characterise these transient species [10].

1.4. Photochemistry: principles and laws

Photochemistry is the branch of chemistry studying the chemical modifications due to the interaction of light with matter. Light is a form of energy and it is defined as a collection of photons propagating through space as electromagnetic waves. Therefore, light exhibits both wave–like and particle–like properties. The speed of light in a vacuum is approximately $3 \times 10^8$ m s$^{-1}$ and, unless reflected or refracted, it is propagated in a straight line [26]. The electromagnetic spectrum is the range of all possible frequencies of electromagnetic radiant. In photochemistry, the wavelength range from 800 nm (near infrared) to 150 nm (far ultraviolet) is the most relevant because it corresponds to the photon energy ranging from 800 to 150 kJ mol$^{-1}$. These energies are much higher than
those associated with thermal motion at ambient temperature and are comparable to the energies of chemical bonds [26].

To understand the interaction between light and matter, it is necessary to assume that the radiation behaves as a particle whose energy is quantised. Also, in matter energy levels are quantised and the consequence is that species exist only in discrete and defined energy states. A transition between certain states of energy must be associated with a fixed energy. As a result of the quantisation of energy, only specific energies and frequencies of radiation can be absorbed or emitted. This behaviour explains the characteristic absorbance or fluorescence bands in the spectra of chemical species. The intensity of light is dependent on the number of photons per unit time. The energy of each photon is directly proportional to the frequency (ν) of the radiation by the expression $E = h\nu$, where $h$ is the Planck’s constant ($6.62607004 \times 10^{-34} \text{ m}^2 \text{ kg} / \text{s}$). Only photons with a definite quantity of energy corresponding to a difference between two allowed energy values can be absorbed. When the absorption occurs, the species involved becomes excited. The assimilation of that energy by the species depends on the wavelength of the radiation. As shown in figure 1.7, the longer the wavelength of incident radiation, the lower the energy [26].

![Electromagnetic (EM) spectrum](image)

**Figure 1.7** Electromagnetic (EM) spectrum.
Absorption is discussed quantitatively by the Lambert – Beer’s law. This law is expressed through the following equation: $T = \frac{I_t}{I_0} = e^{-\varepsilon lc}$. $T$ is the transmittance (the fraction of light that can pass through a sample at a specific wavelength), $I_t$ is the intensity of the transmitted light, $I_0$ is the intensity of the incident light, $\varepsilon$ is the extinction coefficient, $l$ is the path length and $c$ is the concentration of attenuating species in the sample. Initially, the Beer–Lambert law was first developed independently where Lambert’s law stated that absorbance is directly proportional to the thickness of the sample, and Beer’s law stated that absorbance is proportional to the concentration of the sample. The modern law combines the two previous laws and correlates the absorbance to both the concentration ($c$) as well as the thickness ($l$) of the sample. Therefore, the transmittance can be expressed in terms of an absorbance ($A$) which is defined as followed: $A = -\ln\left(\frac{I_t}{I_0}\right)$ and it implies that $A$ becomes linear with the concentration: $A = \varepsilon cl$.

The first law of photochemistry, the Grotthus–Drapper law, asserts that only the light radiations absorbed by the molecule of interest are photoactive and they consequently produce photochemical changes in the molecule. The second one states that during a photochemical reaction (absorption) one particle is excited for each quantum of radiation absorbed. If the process involves a single photon, it must have enough energy for the promotion of chemical change in one molecule [26]. One of the essential concepts to provide information about photochemical reaction mechanisms is the quantum yield ($\Phi$). It is defined as the number of molecules of reactant consumed per photon of light. In the next paragraph this concept will be described in more detail.

1.5. Caged compounds

1.5.1. Caged compounds: definition and properties

Biological processes, such as the activity of genes, proteins and other molecules, are controlled in terms of location and timing [27 to 29]. To study these phenomena it is necessary to achieve a spatiotemporal control of the process using a conditional trigger [1, 2, 3, 28, 29 and 30]. Light is an ideal external trigger signal, since it does not affect the biological environment when it is conveniently selected [29 and 30]. There are several naturally occurring light-dependent processes in biology, such as photosynthesis, light signalling by photoreceptors and a number of light-activated enzymes [31 to 34].
However, the majority of biological reactions do not require light and hence, are thermally-activated. In order to overcome this problem one strategy to obtain light-responsive biologically active molecules is the “caging” technique. This term has been introduced in science, since Kaplan and Hoffman synthesised caged ATP in 1978 [4]. However, many authors prefer to use the term “light-activated” instead of “caged” because this one is considered equivocal [5]. The caging technique is based on the idea that a biologically active substance can be rendered temporarily inactive (or “caged”) by chemical modification with a photolabile protecting group [3, 5, 27 and 28]. A caged compound is described as a bioactive compound whose activity is suppressed by a photoremovable protecting group. It is the inert precursor of an active molecule and the original bioactivity is restored upon photolysis (figure 1.8)[29 and 30].

**Figure 1.8** Photocleavage of the caged group [5].

An ideal photolabile protecting group should be designed based on the following criteria [27]:

- The photo-deprotection should follow a single reaction pathway and occur with high *quantum yield* $\Phi$;
- The by-products should not interfere with the ongoing photolysis, be transparent at the selected wavelength to avoid competitive absorption and be biocompatible. Formation of free radicals should be avoided;
- The photo-release of the bio-agent should be fast, in agreement with the time scale of the analysed biological process;
- Caged molecules should be soluble in the targeted media (usually water) and they should be able, if required, to pass biological barriers or show affinity to specific sites;
• Successful detection of the studied response should depend on the level of activity prior to irradiation;

The first step of the photoreaction is the absorption by the caged compound of a photon at the required energy. After absorbing the photon, the caged molecule is promoted to an excited state, forming highly reactive species that can proceed to stable products. Only the light absorbed by the caged molecule acts as a trigger and, therefore, the higher the proportion of molecules absorbing the photons, the more product will be obtained. In solution, the extinction coefficient of the caged compound should be high at the wavelengths triggering the reaction. Efficiency of photolysis is not only dependent on $\varepsilon$, but it is also determined by the quantum yield $\Phi$.

$$\Phi = \frac{\text{product molecules formed}}{\text{photons absorbed}}$$

The quantum yield is a measure of the effectiveness that an absorbed photon converts a caged compound to its uncaged counterpart. For caged compounds with a high extinction coefficient and high quantum yield, less light would be required to promote the photo-release of the bioactive molecules.

Photolysis of the majority of available caged compounds occurs at wavelengths in the near-UV and visible region [2, 27, 28, 29 and 30]. The efficiency of the photoreaction, the bioactivity of the precursor and the formation of the by-products are influenced by the nature of the molecule being caged and by varying the substituents on the caging group. It is not possible to predict how all these properties will be affected [27].

1.5.2. Design

In general, to design suitable caged compounds two important requirements are: 1) the uncaging step needs to be faster than the process under study; 2) the efficiency of uncaging should be high to easily remove the caging groups. Another main point to consider is that caged compounds must be biologically inert before photolysis. They are usually made using synthetic organic chemistry and from a chemical point of view their design might seem relatively straightforward. Syntheses are multistep for the majority of caged compounds, but some of them are made with one–step “direct” caging. Multistep
syntheses are necessary for natural products with many functional groups showing equivalent reactivity [27].

The two strategies, one-step and multistep, are also referred to as classical and indirect. The first one, the classical, is based on the identification of a key functional group that is biologically relevant for the activity of the molecule and on the covalent modification of that group with a photo-removable moiety. The second one, the indirect route, is based on the notion that the interaction between two or more molecules occurs over a wide molecular surface rendering any particular functional group to the same level in terms of contribution (figure 1.9). The molecules showing this kind of interactions are caged by multisite modifications. Typically, caged small molecules are synthesised employing the classical strategy.

**Figure 1.9** Multistep synthesis and direct caging, both followed by photocleavage [5].

In general, syntheses are made in organic solvents, and only in the final step the caged compounds are rendered water-soluble to be applied to biological systems. It is
important to consider the stability in water of light–activated compounds. Some chemical bonds such as esters are sensitive to hydrolysis, and others are not (ethers, amines and carbamates). As a result of instability the spontaneous hydrolytic release of the caging moiety can often occur [5 and 27].

1.5.3. Caged peptides and proteins

Many biological pathways are controlled in time and space by proteins and peptides. Although there are many problems in producing light–activated macromolecules, a wide assortment of caged peptides and proteins has been designed [35]. Usually, the former consists of inhibitory peptides able to disturb an essential intracellular protein–protein interaction. They are easily made by solid–phase peptide synthesis (SPPS). The latter includes proteins whose activity is rendered inert by covalent modification of a vital amino acid with a photo-removable group [35 and 36]. The caging of proteins is more challenging due to the presence of multiple reactive functional groups on the protein of interest, not all of them having an influence on the enzymatic behaviour [36].

Four different ways of preparation have been described [27 and 37]. The first one is to make these compounds inert by the chemical modification of their reactive amino acid residues. Three methods depending on the type of side chains (amino–, thiol– and acid–reactive) have been developed. Amino groups can be caged by chloroformate or chlorocarbamate and the new urethane bond can be cleaved by UV irradiation (figure 1.10 A). Thiol groups can be modified with 2–nitrobenzyl bromide (figure 1.10 B) and carboxyl groups can be modified with a diazo–derivative (figure 1.10 C). It is necessary to prepare this derivative just prior to use. Normally, this method is direct and easy to use, but on the other hand it is hard to control the position and the number of the modified sites.
Another more complex method is the codon suppression technique. A caged T4 lysozyme, incorporating a nitrobenzyl aspartic acid at Asp20, a vital site for the bioactivity has been synthesised in vitro [37]. The synthesis employs the genetic insertion of unnatural amino acids into the protein. The transfer RNA (tRNA) drives this introduction at a specific codon site. A tRNA suppressor linked to the nitrobenzyl aspartic acid was made and it was shown that the final caged lysozyme was catalytically inert until photo-irradiation with UV light. The advantage of this technique is the possibility to insert photo-removable amino acids at a desired site in any peptide and protein. On the other hand, the synthesis of tRNA linked to a photolabile amino acid is challenging and it is difficult to prepare large amounts of the product in a cell free translation system.

The most common method to obtain caged peptides is via SPPS. This procedure is conventionally employed to make peptides that contain di tert–butyl dicarbonate (Boc-) or fluorenylmethyl carbamate (Fmoc-) amino acids. Boc and Fmoc are protective groups used to avoid the polymerisation of amino acids during the synthesis. Recently, the SPPS technique has been employed to introduce photolabile protecting groups on peptides (figure 1.11). The desired caged peptides can be obtained by reverse–phase high
performance liquid chromatography (HPLC). After illumination with UV irradiation, the uncaged product can be observed by HPLC.

**Figure 1.11** Solid-phase peptide synthesis [37].

The fourth strategy is known as expressed protein ligation (EPL), which is a recent technique that has been applied to several protein engineering problems [38]. As with the codon suppression strategy, EPL is used to insert non-natural amino acids at the site of interest in the protein. By using EPL it is possible to link small pre-made synthetic peptide building blocks (30–50 amino acids) to obtain a final protein product up to 300 amino acids. The assembly of the fragments occurs with high regioselectivity in water at physiological pH. The mechanism of EPL is analogous to the chemical ligation and it consists of three steps. At first, small peptides are made by conventional peptide synthesis and then they are deprotected. The final step is the coupling by chemoselective reaction. After several couplings it is possible to produce long proteins. As shown in figure 1.12, a protein with an N-terminal fusion to intein can be cleaved by thiols, giving an α-thioester derivative. Expression vectors are commercially available and allow the recombination of intein fusion proteins containing chitin-binding domain (CBD). The α-thioester protein can react with a synthetic or recombinant peptide containing a cysteine (Cys) residue, via native chemical ligation to obtain a semisynthetic product. The Cys
residue must be located close to the region where the introduction of the non-natural residue will occur (figure 1.12).

**Figure 1.12** Recombinant protein α-thioesters and EPL [38].

Usually, EPL can be achieved in two ways, both the thiolysis and native chemical ligation reactions are performed in one pot or the α–thioester is isolated first. In the former procedure, the intein must stay folded during the ligation and the type of additives present in the mixture must be limited. In the latter, the chemical ligation is carried out using a wider range of additives, such as detergents, chaotropes and denaturating agents. These additives increase the solubility and consent to obtain polypeptides in high concentrations (millimolar) by improving the ligation yields (figure 1.13).
**Figure 1.13** Tandem ligation procedure. The protecting group (PG) can be a peptide. All the fragments can be produced by synthetic or recombinant methods [38].

EPL has been employed to synthesise different classes of proteins from both eukaryotic and prokaryotic organisms. Examples are kinases, phosphatases, transcription factors, polymerases, ion channels, cytoplasmic signalling proteins and antibodies [35 and 38]. The two main advantages of EPL are the possibility to introduce multiple non-natural amino acids into the protein and that a wider number of modifications are possible. However, it requires more chemical steps than the *in vivo* nonsense suppression mutagenesis.

In conclusion, caged peptides and proteins could be powerful tools in several fields of experimental biology, such as cell biology and molecular biophysics. Even if they have the advantage to control time, dose and site of activation there are at least three points to consider. One is that the photo-removable protecting group should be introduced into the active site of interest to have the desired effect. Another one is the development of new design criteria to use longer wavelengths to prevent tissue damage and to allow tissue penetration by light. UV damages DNA and other cell components in the *in vivo* application. The last one is to employ caged compounds in other fields than physiology, for example nanotechnology [37 and 40].
1.5.4. Caged small molecules

In mainstream applications, the use of caged compounds is influenced by their applicability [1, 2 and 27]. Ideally, a caged molecule is completely inactive and the bioactivity is only fully restored after illumination. Some caged small molecule activators and inhibitors of gene function, such as caged toyocamycin, caged estradiol, caged ecdysone and caged anisomycin, are examples of that scenario [27]. When irradiation cannot quantitatively restore biological activity, it is possible to solve this problem by increasing the concentration of the caged effector. This issue can be due to incomplete uncaging, side reactions, or to the inability of the molecule to reach a bioactive conformation after cleavage. A third situation can arise when the caged molecule still exhibits biological activity before uncaging, but below a threshold essential for having any effects. In this case, the molecule can still be employed. Finally, the worst scenario is when the caged molecule is active enough to promote background activity. These kind of caged molecules can still be used, but only if its concentration is below the biological threshold.

Despite the problems described above, several caged compounds have been developed for studying biological phenomena, such as amino acids, steroids, second messengers, sugars, lipids and enzyme substrates. One of the most common areas in which light–activated molecules have been used is neuroscience. The spatially well–defined flash photolysis of caged agonists or antagonists of neuronal receptors has been extremely useful in investigating kinetics and mechanisms of receptors, transporters and ion channels. Various neuroactive amino acids and neurotransmitters are available [41]. Among them, caged glutamate is the most widely used and several variants have been synthesised by using different chromophores or different caging strategies [42].

Caged ATP and cyclic AMP (cAMP) were the first caged compounds to be prepared and uncaged in living cells. Caged ATP requires three coupling steps and caged cAMP is obtained directly using a diazo approach. Caged ATP can be employed as a source of ATP that cannot be utilised by ATPases until release of ATP due to irradiation [4]. Prior to photolytic cleavage the caged ATP is resistant to the hydrolytic activity of the enzyme; after photolysis the substrate is released and hydrolysis is observed. This caging strategy
also facilitates studies involving the kinetic effects of pre-determined and variable intracellular levels of ATP [4].

Many physiological processes are initiated by an increase of intracellular Ca\textsuperscript{2+} concentration through the opening of Ca\textsuperscript{2+} channels. The inorganic cation calcium cannot form covalent bonds to photolabile groups and therefore a new caging strategy has been realised. This involves the use of chelating agents (BAPTA, EDTA and EGTA) whose affinity for calcium decreases upon illumination. Prior to illumination, when the chelator has high affinity, it can be efficiently loaded with the ion. After photolysis, the affinity is low and a higher concentration of calcium is released from the compound. The caging of calcium and the consequent release after light has been employed to study the role of this second messenger in cellular processes, such as muscle contraction and synaptic transmission [43 to 45].

To track living cells, organelles, and intramolecular molecules in a spatiotemporal way, photo-activatable fluorescent proteins have been produced by labelling the specific protein with synthetic small caged fluorophores, which have been produced by attachment of a fluorescent dye to a photo-removable protecting group. Ideal caged fluorophores should give rise to the fast release of the fluorophore and rapid increase in fluorescence after a brief irradiation. The choice of the fluorophores should be based on their photophysical properties, such as high brightness and weak degradation at selected wavelengths. Typical examples are the photo-activatable fluorescein and rhodamine compounds [46]. They are considered useful calibration systems, but some of them present some limitations in their applications. Caged fluorescein, for example, is very hydrophobic and some proteins labelled with it form aggregates [3 and 46].

Various small caged molecules have been synthesised for analysing protein function or enzyme catalysis. Two caged NADP (nicotinamide adenine dinucleotide phosphate) molecules have been synthesised and characterised for crystallographic studies of isocitrate dehydrogenase (IDH) [52]. One has been projected to be catalytically caged and can bind to IDH prior to photoexcitation without being catalytically active. The second analogue has been designed to be affinity caged in order to inhibit the binding of the compound to IDH prior to photolysis [52]. Three caged NADP cofactors, which showed
different photochemical properties, have been successfully prepared via enzymatic synthesis. The incorporated caged NADP analogues are some of the most sterically hindered groups exchanged using direct enzymatic transglycosidation [53]. Caged o-nitrobenzyl ether has been employed to study the horse liver alcohol dehydrogenase (HLADH) catalysed oxidation of alcohols at -50°C. Despite the removal of the rate-limiting step, the use of caged benzyl alcohol does not yield a detectable H/D kinetic isotope effect. During the fast phase multiple enzyme turnovers have been observed. This phenomenon is due to side reactions, such as the re-oxidation of bound NADH and the dissipation of photo-excitation energy. This experiment has proved that the application of caged compounds to study enzyme kinetic steps can be affected by the presence of NADH when it is involved in the catalytic cycle [54].

Since many biological reactions require oxygen to take place, a cobalt-based caged oxygen molecule \((m\text{-peroxo})(m\text{-hydroxo})\text{bis(bis(bipyridyl)cobalt(III)) nitrate}\) has been synthesised in order to deliver dioxygen in a controlled manner at cryogenic temperatures. Enzymes are often active in the crystalline state and therefore, reaction intermediates may accumulate if catalysis occurs synchronously throughout the crystal formation. Upon cryo-photolysis, the temperature increases and the active compound is released initiating catalysis and as a consequence the accumulation of intermediates can be observed. This caged oxygen molecule is able to release dioxygen at 100 K. This approach has the potential to investigate in detail various oxygen-dependent enzymes [55].

The dynamic behaviour of proteins is another important property of living cells. Different techniques have been developed for data collection and imaging of macromolecules within crystals. Crystallisation often requires several days or weeks and because of that it is not always possible to achieve the co-crystallisation of an enzyme–substrate complex. Therefore, time–resolved X-ray technology has overcome this problem by using a suitable trigger. Caging groups may be employed in combination with Laue crystallography to study the structural changes occurring at an enzyme active site during catalysis. A short laser pulse initiates the reaction and a synchrotron X-ray pulse monitors the reaction steps by time–resolved structure analysis. When the photolabile group is irradiated within the crystal the enzyme turnover is triggered and it is possible to detect
reactive intermediates. For the majority of proteins not containing a naturally light-activatable group, caging is a suitable way to trigger enzyme activity by UV–visible illumination [3, 29 and 56].

Light–activated compounds have been applied to other scientific fields. For instance, nitrobenzyl derivatives have been used in photodirected synthesis of biochips. The caged building blocks are fixed on a solid support and are irradiated through a mask. Therefore, uncaging occurs selectively and then the deprotected functional groups can bind a second caged molecule. Repetition of these illumination and binding steps using masks and varying patterns drives to the formation of the expected set of products. An array of 1024 peptides was successfully synthesised [57].

In the following table (1.1) some commercially available caged compounds are listed.

<table>
<thead>
<tr>
<th>Caged compound</th>
<th>Φ</th>
<th>ε</th>
<th>Φ x ε</th>
<th>Rate (s⁻¹)</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Calcium chelators</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM-nitrophen</td>
<td>0.18</td>
<td>4,300</td>
<td>774</td>
<td>3.8 x 10⁴</td>
<td>Complete</td>
</tr>
<tr>
<td>NP-EGTA</td>
<td>0.23</td>
<td>970</td>
<td>194</td>
<td>6.8 x 10⁴</td>
<td>Complete</td>
</tr>
<tr>
<td>Nitr-S</td>
<td>0.012</td>
<td>5,500</td>
<td>66</td>
<td>2.5 x 10⁴</td>
<td>Complete</td>
</tr>
<tr>
<td>Diazo-2</td>
<td>0.03</td>
<td>22,800</td>
<td>1,596</td>
<td>2.3 x 10⁴</td>
<td>Complete</td>
</tr>
<tr>
<td><strong>Neurotransmitters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNB-Glu</td>
<td>0.14</td>
<td>500</td>
<td>70</td>
<td>4.8 x 10⁴</td>
<td>Fair</td>
</tr>
<tr>
<td>CNB-GABA</td>
<td>0.16</td>
<td>500</td>
<td>70</td>
<td>3.6 x 10⁴</td>
<td>Fair</td>
</tr>
<tr>
<td>CNB-carbamoylcholine</td>
<td>0.8</td>
<td>430</td>
<td>344</td>
<td>1.7 x 10⁴</td>
<td>Excellent</td>
</tr>
<tr>
<td>MNI-Glu</td>
<td>0.085</td>
<td>4,300</td>
<td>366</td>
<td>~10⁵</td>
<td>Excellent</td>
</tr>
<tr>
<td><strong>Phosphates</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPE-IP₃</td>
<td>0.65</td>
<td>430</td>
<td>280</td>
<td>225 and 280</td>
<td>Excellent</td>
</tr>
<tr>
<td>NPE-cAMP</td>
<td>0.51</td>
<td>430</td>
<td>219</td>
<td>200</td>
<td>Fair</td>
</tr>
<tr>
<td>DMNPE-cAMP</td>
<td>0.05</td>
<td>5,000</td>
<td>250</td>
<td>300</td>
<td>Poor</td>
</tr>
<tr>
<td>NPE-cADPribose</td>
<td>0.11</td>
<td>430</td>
<td>271</td>
<td>18</td>
<td>Excellent</td>
</tr>
<tr>
<td>NPE-ATP</td>
<td>0.63</td>
<td>430</td>
<td>271</td>
<td>90</td>
<td>Excellent</td>
</tr>
<tr>
<td>DMNPE-ATP</td>
<td>0.07</td>
<td>5,000</td>
<td>350</td>
<td>18</td>
<td>Fair</td>
</tr>
<tr>
<td><strong>Fluorophores</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bis-CMNB-fluorescein</td>
<td>ND</td>
<td>2,000</td>
<td>ND</td>
<td>ND</td>
<td>Complete</td>
</tr>
<tr>
<td>DMNB-HPTS</td>
<td>ND</td>
<td>5,000</td>
<td>ND</td>
<td>ND</td>
<td>Complete</td>
</tr>
</tbody>
</table>

**Table 1.1** Properties of some commercially available caged compounds [5].
1.5.5. Advantages and issues

Uncaging is a useful strategy compared to other methods for changing the concentration of a molecule inside or outside of living cells. The main reasons for this are that uncaging can be intracellular, rapid, controlled in a spatiotemporal manner, and quantitatively controlled. In general, the light sources employed in this field are classified as either general–purpose light sources, which are used for uncaging and imaging, or specific–purpose light sources that are used only for photolysis. The former consists of Hg, Xe, and metal halide lamps and the latter includes a small number of lasers, such as N₂ gas lasers and solid–state lasers. Lasers can create a specific optical uncaging pathway to generate a limited light spot or a full illuminated field. Release only occurs where light is incident and this is a relevant feature of this technology. In addition, only light is required to cleave the photo-removable protecting groups. Reactants are not necessary and that aspect allows handling very sensitive molecules, which are incompatible with acids or bases. Caging groups with different photophysical properties could bind biochemical activators and inhibitors which are sensitive to distinct wavelengths, making possible the control of both the initiation and termination of a biochemical process in a single experiment. As photo-release is independent of the normal biological source, the timing of uncaging is strictly dictated by irradiation. The caging strategy gives a precise time zero offering the possibility to initiate a biological response with high temporal resolution.

A significant application of the uncaging technique is in the intracellular release. Light can pass through the plasma membrane promoting changes in concentration. Using this approach it is possible to vary solute concentrations, and it is more powerful compared to other methods. Quantification of photolysis in situ is achievable using the caging strategy. Usually, the amount of photo-release can be studied and quantified by combining a fluorescent probe to the uncaging event [5, 27 and 28]. Previously, some issues related to the use of caged compounds were described. The caged molecule itself can have biological activity prior to photolysis, the released by-products may also be active and the light pulse itself can trigger the reaction without a caged molecule [30]. Another limitation in the application of caging technology is that it is hard to distinguish between photolabile groups on the basis of wavelengths [58].
1.6. B$_{12}$-systems

In this thesis various vitamin B$_{12}$ derivatives and a B$_{12}$-dependent enzyme have been investigated and are introduced below.

1.6.1. Cobalamins

Vitamin B$_{12}$, also called cobalamin, is a complex molecule that was discovered in the late 1940s. Its role is to maintain healthy nerve cells and to help in the production of DNA and RNA. Together with B$_{9}$ (folic acid), B$_{12}$ helps the formation of red blood cells, and it works together with both B$_{6}$ and B$_{9}$ to control blood levels of the amino acid homocysteine. High levels of homocysteine are associated with heart disease. B$_{12}$ deficiency can cause a range of symptoms, including pernicious anaemia, caused by an impaired uptake from food. Vitamin B$_{12}$ supplements in high doses, either given as injections or orally, are prescribed to treat this disease. For this reason B$_{12}$ is also known as antipernicious anaemia factor and it is required for human and animal metabolism, even if it can only be derived from the diet. Bacteria and archaea are the only natural source of B$_{12}$-derivatives, whereas several biological reactions in many different organisms depend on them [59 and 60].

The structures of vitamin B$_{12}$ and its derivatives were elucidated by X-ray crystallography in the laboratory of Dorothy Hodgkin in the late 1950s [61]. Vitamin B$_{12}$ is the only biomolecule to have a stable metal-carbon bond (C-Co). In addition, it has a corrin ring, consisting of four pyrrolic units, three methylene bridges and a central cobalt ion (figure 1.14). There are four main cobalamins, namely cyanocobalamin, hydroxocobalamin, methylcobalamin and adenosylcobalamin. Cyanocobalamin contains a cyanide group as the axial ligand and does not have any direct cofactor role due to its inert Co(III)-corrin [62]. Hydroxocobalamin (OHCObl) is most generally used for vitamin B$_{12}$ replacement therapy and contains a hydroxyl ligand. The physiologically relevant cofactors are AdoCbl (5’-deoxy-5’adenosylcobalamin) and MeCbl (methylcobalamin), which are both light sensitive and more chemically labile (figure 1.14). Enzyme catalysis mediated by AdoCbl and MeCbl has a crucial role in various biological processes, with different modes of action [60]. All AdoCbl- dependent enzymes involve homolytic cleavage of the Co-C bond upon substrate binding to form a 5’-deoxyadenosyl / cob(II)alamin radical pair that
mediates catalysis. The role of MeCbl in catalysis differs significantly to that of AdoCbl. MeCbl-dependent catalysis involves the transfer of a methyl group from a methyl donor to a methyl acceptor by so-called methyltransferases, which are involved in important metabolic pathways in humans, animals and bacteria. Several substrates act as sources of methyl groups, such as methanol, aromatic methyl ethers, methyl amines or N5-methyltetrahydropterins (such as N5-methyltetrahydrofolate). Thiols are the methyl group acceptors in methanogenesis and in methionine synthesis.

![Structure of cobalamin: A) Methylcobalamin, B) Adenosylcobalamin.](image)

**Figure 1.14** Structure of cobalamin: A) Methylcobalamin, B) Adenosylcobalamin.

### 1.6.2. Ethanolamine ammonia lyase (EAL)

Ethanolamine ammonia lyase (EAL) is a coenzyme B_{12}- or AdoCbl–dependent enzyme that catalyses the formation of acetaldehyde and ammonia (NH$_3$) from 2-aminoethanol or ethanolamine (figure 1.15).
Figure 1.15 EAL (holoenzyme) catalyses the conversion of 2-aminoethanol into acetaldehyde and ammonia.

In general, lyases are enzymes that break chemical bonds by means other than hydrolysis and oxidation. EAL converts ethanolamine into the pre-product intermediate carbinolamine via a 1,2-rearrangement reaction, which is typical of AdoCbl-dependent isomerases (figure 1.16). Carbinolamine then dissociates into the final products acetaldehyde and ammonia [6 and 63].

Figure 1.16 General mechanism of AdoCbl-dependent isomerases. Substrate binding initiates homolysis and the Ado radical promotes the formation of the substrate radical by H-abstraction followed by rearrangement to the product radical. The full mechanism of EAL-B₁₂ in the presence of ethanolamine is illustrated in figure 4.7.

The structure of EAL from Escherichia Coli (E. coli), including bound cofactor and substrate, was elucidated via X-ray crystallography in 2010. EAL from Salmonella enterica has also been studied in detail and is composed of two subunits: the largest one (EutB) consists of 453 residues and has a molecular weight of 49.4 kDa, whereas the smallest subunit (EutC) consists of 298 residues and has a molecular weight of 32.0 kDa. The EutB-
EutC heterodimer is the key structural unit of EAL. EutB is folded into a \((\beta\alpha)_8\) TIM barrel with the substrate-binding site at the C-terminal end of the \(\beta\)-barrel. EutC covers the C-terminal end of the \(\beta\)-barrel and the cofactor binding site. The enzyme is a hexamer of dimers \(\alpha_6\beta_6\) having six active sites. The very high molecular weight of active EAL (488 kDa) means it has a low solubility (<2 mg/ml) [64].

EAL has become an important model system to understand the catalytic mechanism of coenzyme B\(_{12}\)-dependent enzymes in general. In particular, it has been used to study the relationship between catalysis and enzyme–substrate complex geometry, and the properties of the active site [6, 63 and 64]. EPR studies, in conjunction with UV-vis spectroscopy, have been used to follow the oxidation state of cobalt during the reaction and to propose a catalytic mechanism of EAL. These experiments allowed the chemical state of the AdoCbl to be characterised, but were not able to uncover the role of the protein in catalysis. Stopped-flow measurements of EAL in the presence of different substrates were performed along with kinetic isotope effect (KIE) studies and showed that Co-C homolysis is coupled to subsequent hydrogen abstraction from the substrate [65]. Magnetic field effect (MFE) continuous wave photolysis of free EAL and EAL bound to AdoCbl revealed that the AdoCbl-binding site acts as a cage favouring geminate recombination [66 and 67]. Despite all the recent findings about EAL, a full understanding of the protein contribution during catalysis nor information about the 5′-deoxyadenosylcobalamin (Ado) radical lifetime, its interaction with the enzyme and the detailed role of the protein during Co-C homolysis have been clarified yet [68]. EAL is a suitable model system to investigate the mechanism of catalysis by this cofactor as it can be easily produced in \textit{E. coli}. Hence, in this thesis using a combination of RFQ and caging strategies I have tried to spectroscopically characterise the Ado radical.

1.6.3. Caging of amines

In this thesis a caging approach was applied to the ethanolamine substrate as a tool for triggering EAL catalysis by light. The most widely used caging groups are 2-nitrobenzyl or 2-nitrophenyl derivatives due to their relative robustness and their commercial availability. These groups are connected to the biologically active molecule by bonding to a hetero-atom (usually O, S or N) as an ether, thioether, ester (including phosphate or
thiophosphate esters), amine or similar functional group. The caged compounds are made via organic synthesis and direct caging. Both the structure of the nitrobenzylic/phenylic compound and the atom to which it is linked affect the efficiency and wavelength required for photo-removal [1, 27 and 28]. In the current work, 2-nitrophenylpropylchloroformate (NPPOC-Cl) was used to cage ethanolamine. The synthesis is described schematically in figure 1.17, while the uncaging mechanism is presented in the result section.

![Diagram](image.png)

**Figure 1.17** Direct caging of 2-aminoethanol with 2-nitrophenylpropylchloroformate.

1.7. Thesis aim

A complete understanding of an enzyme’s catalytic mechanism requires a full kinetic characterisation, which describes all the steps of the reaction together with their rate constants, and a structural description of all intermediates that are formed during the reaction. These intermediates are often characterised by short lifetimes, distinctive light absorption features, and in some cases vibrational signatures too and, while their kinetics are mainly carried out by stopped-flow techniques, a full structural description normally requires multiple biophysical methods [8 and 10]. The magnetic resonance of the unpaired electron spin in a magnetic field is a more selective method of detection which also provides a great deal of information about the electronic environment. This is the basis for electron paramagnetic resonance (EPR) spectroscopy. However, EPR techniques typically require relatively long measurement times and very often, low temperatures to fully characterise these short-lived species [23]. Therefore, common rapid mixing techniques, such as stopped-flow or quench-flow are not directly suitable. However, the combination of rapid freeze-quench (RFQ) followed by EPR analysis provides the ideal approach to kinetically trap and spectroscopically characterise these transient radical species. The development of a rapid freeze-quench device combined with flashing capabilities has exciting potential to provide a detailed understanding of the catalytic cycle of many different enzyme systems. This unique device enables the generation and
trapping of radical species *in situ*, which can then be analysed via EPR. The project goal is to calibrate and improve a RFQ prototype and then apply it to important biological systems (figure 1.18). In this case, it has been used to study the mechanism and to identify any short-lived intermediates in the $\text{B}_{12}$-dependent enzyme EAL.

The caging strategy is another approach to study short-lived intermediates (figure 1.18). It has been proved to work efficiently in several fields [27 to 32]. Caged compounds of amino acids, steroids, second messengers, sugars, lipids have been synthesised and used for studying biological phenomena. In this work, the caging strategy was applied to ethanolamine ammonia lyase (EAL), a $\text{B}_{12}$-dependent enzyme, in order to identify the intermediates and regions of the protein that are involved in the catalytic cycle.

**Figure 1.18** Project goals summary.
### 2. Materials and methods

#### 2.1. Reagents, chemicals and solvents

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
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<tr>
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<tr>
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<tr>
<td>5'-deoxyadenosylcobalamin (AdoCbl)</td>
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</tr>
<tr>
<td>β-mercaptoethanol (βME)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Coomassie Blue stain</td>
<td>Expedeon</td>
</tr>
<tr>
<td>Deuterated ethanolamine</td>
<td>Sigma Aldrich</td>
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<tr>
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<tr>
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### Table 2.1 List of reagents, chemicals and solvents suppliers.

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<th>Enzymes and vectors</th>
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<td>Myoglobin</td>
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<tr>
<td>pET SEAL</td>
<td>Eurofins</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
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<table>
<thead>
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<th>Solvents</th>
<th>Suppliers</th>
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<td>Deuterated chloroform (CDCl₃)</td>
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<td>Ethanol (EtOH)</td>
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<td>Ethylacetate (AcOEt)</td>
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<td>Hexane</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>Tetrahydrofuran (THF)</td>
<td>Fisher Scientific</td>
</tr>
</tbody>
</table>

#### 2.2. Production and biochemical characterisation of ethanolamine ammonia lyase

#### 2.2.1. Expression

Ethanolamine ammonia lyase was expressed and purified in *Escherichia Coli* using the plasmid pET SEAL, containing genes encoding both the 31 kDa β and 50 kDa α subunits [64]. Cells were grown in 2-YT growth which contains 16 g/L tryptone, 10 g/L yeast extract and 5 g/L NaCl. To every 31 g of media powder 1 L of distilled water was added and then the medium was autoclaved at 2 Bar, 121°C for about 20 minutes. After cooling ampicillin was added to reach a final concentration of 100 µg/mL. The LB agar plates were prepared under sterile conditions using 40 mL agar medium with ampicillin at a final concentration of 100 µg/mL. 100 µL of transformant mixture were added per plate and incubated overnight at 37°C. The transformant mixture was made by adding 1 µL of pET SEAL to 50 µL of competent cells BL21(DE3) followed by incubation on ice for 30 minutes, heat shock for 45 seconds at 42°C and incubation at 37°C for 1 h. A starter culture was used for inoculation of large culture (30 x 600 mL). 6 mL of the starter culture were added to each autoclaved flask containing sterile media and ampicillin at a final concentration of 100 µg/mL. Then, cultures were incubated at 37°C in a stirring incubator (Infors Ltd) at 220 rpm for 2 hours. After 2 hours, 1 mL of the culture was removed under sterile conditions and the optical density was measured. When the optical density reached between 0.8-1.0 A.U. the cultures were ready for induction. All the flasks were removed from the incubator and IPTG at a final concentration of 100 µM was added to each flask. The
cultures were incubated overnight at 25°C. Cultures were centrifuged at 6000rpm for 20 minutes at 4°C and then stored at -20°C.

2.2.2. Purification

The pellets were resuspended in 100ml of pH 7.5 50 mM HEPES/glycerol (10%) buffer. Magnesium chloride solution (final concentration 1 mM) and one tablet of Complete™ EDTA-free protease inhibitor tablets (Roche diagnostics) were added. Once resuspension has been achieved, 1mg DNAse and 50mg lysozyme were added. The suspension mixture (light brown in colour) was sonicated with 15 x 20 second blasts at 40-45% intensity. The suspension was spun at 2500 rpm for 30 minutes.

The pellet was resuspended in 250ml 50 mM HEPES pH 7.5 buffer and a protease inhibitor tablet was added. The suspension was spun again at 2500 rpm for 30 minutes and the supernatant (pale-yellow in colour) was retained. Ammonium sulphate (84 g/L) was added to the supernatant. The pellet was resuspended in 100ml 10mM HEPES pH 7.5 buffer. Dialysis against 5 L of 10mM HEPES was performed overnight with stirring. Dialysis was repeated another two times for 4 and 1 hours respectively. The suspension was spun at 2500 rpm for 30 minutes and the supernatant corresponding to EAL was retained. The EAL solution was concentrated down by centrifugation using 4 x 100,000 MWCO centrifugal-filter devices (Millipore) at 30,000 rpm for 30 minutes. Aliquots of concentrated sample were snap frozen in liquid nitrogen and stored at -80°C.

To monitor the efficiency of the purification protocol and to check the purity of the final enzyme sample SDS-PAGE gel electrophoresis was performed on all retained samples ((Mini-Protean TGX Stain-Free™ Gel with Precision Plus Protein™ Unstained Standards, BioRad). β-mercaptoethanol (βME) was used as the denaturating agent. Samples were mixed with concentrated SDS loading dye and heated for 5 min at 100°C before loading on the gel. The gel was run in SDS buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1 % SDS) at 300 V for ~ 15 min. The gels were stained afterwards using Coomasie Blue stain and visualised with a Bio-Rad Gel Doc EZ imager.
2.2.3 Binding protocol of EAL with the AdoCbl cofactor

To bind EAL to its AdoCbl cofactor an Econo-Pac 10DG (Biorad) was employed. The column was equilibrated with HEPES (20 mM, pH 7.5) while EAL apoenzyme was incubated with B12 (in large excess, 10 μL from a 10 mM stock for 1 mL of apoenzyme) on ice for about 10 minutes. Then, the sample volume (usually 1 up to a maximum of 2 mL) was added on the column. 3 x (sample volume) mL of buffer were added into the column and allowed flowing to waste. 1.5 x (sample volume) mL were added to the column and collected. The final concentration of the enzyme was then determined by Lambert-Beer using the absorbance at 525 nm and ε= 8 mM⁻¹ cm⁻¹. Once finished, a red band corresponding to the unbound AdoCbl was visible halfway through the column.

2.2.4. Steady-state activity assay

EAL bound to AdoCbl catalyses the conversion of 2-aminoethanol (2-AE) to acetaldehyde and ammonia. This reaction is coupled to yeast alcohol dehydrogenase (YADH), an enzyme that employs NADH as an electron donor to convert acetaldehyde to ethanol forming NAD⁺ as a by-product. The amount of reagents involved in the assay was determined by UV-vis spectroscopy (Cary Varian 50 spectrophotometer).

YADH: 100 units are desired in the final reaction vessel. Therefore 1 mg was added to 1 ml of HEPES (20 mM pH 7.5) and the absorbance of a 1 in 10 dilution was measured at 280 nm. To obtain the desired concentration the following calculations were made:

\[
\text{Determination of } mg/ml: \quad \frac{(Absorption\ Value)}{14.6} \times 100 = x \text{ mg/ml}^{-1}
\]

\[
\text{Determination of units: } \quad x \text{ mg/ml}^{-1} \times 340 \text{ units mg}^{-1} x \text{ units ml}^{-1}
\]

\[
\text{Determination of volume: } \quad \frac{100 \text{ units}}{x \text{ units ml}^{-1}} \times 1000 = \text{Number of } \mu l \text{ required}
\]

The number of units/mg used in the calculations is taken from the supplier’s specifications sheet. Whereas, 14.6 is the extinction coefficient expressed in mM⁻¹ cm⁻¹.

NADH: The desired final concentration is 150 nM, therefore 3.82 mg were added to 500 μl of HEPES (20 mM pH 7.5) and the absorbance of a 1 in 166.7 dilution (6 μl in 1000 μl)
was measured at 340 nm ($\varepsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$). To obtain the desired concentration the following calculations were made:

\[
\text{Determination of concentration: } \frac{\text{Absorption Value}}{6.22} \times 166.7 = x \mu M
\]

\[
\text{Determination of volume: } \frac{150 \text{ nM}}{(x \mu M \times 1000)} \times 1000 = \mu l \text{ required}
\]

Ethanolamine: The desired final concentration is 1 mM. Therefore 0.84 µL was added to 7 mL of HEPES (20 mM pH 7.5) and then 500 µL was added to the reaction vessel.

EAL: The desired final concentration is 100 nM. Before adding to the cuvette, the EAL apoenzyme was incubated with $\sim$ 10x excess of 5’deoxyadenosylcobalamin (AdoCbl) on ice for 10 minutes to ensure binding of the cofactor. The final concentration of the enzyme was determined by Lambert-Beer using the absorbance at 525 nm and $\varepsilon = 8 \text{ mM}^{-1} \text{ cm}^{-1}$ after removing the unbound AdoCbl as previously described.

After calculating the volumes of each of the reagents required, they were all added to the 1 ml cuvette except EAL holoenzyme which was added last. A steady decrease in absorbance at 340 nm should be observed, with the greatest rate of increase at the start of the reaction (the initial velocity). The initial decrease in absorption (abs/min) was measured using the kinetics software on the Cary 50 spectrophotometer. This protocol was employed with the same concentration for caged-ethanolamine in order to check the activity of the enzyme with the caged substrate.

2.3. Rapid mixing techniques

Two main rapid mixing techniques were employed in this work. The first technique is correlated with the development of a rapid freeze-quench (RFQ) device (TgK Scientific), which is fully described in Chapter 3. In addition, stopped-flow experiments were carried out by using an Applied Photophysics SX20 stopped-flow instrument.

2.3.1. Chemical calibration of the rapid freeze-quench instrument

The mixing efficiency of the new RFQ instrument has been assayed by studying the alkaline hydrolysis of 2,4-dinitrophenylacetate (DNPA) with NaOH (1 M) (Figure 2.1). This reaction was performed at room temperature and studied under pseudo first order reaction conditions ([NaOH] >> [DNPA]). About 10 mg of DNPA was dissolved in 1 mL of
ethanol and then, the solution was made up to 50 mL in HCl (2 mM). The blanks were prepared manually as follow: $t_{\text{inf}}$, which corresponds to the reaction at completion, was prepared by mixing 1 mL of stock solution containing DNPA (0.88 μM) and 1 mL of NaOH (1 M) up to 10 mL of HCl (2 M). For $t_0$, which corresponds to unreacted DNPA, the 1 mL of solution containing DNPA (0.88 μM) was mixed with 5 mL of HCl (2 M) and 1 mL of NaOH (1 M) was added. At the end, the solution was made up to 10 mL of HCl (2 M). The spectra were measured between 240 and 420 nm by using a Cary Varian 50 UV-visible spectrophotometer. The formation of the final product, dinitrophenol (DNP), was followed spectrophotometrically (Cary Varian 50 spectrophotometer) in the UV region by monitoring the absorbance change at 294 nm.

![Diagram](image)

**Figure 2.1** Alkaline hydrolysis of DNPA with NaOH. 1) Nucleophilic attack of OH$^-$ at the electrophilic C of the ester leading to a tetrahedral intermediate. 2) The intermediate collapses forming acetic acid and the alkoxide. 3) Acid/base reaction where the alkoxide works as a base deprotonating the acetic acid. DNP and the sodium salt of the acetic acid are formed.

Samples of the reaction with different ageing times were obtained by mixing the reagent solutions with the apparatus and spraying the final mixture directly into a quenching solution of HCl (2 M, 2 mL)[10 and 21].

The absorbance spectra of the quenched solutions were measured (Cary Varian 50 UV-visible spectrophotometer) and the ratio between the absorbance at 294 nm and 260 nm ($A_{294}/A_{260}$) was used to calculate the fraction of sample reacted as follows:

$$\frac{[(A_{\text{ratio}})_t - (A_{\text{ratio}})_0]}{[(A_{\text{ratio}})_{\text{inf}} - (A_{\text{ratio}})_0]}$$

where $(A_{\text{ratio}})_t$ is the ratio $A_{294}/A_{260}$ as a function of time; $(A_{\text{ratio}})_0$ and $(A_{\text{ratio}})_{\text{inf}}$ correspond to the ratio of absorbances at $t=0$ and at completion of the reaction respectively. 260 nm is the isosbestic point and 294 nm corresponds to the formation of DNP.
2.3.2. Stopped-flow experiments

2.3.2.1. Stopped-flow experiment of myoglobin against sodium azide

Myoglobin and sodium azide were used without further purification. The reaction was studied via stopped-flow by measuring the increase in absorption at 575 nm at 25°C and using Tris buffer (50 mM, pH 7). Sodium azide was used in excess in order to study the binding reaction with a pseudo-first order kinetic.

\[
\frac{[HS]_t}{[HS]_0} = e^{-k_{\text{obs}} \times t}
\]

\[
k_{\text{obs}} = k \times [N_3^-]
\]

where \([HS]\) and \([N_3^-]\) are the concentrations of the high-spin conformation of myoglobin and azide, respectively; \(k_{\text{obs}}\) is the apparent rate constant and \(k\) is the rate constant.

The initial concentration of myoglobin was 70 μM and sodium azide was used as follows: 0.35 mM, 0.70 mM, 2 mM and 5 mM [21].

2.3.2.2. Stopped-flow experiment of ethanolamine against substrates

EAL (holoenzyme) was mixed with its natural substrate ethanolamine (2-AE) and aminopropanol (2-AP) in the stopped-flow to follow the conversion of cob(III)alamin to cob(II)alamin over a few milliseconds. The experiments were carried out by recording the absorbance change at 525 nm at 5°C using final concentrations of 15 μM EAL, 2.5 mM 2-AE and 2-AP at different concentrations (0.5 mM, 1 mM and 2.5 mM).

2.4. Procedure to prepare NPPOC-protected amines

2.4.1. Synthesis

In a round-bottom flask purged with N₂, Na₂CO₃ (2 mmol) was added to a solution of 2-aminoethanol or the selectively deuterated form (1 mmol) in a 1:1 ratio of H₂O:1,4-dioxane (or only 1,4-dioxane) at 0°C. NPPOC-Cl (1 mmol) in THF was added by dropwise addition. After 20 minutes the ice bath was removed and stirring was continued for 24h. The reaction mixture was dried by rotary vaporisation. Then, 3 mL of H₂O was added and the mixture was extracted with ethylacetate (3x10mL) to remove NPPOC-Cl and its hydrolysed product. The desired product was in the organic layer. HCl formed during the caging was neutralised with Na₂CO₃.
2.4.2. Purification and characterisation

The caged compounds were purified via a silica column with ethyl acetate and hexane (1:1) as the running solvents. The fractions were monitored by thin layer chromatography (TLC) and then characterised by proton magnetic resonance (\(^1\)H-NMR, Bruker 400 MHz) in deuterated chloroform (CDCl\(_3\)).

2.5. Photolysis

Different photolysis experiments were performed using either LED (ThorLabs) or laser (Quantel Brilliant B) excitation. Most photolysis measurements were monitored spectroscopically (Cary Varian 50 spectrophotometer). In the laser set up the light pulse is provided by a neodymium-doped yttrium aluminium garnet (Nd:YAG) laser which generates an output beam in the IR region of the spectrum at 1064 nm. The probe beam is provided by a 150 W Xenon Arc flash lamp and adjusted to the probing wavelength with a monochromator. Then, the absorbance change is measured using a photomultiplier tube detector (PMT) and recorded by a digital oscilloscope (Applied Photophysics). Details of each experiment are also described in the results chapters. In this section they are listed below:

- Caged-2-AE (stock solution in water 5% EtOH (v/v) and then diluted in HEPES 20 mM pH 7.5 to a final concentration of 230 \(\mu\)M) was illuminated with LEDs at 365 nm (a), 280 nm (b) and 265 nm (c). The illumination steps were regulated via oscilloscope (Tektronix TDS 3032C) as follows: ten pulses of 100 ms each, twenty-one pulses of 500 ms each and 17 pulses of 1 s each.

- Caged-2-AE and caged-2-\(^2\)H\(_4\)-AE (stock solutions in water 5% EtOH (v/v) and then diluted in HEPES 20 mM pH 7.5 to a final concentration of 90 \(\mu\)M) were illuminated with a 365 nm LED. The illumination steps were regulated via oscilloscope (Tektronix TDS 3032C) as follows: ten pulses of 100 ms each, thirty pulses of 500 ms each and twenty-one pulses of 1 s each.

- Flow-cell experiments on caged-2-AE (6 mM in HEPES 20 mM pH 7.5) were measured at multiple wavelengths (600 to 270 nm) and irradiated at: 266 nm (30 mJ) and 355 nm (30 mJ and 100 mJ) with laser.
Kinetic transients of caged-2-AE (1 mM) in the presence of EAL (15 μM) with the laser flash photolysis instrument and selectively deuterated caged-2-AE (1 mM) in the presence of EAL (15 μM) were recorded upon illumination with laser at 355 nm (100 mJ) and monitored at 420 nm.

Kinetic transients of caged-2-AE in (1 mM) the presence of EAL (15 μM) and deuterated caged-2-AE (1 mM) in the presence of EAL (15 μM) were also recorded upon illumination with laser (355 nm excitation, 100 mJ) and monitored at 525 nm.

77 K absorbance spectra showing the uncaging of caged-2-AE (1 mM) in the presence of EAL (15 μM) were measured after illumination at 100 K. Spectra were recorded at 77 K in sucrose (100% w/v in HEPES 20 mM pH 7.5) as cryo-protectant after dark incubation at 150 K, 160 K, 170 K, 180 K, 190 K and 200 K.

2.6. EPR measurements

All the EPR experiments were performed as continuous wave experiments with a Bruker E580X EPR instrument with a Bruker ER4118X-MD5 dielectric resonator. Details of each experiment are also described in the results chapters. In this section they are listed below:

CW-EPR spectra of Mb (200 μM) vs azide at different concentrations (500 μM, 1 mM, 2 mM) were measured at 12 K, centre field 2600 G, modulation amplitude 5 G, sweep width 5000, number of points 2048, microwave power 25 dB.

CW-EPR spectra of Cyt c and its reduced form (by reaction with sodium dithionite) at different concentrations (500 μM, 1 mM, 2 mM) were measured at 12 K, centre field 2600 G, modulation amplitude 5 G, sweep width 5000, number of points 2048, microwave power 25 dB.

CW-EPR spectra of Cyt c (1 mM final concentration) mixed against NADH/NMN (2 and 50 mM respectively) and illuminated with LED (ThorLabs) at λ_{exc}=365 nm measured at 12 K at different temperatures and ageing times: 4°C (loop 1, different flow-rates: 6 (11.7 ms), 5 (12.6 ms), 4 (14.0 ms), 2 (20.7 ms)); 15°C (loop 1 flow-rates: 6 (11.7 ms), 5 (12.6 ms), 4 (14.0 ms), 2 (20.7 ms), loop 2 flow-rate 2 (28.2 ms) and loop 3 flow-rates: 4 (43.2 ms), 3 (55.2 ms); 20°C (loop 1 flow-rates: 6
(11.7 ms), 5 (12.6 ms), 4 (14.0 ms), 2 (20.7 ms), loop 2 flow-rate 2 (28.2 ms) and loop 3 flow-rates: 4 (43.2 ms), 3 (55.2 ms).

- **CW-EPR spectra of** MnCl$_2$ (200 and 400 μM) **in** HEPES (20 mM, pH 7 were measured at 20 K, centre field 3000 G, modulation amplitude 5 G, sweep width 5000, number of points 2048, microwave power 30 dB. With the same conditions were collected spectra in the presence of DMNP-EDTA (400 μM) and pyruvate kinase (250mg X mL).

- **CW-EPR spectra of** MeCbl (200 μM, 400 μM and 1 mM) were measured at 12 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000 G, number of points 2048, microwave power 25 dB.

- **CW-EPR spectra of** EAL (48 μM) **in** the presence and absence of substrates (2-aminoethanol 10 mM and 2-aminopropanol 10 mM) were measured at 20 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000 G, number of points 2048, microwave power 30 dB.

- **CW-EPR spectra of** EAL (60 μM) were measured at 20 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000, number of points 2048, microwave power 30 dB before and after illumination.

- **CW-EPR spectra of** caged-2-AE (5 mM) **in** the presence of EAL (60 μM) were measured at 20 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000, number of points 2048, microwave power 30 dB after annealing at 177.15K, 187.15 K, 199.15 K, 209.15 K, 218.15 K, 238.15 K, 245.15 K, 253.15 K, 263.15 K, 253.15 K. Some of the previously described samples were recorded at 20 K, centre field 3250 G, modulation amplitude 10 G, sweep width 1500, number of points 2048, microwave power 30 dB.
3. Development of a novel rapid freeze-quench instrument for EPR studies

3.1. Introduction

In this chapter the development of a novel rapid freeze-quench (RFQ) device combined with flashing capabilities is described. The rapid freeze-quench technique will be applied to biological samples and coupled to EPR spectroscopy in order to identify reaction intermediates. As with all rapid mixing techniques, the RFQ technique involves driving two solutions into a mixing chamber at high speed. Then, the new solution is sprayed through a loop into a cryogenic medium. The resulting frozen sample is collected and usually analysed via X-band (9.5 GHz) and continuous wave (CW) EPR. This method has allowed the investigation and spectral characterisation of short-lived intermediates involved in xanthine-oxidase- and cytochrome-oxidase-catalysed reactions [8, 11 and 21]. A recent application of RFQ-EPR revealed the formation of tyrosine radicals as intermediates of the reaction of cytochrome P450cam with peroxy acids [71]. Also, unique haem and radical intermediates were resolved in the reaction of Mycobacterium tuberculosis KatG with hydrogen peroxide using a commercially available rapid-mixing device (Update Instruments), which sprayed the reaction mixture onto two copper wheels immersed in liquid nitrogen [72]. In the last decade RFQ devices have been improved to reduce sample volume, mixing time and to simplify the packing procedure [12 to 19]. Despite all of these improvements, a RFQ apparatus combined with flashing capabilities to allow EPR analysis of both thermally-activated and light-activated reactions has not yet been developed. In the current work an innovative device that enables the generation and trapping of radicals in both types of reactions is described.

3.2. Results and Discussion

3.2.1 Apparatus design

The RFQ device that has been developed and used in this work (figure 3.1) contains a stopped-flow driving unit (Hi-Tech HSU-63, now TgK Scientific) that consists of a two syringes system driven by a type 34 stepper motor (McLennan TS368N50E3). The control of the motor is achieved using an electronics control unit (MSU-63SF Module Support Unit), which houses both power supply and stepper motor drive units. The flow-rates are set-up with the KinetaDrive software designed by TgK Scientific. The two syringes (2.5 mL
NA5050 for RQF-63 Quench Flow Apparatus) are driven simultaneously by a common push plate and both filling and driving of the two reagents is controlled by individual distribution valves. Samples are loaded from syringes above each drive syringe and valve. When the syringes are pushed, the reagents are carried through a thermostated umbilical connection in PEEK lines. This umbilical connects the lines through a mixer and into an optical quartz cell (Hellma) for illumination of the reaction sample by high-power LEDs at the desired wavelength. The reaction sample then passes through one of five ageing loops, which have different sample volumes and different ages times. Finally, the reacted solution passes through a spray nozzle and is sprayed onto a rotating plate made of aluminium, due to its high thermal conductivity (150 W/(mK)) and the fact that it is EPR silent. The plate is temperature-equilibrated at 77 K in liquid nitrogen and placed on a rotating wheel, also made of aluminium. In general, the use of a rotating plate has two main advantages compared to the chilled isopentane bath that has typically been used previously for such measurements. Firstly, the freezing-time is faster [21] and secondly the frozen samples obtained are likely to be more homogeneous due to the fact that they form a single layer with the same thickness and freezing-time. The frozen samples are then scraped from the surface of the plate and packed into an EPR tube, which is connected to a funnel via heat shrinking tube and placed in a Dewar full of liquid nitrogen, by using a packing rod (Bio-Logic Science Instruments). The characterisation of the instrument and optimisation/modification of different parts of the experimental design (e.g. the rotating wheel or the packing procedure) are discussed in the following sections.
Prior to using the RFQ device it was first necessary to characterise the age-time of the ageing loops (Figure 3.2). The ageing loops used in the current instrument are made of Tefzel tubing 0.04” I/D x 1/16” (1 mm x 1.6 mm). To determine the correct volume of each ageing loop they have all been weighed empty and then filled with a solution of bromophenol blue (Sigma Aldrich) in distilled water (to improve visibility) and weighed again (1 mg = 1 μL). Before filling with the dye all the loops have been carefully washed with distilled water and cleared with gas nitrogen and an air duster each time. This procedure has been repeated ten times for each loop and used to accurately determine their volumes. Based on these volumes it was then possible to calculate the theoretical ageing times for each loop at all of the different flow-rates used (table 3.1)
Figure 3.2 The ageing loops that have been used in the RFQ instrument. a) Nozzle; b) loop 1, volume 27 μL; c) loop 2, volume 42 μL; d) loop 3, volume 144 μL; e) loop 4, volume 238 μL; f) loop 5, volume 312 μL. All the loops are made of Tefzel tubing and the metal holders are made of stainless steel.

<table>
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<td>6.0</td>
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</tbody>
</table>

Table 3.1 Ageing times in milli-seconds calculated for each loop and flow-rate. The ages times not reported indicate that those flow-rates cannot be used with the stated loops based on experimental evidences from TgK Scientific.

3.2.3. Optimisation of the rotation system

Prior to calibrating the freeze quench instrument, the rotating wheel that is used for rapidly freezing the samples was optimised and re-designed to improve its performance. The initial concept (designed and built by TgK Scientific) consisted of a cold plate which would rapidly freeze a sprayed sample to form a fine, frozen powder that is easy to handle for subsequent packing into EPR tubes. For this to work efficiently it was thought that the sample should be frozen in a thin, layer by layer approach. The original design idea involved the cold surface rotating across the stream of sample volume emitting from the nozzle, rather than moving the spray nozzle across a cold surface. Hence, a saucer shaped Teflon coated aluminium alloy plate was designed (P/N NA7760 TgK Scientific Ltd, figure 3.3). In order for the plate to be cooled effectively it needs to maintain an efficient thermal connection to a liquid nitrogen reservoir in the polyurethane Dewar below it.
(Figure 3.4). For this purpose, a circular heatsink, made from aluminium alloy was chosen (Fischer Elektronik SK584 60 SA), and machined to reduce some of its mass. In addition, it was modified to make a pocket into which a stirrer bar could be placed and sealed by a polyoxymethylene (POM) closure plate (figure 3.3). To make the plate rotate without the need of physically joining through the Dewar, a rotating system based on magnetic coupling was designed using a low-cost lab stirrer (non-heating) from CAT M5 (M. Zipperer GmbH, 79282 Ballrechten-Dottingen, Germany, figure 3.4a), which removes the need for mechanical motorised rotation. However, upon using this setup it soon became apparent that problems arose with the build-up of ice underneath the wheel which prevents it from rotating freely at cryogenic temperatures. In order to avoid ice formation at low temperatures a constant stream of nitrogen or argon gas was blown over the wheel. Unfortunately, this still did not prevent ice build-up and therefore, a new improved rotation system was deemed necessary.

**Figure 3.3** Technical drawing of the initial rotating wheel that was used in the RFQ instrument. Adapted from TgK Scientific. The Teflon coated aluminium alloy plate and the original rotating wheel are shown.
The initial rotating system provided by TgK Scientific for the RFQ instrument: a) Rotating system based on magnetic coupling was designed using a low-cost lab stirrer (non-heating) from CAT M5 (M. Zipperer GmbH, 79282 Ballrechten-Dottingen, Germany); b) Rotating wheel inside the polyurethane Dewar in which the liquid nitrogen is poured.

Consequently, a new improved rotation system was designed and optimised, based on a different magnetic coupling concept (figures 3.5 and 3.6). This wheel is made of aluminium and is composed of two parts (figures 3.5 and 3.6) in which there are two opposing magnets with the same charge. The upper part floats on the base and the rotation between the two strictly depends on the flow of nitrogen gas around the upper part. By using this innovative rotating system a better rotation was obtained, although some condensation, leading to friction, was still present. Condensation could be reduced by warming up the wheel with a heat-gun and wiping it carefully between each measurement. It is also very easy for the wheel to become unbalanced during the rotation due to misalignment of the nitrogen gas lines and therefore, it has been necessary to carefully check their alignment before each experiment. In addition, it has often been beneficial to spray some lubricant on the wheel and let it rotate at room temperature. Before starting any experiment, the lubricant was removed. In this way it ensured that no metal residues were released due to any excess friction between walls of the wheel. By using these simple optimisation steps a smooth rotation of the system could be observed, even at very high speed, although unfortunately, it was not possible to determine a correlation between gas flow pressure and rotation speed.
Figure 3.5 The new magnetic coupling rotating system used in the RFQ instrument: a) Rotating disc and stand set-up; b) Set-up inside the polyurethane Dewar; c) Upper part where the Teflon coated aluminium alloy plate is placed; d) Upper part where one of the magnets is placed; e) Stand; f) Stand where the second magnet is placed.
3.2.4. Calibration steps

In order to fully determine the efficiency of the rapid mixing instrument three calibration steps have been used to validate the chemical-quenching, freeze-quenching and flash-freeze-quenching capabilities of the instrument. The first two have been previously described in the literature [13, 15, 16, 19 and 20], whereas it was necessary to design and develop a new assay to test the efficiency of the flashing part of the instrument, which is unique to this particular device (figure 3.7).
3.2.4.1. Chemical calibration: mixing efficiency and instrumental dead time

All rapid quench-flow / freeze quench devices require accurate calibration of the ageing loops and instrumental dead time by measuring a pseudo-first-order reaction on the time scale of interest [10]. Hence, the mixing efficiency of the new RFQ instrument has been assayed by studying the alkaline hydrolysis of 2,4-dinitrophenylacetate (DNPA) with NaOH (1 M) (Figure 3.8). This reaction was performed at room temperature and studied under pseudo first order reaction conditions ([NaOH]>>[DNPA]), where the concentration of DNPA prior to mixing was 0.88 μM). The formation of the final product, dinitrophenol (DNP), was followed spectrophotometrically in the UV region by looking at the absorbance change at 294 nm (Figure 3.9).

Figure 3.8 Alkaline hydrolysis of DNPA with NaOH. 1) Nucleophilic attack of OH\(^-\) at the electrophilic C of the ester leading to a tetrahedral intermediate. 2) The intermediate collapses forming acetic acid and the alkoxide. 3) Acid/base reaction where the alkoxide works as a base deprotonating the acetic acid. DNP and the sodium salt of the acetic acid are formed.
Figure 3.9 Absorbance spectra of DNPA solutions representing the unreacted sample $t_0$ and the fully reacted sample $t_{inf}$. About 10 mg of DNPA was dissolved in 1 mL of ethanol and then, the solution was made up to 50 mL in HCl (2 mM). The blanks were prepared manually as follow: $t_{inf}$ was prepared by mixing 1 mL of stock solution containing DNPA (0.88 μM) and 1 mL of NaOH (1 M) up to 10 mL of HCl (2 M). For $t_0$ 1 mL of solution containing DNPA (0.88 μM) was mixed with 5 mL of HCl (2 M) and 1 mL of NaOH (1 M) was added. At the end, the solution was made up to 10 mL of HCl (2 M). The spectra were measured between 240 and 420 nm by using a Varian Cary 50 UV-visible spectrophotometer.

Absorbance spectra over a 90 minutes period confirmed that there was minimal background hydrolysis of DNPA during the course of the experiment (figure 3.10).

Figure 3.10 Hydrolysis test of the DNPA stock solution obtaining as follows: 20 mg of DNPA were dissolved in 2mL of ethanol and made up to 100 mL in HCl (2 mM). Then, the traces were recorded every 2 minutes for 90 minutes.

Samples of the reaction with different ageing times were obtained by mixing the reagent solutions with the apparatus and spraying the final mixture directly into a quenching
solution of HCl (2 M, 2 mL) [10 and 21]. At this stage, only the driving unit and the loops were used in the calibration procedure, thus avoiding any potential problems caused by the downstream freezing and packing processes. As the instrument is not designed as a conventional quench flow a number of different collection vessels were investigated to optimise the quenching process (Table 3.2). Several of these were deemed to be unsuitable as they resulted in an inefficient quenching of the reaction (example data are shown in figures 3.11 and 3.12). However, a suitable collection vessel (PYREX Griffin beaker) was eventually identified and used for the subsequent calibration analysis.

<table>
<thead>
<tr>
<th>Container</th>
<th>Characteristics</th>
<th>Issues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disposable plastic bijou</td>
<td>Volume: 7 mL, Height: 50 mm, Inner diameter: 22 mm, Distance between nozzle and HCl: 3 cm</td>
<td>Sprayed solution not quenched properly due to drops staying on the container walls and keeping reacting</td>
</tr>
<tr>
<td>(Sigma-Aldrich)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plastic beaker (Sigma-Aldrich)</td>
<td>Volume: 50 mL, Height: 57 mm, Inner diameter: 49 mm, Distance between nozzle and HCl: 2 cm</td>
<td>Sprayed solution not quenched properly due to the container convex bottom</td>
</tr>
<tr>
<td>Plastic beaker (Sigma-Aldrich)</td>
<td>Volume: 25 mL, Height: 49 mm, Inner diameter: 38 mm, Distance between nozzle and HCl: 2 cm</td>
<td>Sprayed solution not quenched properly due to the container convex bottom</td>
</tr>
<tr>
<td>PYREX Griffin beaker (Sigma-Aldrich)</td>
<td>Volume: 10 mL, Height: 32 mm, Inner diameter: 25 mm, Distance between nozzle and HCl: 2 cm</td>
<td>Optimal conditions. Flat bottom</td>
</tr>
</tbody>
</table>

Table 3.2 Details of the different collection vessels used for testing the chemical quench reaction. PYREX Griffin beakers (volume= 10 mL) were identified as optimal containers for this process.
Figure 3.11 Example quench flow data obtained using different collection vessels in the RFQ instrument. a) UV absorbance spectra of DNPA (0.88 μM) sprayed against NaOH (1 M) via loop 1 and 2 at different flow-rates and collected in disposable plastic bijous containing HCl (2 M); b) Fractions of sample reacted obtained from the absorbance spectra shown in a) and calculated as follows: \( \frac{(A_{\text{ratio}})_{t} - (A_{\text{ratio}})_{0}}{(A_{\text{ratio}})_{\text{inf}} - (A_{\text{ratio}})_{0}} \), where \((A_{\text{ratio}})_{t}\) is the ratio \(A_{294}/A_{260}\) as a function of time; \((A_{\text{ratio}})_{0}\) and \((A_{\text{ratio}})_{\text{inf}}\) correspond to the ratio of absorbances at t=0 and at completion of the reaction respectively. 260 nm is the isosbestic point and 294 nm corresponds to the formation of dinitrophenol; c) UV absorbance spectra of DNPA (0.88 μM) sprayed against NaOH (1 M) via loop 1 and 2 at different flow-rates and collected in disposable plastic bijous containing HCl (2 M); d) Fractions of sample reacted obtained from the absorbance spectra shown in c) and calculated as described in b).
Figure 3.12 Values of sample reacted obtained with different loops and flow-rates (plastic beaker 25 mL). Fractions of sample reacted calculated as follows: 
\[
\frac{[(A_{\text{ratio}})_t - (A_{\text{ratio}})_0]}{[(A_{\text{ratio}})_{\text{inf}} - (A_{\text{ratio}})_0]},
\]
where \((A_{\text{ratio}})_t\) is the ratio \(A_{294}/A_{260}\) as a function of time; \((A_{\text{ratio}})_0\) and \((A_{\text{ratio}})_{\text{inf}}\) correspond to the ratio of absorbances at \(t=0\) and at completion of the reaction respectively; 260 nm is the isosbestic point and 294 nm corresponds to the formation of dinitrophenol.

After finding the optimal quenching vessel (PYREX Griffin beaker), the absorbance spectra of the quenched solutions were measured (Cary Varian 50 UV-visible spectrophotometer) and the ratio in absorbance at 294 nm and 260 nm \((A_{294}/A_{260})\) was used to calculate the fraction of sample reacted as follows:

\[
\frac{[(A_{\text{ratio}})_t - (A_{\text{ratio}})_0]}{[(A_{\text{ratio}})_{\text{inf}} - (A_{\text{ratio}})_0]}
\]

where \((A_{\text{ratio}})_t\) is the ratio \(A_{294}/A_{260}\) as a function of time; \((A_{\text{ratio}})_0\) and \((A_{\text{ratio}})_{\text{inf}}\) correspond to the ratio of absorbances at \(t=0\) and at completion of the reaction respectively. 260 nm is the isosbestic point and 294 nm corresponds to the formation of DNP. The resulting fraction of reacted sample was plotted at different timepoints and fitted to a single exponential function to obtain a pseudo-first-order rate constant of \(k = 25.3 \pm 4 \text{ s}^{-1}\) (figure 3.13a). A quenching dead-time of 2 ms could be determined by comparison with a commercially available stopped-flow instrument (TgK Scientific, \(k = 26.50 \pm 0.06 \text{ s}^{-1}\), figure 3.13b).
Figure 3.13 Determination of the rate constant of DNPA hydrolysis. a) The fraction of reacted sample was measured with different loops and flow-rates (PYREX Griffin beaker, volume=10 mL) and plotted against time. Fractions of sample reacted calculated as follows: \( \frac{(A_{\text{ratio}})_t - (A_{\text{ratio}})_0}{(A_{\text{ratio}})_{\text{inf}} - (A_{\text{ratio}})_0} \), where \((A_{\text{ratio}})_t\) is the ratio \(A_{294}/A_{260}\) as a function of time; \((A_{\text{ratio}})_0\) and \((A_{\text{ratio}})_{\text{inf}}\) correspond to the ratio of absorbances at t=0 and at completion of the reaction respectively; 260 nm is the isosbestic point and 294 nm corresponds to the formation of DNP. b) Stopped-flow trace (TgK Scientific) of DNPA (1 mL of stock solution 0.88 μM up to 10 mL in HCl 2 mM) against NaOH (1 M). Absorbance changes were measured at 310 nm. The red lines show the fit to a single exponential function \(y = y_0 + A \cdot \exp(-R_0 \cdot x)\) to obtain the pseudo-first-order rate constants.

3.2.4.2. Freeze-quench test to determine quenching-time

In a RFQ experiment the quenching-time \(t_Q\) is the time interval from the beginning of a reaction to its quenching by freezing. The quenching-time corresponds to the contribution of the mixing-time, the transport-time and the freezing-time (equation 3.1) [20].

\[
t_Q = t_M + t_T + t_F
\]

Equation 3.1 Quenching-time described by the sum of the mixing-time \(t_M\), the transport-time \(t_T\) and the freezing-time \(t_F\).

The mixing-time is the time required for the reagents to mix after passing through the mixer. Then, during the transport time \(t_T\) the reaction chemistry occurs as the new solution exits the loop and reaches the cryogenic medium. The freezing time \(t_F\) is more difficult to calculate as it combines the time of the sample cooling from room temperature to the designated freezing temperature, together with the time required for the sample to transition from the liquid phase to a solid. In an ideal RFQ device \(t_M\) and \(t_F\)
are close to zero [8]. Usually the freezing-time is determined by measuring a reaction test of known kinetics.

In the freeze-quench experiment the time resolution is also dependent on the distance between the spraying loop and the rotating plate. Another parameter to consider is the air friction which can affect the jet speed. Therefore, a direct calibration is necessary to obtain a reliable sample-ageing time. As a model system for EPR analysis the binding reaction of sodium azide (NaN₃) to myoglobin (Mb) has previously been employed [12, 13, 16, 19 and 20]. The effect of this ligand on myoglobin has been extensively studied [73 and 74]. Myoglobin exists at neutral pH in a high-spin iron form (Fe³⁺, S=2/5) and when it binds to sodium azide the conversion to low-spin configuration (Fe²⁺, S=1/2) can be detected via EPR. The azide replaces a water molecule, which is a weaker ligand, leading to a six-coordinated conformation due to its strong ligand field effect. The same reaction has now been studied here using a conventional stopped-flow instrument and the newly developed RFQ under the same temperature (25°C) and pH conditions (Tris buffer 50 mM, pH 7). Sodium azide has been used in excess in order to study the binding reaction with a pseudo-first order kinetic.

\[
\frac{[HS]t}{[HS]_0} = e^{-k_{obs} \times t}
\]

\[
k_{obs} = k \times [N_3^-]
\]

where \([HS]\) and \([N_3^-]\) are the concentrations of high-spin conformation myoglobin and azide, respectively; \(k_{obs}\) is the apparent rate constant and \(k\) is the rate constant.

To follow the formation of the myoglobin-azide complex a series of stopped-flow experiments were initially performed, prior to the freeze quench experiments, by measuring the increase in absorption at 575 nm. The initial concentration of myoglobin was 70 μM and sodium azide was used as follows: 0.35 mM, 0.70 mM, 2 mM and 5 mM [21]. The transients were all fitted to a single exponential equation to obtain the apparent constants \(k_{obs}\) (figure 3.14). The plot of \(k_{obs}\) against the azide concentration showed a linear behaviour (figure 3.15), and the fitting gave a second order rate constant \(k\) of 6984 (± 130) M⁻¹ s⁻¹.
Figure 3.14 Stopped-flow absorbance transients at 575 nm upon mixing of myoglobin (70 μM) with sodium azide at different concentrations: a) [NaN₃] = 0.35 mM; b) [NaN₃] = 0.70 mM; c) [NaN₃] = 2 mM; d) [NaN₃] = 5 mM. All data were fitted to a single exponential equation to obtain the k_{obs} values (red lines).
Figure 3.15 Plot of the observed rate constants ($k_{obs}$) against the azide concentration. A second order rate constant $k$ of 6984 (± 130) M$^{-1}$ s$^{-1}$ was obtained by fitting the data to a straight line.

Prior to studying the kinetics of myoglobin-azide complex formation by RFQ, the EPR spectra of the free and bound forms were measured to determine the $t_0$ and $t_{inf}$ spectra. Myoglobin by itself, corresponding to the $t_0$ of the reaction, and myoglobin fully bound to azide ($t_{inf}$) were prepared and pipetted directly into the EPR tubes (inner diameter 3 mm) to avoid any issues with manual packing. EPR spectra were measured at 12 K using a CW-EPR (Bruker) as described in the Materials and Methods chapter (figure 3.16). Myoglobin in high-spin configuration (Fe$^{3+}$, $S$=2/5) binds a water molecule as the axial ligand and exhibits a dominant sharp signal with $g_{xy}$=6 and a secondary one with $g_{x}$=2. In the presence of NaN$_3$, the water molecule is promptly replaced and Mb turns to a six-coordinated low-spin conformation which exhibits a different EPR signal with $g_1$=2.59, $g_2$=2.18, $g_3$=1.83.
Figure 3.16 CW-EPR spectra of free and azide-bound myoglobin. a) CW-EPR spectrum of myoglobin in high-spin conformation (Fe$^{3+}$, $S$=2/5, $g_{xy}$=6, $g_z$=2) measured at 12 K; b) CW-EPR of the myoglobin-azide complex in low-spin conformation (Fe$^{2+}$, $S$=1/2, $g_1$=2.59, $g_2$=2.18, $g_3$=1.83) measured at 12 K.

The drive unit of the RFQ device was moved into an anaerobic glovebox prior to starting the freeze-quench test to try to eliminate any potential problems caused by the presence of oxygen. However, a number of other problems were observed during the initial freeze-quench experiments, and measurements were repeated several times using different experimental conditions to overcome these various issues (see figure 3.17 for summary). In some samples manganese and copper contaminations were found to be present, which is a major problem as both metals give distinctive and strong EPR signals (figure 3.17a). However, the buffer and EPR cavity were ruled out as potential causes of the contamination signals and as a temporary solution to the problem ethylenediaminetetraacetic acid (EDTA) was added to all solutions. EDTA is a chelating agent able to trap metals and is EPR silent. An additional difficulty encountered was the presence of an unknown peak, which was detected with different intensities in some samples (figure 3.17b), and again both buffer and EPR cavity were ruled out as potential sources of contamination. The origin of the signal was eventually identified to certain isolated plates, where the scratching of the samples has damaged the Teflon coating and caused the appearance of the additional peak in some EPR spectra. The damaged plates were discarded and plastic spatulas/tweezers were subsequently used to gently collect the frozen samples from the plates to prevent further scratches.
Figure 3.17 Summary of the problems encountered during testing of the RFQ instrument. 
a) CW-EPR spectrum of Mb-azide complex at 12 K (loop 5, flow-rate 5) showing a strong 
manganese signal; b) CW-EPR spectra of Mb-azide complex at 12 K (loop 1, flow-rate 7) 
showing oxygen background noise and its reduction by blowing helium in the sample; c) 
CW-EPR spectrum of Mb-azide complex at 12 K (loop 2, flow-rate 2) showing an unknown 
peak.

The other major problem involved the presence of a large oxygen background signal, 
which affected most of the samples, often entirely covering the area of interest in the 
spectrum. Consequently in some samples it was impossible to see the Mb signals (figure 
3.18). In some cases the amount of oxygen could be reduced by blowing helium gas into 
the EPR tubes that contained the samples (figure 3.17b).
Figure 3.18 CW-EPR of Mb-azide complex measured at 12 K (loop 2, flow-rate 6). Oxygen background noise covering entirely the signal of interest.

However, in order to fully remove the trapped oxygen a new packing set-up was developed and used for all subsequent measurements. The amount of sample in a tube and the way in which the sample is compressed significantly affect the quality of the resulting EPR spectrum. Therefore, the packing step is crucial for obtaining optimal EPR spectra and for ensuring a good signal-to-noise-ratio. Hence, the packing set-up has been modified to improve the packing efficiency. The initial packing strategy consisted of scraping the frozen sample from the plates inside a foam Dewar (Spearlab) filled with liquid nitrogen. The frozen sample, now in powder form, was then transferred to a funnel attached to an EPR tube that is placed on top of a second foam Dewar filled with liquid nitrogen and the sample was pushed into the EPR tube using packing rods (figure 3.19). The funnel was made of aluminium in order to avoid temperature fluctuations and the EPR tubes were connected to it via a heat-shrinking tube. After filling the EPR tubes, the heat-shrinking tube was cut with a scalpel to store the sample into a portable cryogenic storage Dewar. To improve the packing efficiency and remove as much oxygen as possible the EPR tubes were subsequently connected to a vacuum pump (Watson-Marlow sciQ 400) via a silicon tube. The bottom of the EPR tubes were cut off and sealed with a customised filter (figure 3.19). The pump helped to compress the sample grains more efficiently and as a result, the oxygen that used to accumulate among the grains was
This approach also enables the successful collection of smaller frozen particles, which are generated at higher flow rates.

**Figure 3.19** Schematic representations of the two packing systems used in the RFQ instrument.

After the technical improvements, Mb-azide complex EPR spectra were carefully selected to calculate the instrument freezing-time (figure 3.20). The rotating plate was kept at a distance of 2 cm from the spray nozzle. The freezing-time was then calculated by plotting $\ln(f_{HS_t}/f_{HS_0})$ against time and extrapolating the fit to work out the intercept on the x-axis. The value of $f_{HS_t}$ was calculated as the height of the main peak in the high spin region divided by $(HS \text{ height} + HS \text{ scale factor } \times LS \text{ height})$, whereas the value of $f_{HS_0}$ corresponds to HS at $t=0$. Under these conditions the freezing-time that was calculated from the plot (figure 3.21a) was $31.6 \pm 3.9$ ms. This value is about six times longer than the freezing-times described in the literature for other RFQ instruments [13, 15, 17, 19, 20 and 21]. The most likely reason for this slow freezing-time was thought to be the sample-transport-time prior to freezing. Therefore, in order to improve this transport time the distance between the rotating plate and the spray nozzle was reduced down to 7 mm and the measurements repeated again to calculate the freezing time (figure 3.21b). As a result, a freezing-time of $5.2 \pm 3.8$ ms was obtained (figure 3.21b). This value is in
perfect agreement with the freezing-times of the RFQ devices described in the literature which show values between 5 and 7 ms [19 to 21].

Figure 3.20 Selected CW-EPR spectra of the Mb-azide complex measured at 12 K and sprayed at a distance of 7 mm between the spray nozzle and the rotating plate: a) loop 1, flow-rate 7, 3.9 ms; b) loop 2, flow-rate 4, 10.5 ms; c) loop 2, flow-rate 2, 21.0 ms; d) loop 3, flow-rate 5, 28.8 ms; e) loop 5, flow-rate 5, 62.4 ms; f) loop 5, flow-rate 4, 78.0 ms. Different signal intensities are due to the level of sample that was obtained in the EPR tubes.
Figure 3.21 Calculation of the freezing time from the freeze-quench test upon formation of the Mb-azide complex: a) Plot of $\ln(f_{HS_t}/f_{HS_0})$ against time for freeze-quench experiments carried out with the rotating plate at a distance of 2 cm from the nozzle; b) Plot of $\ln(f_{HS_t}/f_{HS_0})$ against time for freeze-quench experiments carried out with the rotating plate at a distance of 7 mm from the nozzle. The data were fitted to a straight line and the intercept on the x-axis was used to calculate the freezing times. The value of $f_{HS_0}$ corresponds to HS at t=0.

These data show that the RFQ instrument developed here is comparable to other known RFQ instruments. The packing strategy, coupling a vacuum pump and packing rods, has shown several advantages for the collection of frozen particles. Similar approaches were developed by other research groups, but they involved the use of chilled isopentane to freeze-quench the sample particles which then were filtered under pressure through a stainless funnel connected to an EPR tube fitted with a porous disk at its bottom [18 and 19]. Isopentane is extremely flammable and in certain vapour-air proportions can be explosive. In our device only nitrogen has been employed which has the advantage of being inert. As explained previously the obtained freezing-time of $5.2 \pm 3.8$ ms is similar to the times of the RFQ devices described in the literature which show values between 5 and 7 ms [19 to 21]. In the following table the quenching time for each loop and flow-rate is shown (table 3.3).
Table 3.3 Quenching times in milli-seconds calculated for each loop and flow-rate. The values not reported indicate that those flow-rates cannot be used with the stated loops based on experimental evidences from TgK Scientific.

However, despite these promising results the RFQ instrument described here could be improved further to obtain more consistent data sets. The reproducibility is influenced by both the speed and stability of the plate during rotation (figure 3.22). When the rotation speed varies the sample freezes at different times and the data is not comparable with other samples collected using the same conditions (i.e. same flow-rates and loops). On occasions the plate can suddenly stop rotating and the sample is then sprayed over the same area of the plate. This results in layers of sample with variable freezing-times and this is reflected in inconsistencies in the EPR data obtained. In addition, the plate can also become unbalanced while rotating, causing the sample to splash inhomogenously on the surface of the plate. Again, this results in some of the sample freezing slower than others. However, as the device is still a prototype several improvements could still be introduced and some of these are described in the conclusions section at the end of the chapter.
Figure 3.22 Summary of the problems in obtaining reproducible data from the RFQ instrument. Both CW-EPR spectra in a) and b) of the formation of the Mb-azide complex were collected under identical conditions (loop 1, flow-rate 7, 12 K).

3.2.4.3. Development of a light-activated assay to test the flashing capabilities of the new RFQ instrument

As mentioned above, a freeze-quench apparatus combined with photoexcitation capabilities has never been reported previously. In the present RFQ instrument the mixing-chamber is linked to a quartz optical cell (design TgK Scientific, production Hellma, 2 mm path), which can be illuminated via interchangeable LEDs (figure 3.23). In order to prove the feasibility of the approach and the applicability of the device it was firstly necessary to develop a suitable light-activated assay that could be used in the instrument. Ideally, the assay should give rise to changes in the time range achievable with the device (ms), provide changes in the measured EPR signals and have a high quantum yield as the sample will only be illuminated transiently as it passes through the cell (~1 ms). Three different light-activated assays have been investigated and applied to the RFQ instrument and these are described below.
3.2.4.3.1. Photo-induced reduction of cytochrome c in the presence of NADH and NMN

Cytochrome c (Cyt c) is a small water-soluble haem protein that is a highly efficient electron-transporter. Initial photo-reduction experiments involved using NADH as a photo-excitable electron donor to transfer electrons to the oxidised form of cytochrome c, often referred to as ferricytochrome c [75], with nicotinamide mononucleotide (NMN) included as a mediator. It has been shown previously that Cyt c can be reduced by NAD\(^{−}\) radicals and hydrated electrons that are generated upon excitation of NADH (figure 3.24)[75 and 76]. The reduction can occur directly or through mediation by a radical species of NMN [77].

\[
\text{NADH} \xrightarrow{hv} \text{e}^{−}(\text{aq}) + \text{NAD}^{+} + \text{H}^{+} \\
\text{e}^{−}(\text{aq}) \cdot \text{Fe}^{3+} \rightarrow \text{Fe}^{2+} \\
\text{NAD}^{+} + \text{Fe}^{3+} \rightarrow \text{NAD}^{+} + \text{Fe}^{2+}
\]

Figure 3.24 Photo-induced reduction of Cyt c in the presence of NADH. Excitation of NADH produces NAD\(^{−}\) radicals and solvated electrons and facilitates the reduction of ferric Cyt c to ferrocytochrome.
Prior to starting the experiment, the cytochrome c was treated with potassium ferricyanide (K₃Fe(CN)₆) to ensure that the protein was fully oxidised [78]. Samples of Cyt c by itself (2, 1, 0.5 mM) and its reduced form with sodium dithionite (DT) were used as references (figures 3.25). As expected the oxidised form of ferricytochrome c is low-spin ferric (S=1/2) while the reduced form (ferrocytochrome) is diamagnetic [79]. The oxidised form exhibits a strong signal with $g_z=3.06$, a second one with $g_y=2.24$ and a minor one with $g_x=1.24$. When Cyt c is chemically reduced all the EPR signals disappear because it becomes diamagnetic. The flash-test experiment was carried out anaerobically by mixing Cyt c (1 mM final concentration) against NADH (2 mM final concentration) and NMN (50 mM final concentration) and illuminating the mixed sample with an LED (ThorLabs) at $\lambda_{exc} = 365$ nm. Different ageing times and temperatures (4, 15 and 20°C) were used. As this test was aimed at investigating the flashing capabilities of the instrument and because the reduced form of Cyt c is stable [75], it was not performed as a freeze-quench experiment due to the inconsistencies observed with the packing process. Instead, all the solutions were sprayed directly into black Eppendorf tubes and then transferred into EPR tubes to be stored in liquid nitrogen and analysed.

![Figure 3.25](image-url) EPR spectra of oxidised and reduced Cyt c. a) CW-EPR spectrum of Cyt c at different concentrations (500 μM, 1 mM, 2 mM) were measured at 12 K, centre field 2600 G, modulation amplitude 5 G, sweep width 5000, number of points 2048, microwave power 25 dB. Oxidised ferricytochrome c is low-spin ferric (S=1/2), but at a concentration of 2 mM a high-spin signal for haem proteins was detected (at 1130 G $g=5.9$); b) CW-EPR spectrum of reduced Cyt c at different concentrations (500 μM, 1 mM, 2 mM) measured under the same conditions as in a). As expected the reduced form is diamagnetic.
The EPR spectra from this flash-test experiment are shown in figure 3.26 and were measured under a range of conditions: different loops (L) and flow-rates (FR). Although there are changes in the EPR signals, it is difficult to understand if the photo-reduction worked in the time range of interest. The variations of the intensities in the EPR signals are probably due to differences the heights of the samples inside the tubes. All the samples collected through the RFQ device exhibit the same signals and line shapes of the oxidised form (figure 3.26). From the comparison in figure 3.26d the reaction seems too slow to be used as a flash-test. The sample illuminated continuously for 5 minutes with an LED (365 nm) shows less intense signals compared to the not illuminated sample, but it confirms the inefficiency of the reaction (figure 3.26d).
Figure 3.26 CW-EPR spectra of Cyt c (1 mM final concentration) mixed against NADH/NMN (2 and 50 mM respectively) and illuminated with LED (ThorLabs) at $\lambda_{\text{exc}}=365$ nm measured at 12 K at different temperatures and ageing times: a) 4°C (loop 1, different flow-rates: 6 (11.7 ms), 5 (12.6 ms), 4 (14.0 ms), 2 (20.7 ms)); b) 15°C (loop 1 flow-rates: 6 (11.7 ms), 5 (12.6 ms), 4 (14.0 ms), 2 (20.7 ms), loop 2 flow-rate 2 (28.2 ms) and loop 3 flow-rates: 4 (43.2 ms), 3 (55.2 ms); c) 20°C (loop 1 flow-rates: 6 (11.7 ms), 5 (12.6 ms), 4 (14.0 ms), 2 (20.7 ms), loop 2 flow-rate 2 (28.2 ms) and loop 3 flow-rates: 4 (43.2 ms), 3 (55.2 ms)); d) Comparison of Cyt c (1 mM oxidised), Cyt c reduced with DT and Cyt c in the presence of NADH and NMN after 5 minutes of continuous illumination with an LED (365 nm) at room temperature.

A probable reason for the photochemical inefficiency is that the high concentrations of reagents used in the experiments have a very high absorbance and may prevent the light penetrating through the optical cell. In order to determine if this is the case the UV-vis spectra of samples under different illumination conditions were recorded (Cary Varian 50 spectrophotometer, figure 3.27). The oxidised form of Cyt c exhibits the characteristic Soret band at 400 nm for haem proteins, whereas the reduced form has an additional peak at 550 nm. This latter feature only starts to become visible after a few seconds of illumination (figure 3.27b) and hence it does not appear that it is possible to reduce the
Cyt c with short ms pulses of light. These data suggest that the quantum yield of this photoreduction is too low to be measured using the short illumination times available (ms) with the RFQ instrument and therefore, other systems were tested.

**Figure 3.27** UV-vis absorbance spectra to monitor the photoreduction of Cyt c upon illumination of NADH. a) Spectra of oxidised Cyt c (1 mM), Cyt c reduced with sodium dithionite and of samples collected in black Eppendorf after rapid mixing with NADH/NMN and illumination through the RFQ device (loop 1, different flow-rates: 6 (4.5 ms), 5 (5.4 ms), 4 (6.8 ms), 2 (13.5 ms)). There are not significant changes in the samples illuminated via LED (365 nm); b) Difference traces of Cyt c collected as in a) using the non-illuminated sample as the blank; c) Spectra of Cyt c (500 μM) in the presence of NADH/NMN after progressive illumination inside the UV-vis Cary 50 spectrophotometer by an LED (365 nm). The characteristic peak of the reduced form is visible (550 nm), but only after prolonged illumination; d) Difference traces of Cyt c collected as in c) using the non-illuminated sample as the blank.

### 3.2.4.3.2. 'Uncaging' of free Mn$^{2+}$ from DMNP-EDTA followed by binding to pyruvate kinase

The second system that was used as a flash-test involved the binding of manganese (Mn$^{2+}$) to pyruvate kinase. In order to temporally-control this process 1-(4,5-dimethoxy-2-
nitrophenyl)-1,2-diaminoethane-\(N,N,N',N'\)-tetraacetic acid (DMNP-EDTA) was used to cage Mn\(^{2+}\) and release it upon photolysis. DMNP-EDTA is a photolabile derivative of EDTA (a chelator) and it is usually employed to cage either calcium (Ca\(^{2+}\)) or magnesium (Mg\(^{2+}\)). Photolysis by UV light decreases its affinity for both cations and therefore, they become physiologically available in microseconds. By using this approach regulatory effects of calcium and magnesium on cellular processes have been investigated [5 and 80]. In particular, secretory processes in neuronal and non-neuronal cells have been extensively studied by the gradual photorelease of Ca\(^{2+}\) [43 and 44]. DMNP-EDTA is also useful for the photolytic release of other divalent cations and has been used here to cage Mn\(^{2+}\) (figure 3.28). Mn\(^{2+}\) is known to give characteristic EPR signals and consequently EPR spectroscopy has been used to provide information about the local environment of Mn\(^{2+}\)-enzyme complexes [81 and 82] and to study metal ion activation in certain enzymes [83 and 84]. In order to avoid any back-bonding with DMNP-EDTA after photolysis, the released Mn\(^{2+}\) was trapped by binding to pyruvate kinase. Pyruvate kinase catalyses the last step of glycolysis by transferring a phosphate group from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP), leading to the formation of pyruvate and one molecule of adenosine triphosphate (ATP), and as it requires a divalent cation for activation it has been studied via EPR in the presence of Mn\(^{2+}\) [83 and 84].

![Figure 3.28](image)

**Figure 3.28** Photo-cleavage of DMNP-EDTA yielding free Mn\(^{2+}\) and two iminodiacetic acid photoproducts.

Again, this test reaction was not performed as a freeze-quench experiment due to the inconsistencies observed with the packing process and hence, all the solutions have been mixed into black Eppendorf tubes prior to transferring into EPR tubes. Prior to carrying out the experiment through the RFQ device, some benchmark tests have been used to
monitor if any changes in the EPR spectra were detectable. For this purpose, samples of \( \text{Mn}^{2+} \) (in HEPES 20mM, pH 7) by itself (0.4 and 0.2 mM) and of DNMP-EDTA caged manganese have been collected manually (figure 3.29a and b). In both a) and b) it is possible to observe the spectrum for free manganese (black trace) which exhibits six signals. When it binds pyruvate kinase (250mg x mL) the EPR signals tend to broaden (blue trace). DMNP-EDTA caged manganese (red trace, 400 μM) shows a small “hump” at 2200 G and after 1 minute of continuous illumination with an LED (265 nm) part of the manganese is released (figure 3.29a green line). However, comparing the EPR spectra (figure 3.29) there are no appreciable changes between the caged manganese and manganese trapped with pyruvate kinase. Although there should be changes in the composition and geometric arrangement of ligands in the coordination sphere of the cation there is neither an evidence of significant variations in the position nor in the line shape of the EPR signals. This photochemical reaction was considered unsuitable for testing the flash part of the instrument due to the fact that the EPR spectra of the species of interest are too similar.

**Figure 3.29** CW-EPR spectra of caged, uncaged and enzyme bound Mn\(^{2+}\)measured at 20 K, centre field 3000 G, modulation amplitude 5 G, sweep width 5000, number of points 2048, microwave power 30 dB: a) Samples containing MnCl\(_2\) (200 μM) in HEPES (20 mM, pH=7) are shown; b) Samples containing MnCl\(_2\) (400 μM) in HEPES (20 mM, pH=7) are shown.
3.2.4.3.3. Photolysis of methylcobalamin

Methylcobalamin (MeCbl) is a vitamin B\textsubscript{12} derivative in which the cobalt is bound to a methyl group (figure 3.30). MeCbl is a physiologically relevant cofactor in various biological processes [60]. MeCbl-dependent enzyme catalysis involves the transfer of a methyl group from a methyl donor to a methyl acceptor by so-called methyltransferases, which are involved in important metabolic pathways in humans, animals and bacteria. Several substrates act as sources of methyl groups, such as methanol, aromatic methyl ethers, methyl amines or N5-methyltetrahydropterins (such as N5-methyltetrahydrofolate). Thiols are the methyl group acceptors in methanogenesis and in methionine synthesis. In addition to MeCbl playing a crucial role as a bioinorganic cofactor, it also displays complex photophysical and photochemical properties. These properties have been extensively studied for many corrinoids in order to address some mechanistic questions about B\textsubscript{12} enzymatic catalysis by various techniques, such as laser-flash [85 and 86], continuous wave photolysis [66 and 87], kinetic magnetic field effects (MFE) [67 and 88], chemically induced dynamic electron polarisation (CIDEP) [89], chemically induced dynamic nuclear polarisation (CIDNP) [90] and time-dependent density functional theory (TD-DFT) [91 to 93]. The photochemistry of the B\textsubscript{12} derivatives involves cobalt (III) complexes and the bond breaking between the cobalt ion and an axial ligand [94 and 95]. The results obtained for MeCbl show that its photolysis is wavelength-dependent [93 and 96]. Upon excitation at 400 nm, 25% of the initially excited MeCbl molecules undergo bond homolysis, forming a methyl radical and cob(II)alamin, while the remaining molecules form a metastable photoproduct, which has an absorption spectrum similar to that of a cob(III)-alamin species bound to a very weak axial ligand. Approximately 12% of the photoproduct then undergoes photolysis and the remaining molecules recover to the ground state. Excitation at 520-530 nm only yields the metastable intermediate, about 14% of which undergoes photolysis and ground state recovery [96 and 97]. In this work, MeCbl has been employed as a flash-test with the only aim to prove the applicability of the device on a suitable biological sample.
Figure 3.30 Structure of cobalamin. The structure consists of a corrin ring, containing four pyrrolic units, three methylene bridges and a central cobalt ion. In methylcobalamin the Co$^{3+}$ is bound to a methyl group.

Prior to carrying out the experiments with the RFQ device EPR spectra were recorded of MeCbl solutions (0.4, 1 and 2 mM in HEPES 20 mM pH 7) with no illumination. Samples were also illuminated with LEDs at 365 nm and 530 nm in order to establish the optimal experimental conditions (figures 3.31 and 3.32). Sample preparation and photolysis were carried out aerobically as oxygen significantly increases the rate of photolysis of alkylcobalamins compared to a nitrogen atmosphere. O$_2$ reacts rapidly with alkyl radicals but not with Co$^{2+}$ (about 20 minutes) and leads to an irreversible product formation (aquocobalamin). In the absence of oxygen an efficient regeneration of MeCbl is known to occur [94 and 97]. EPR spectra of all of these MeCbl samples show that it is diamagnetic prior to illumination and upon photolysis gives a sharp signal in the low-spin region at 2907 G with g=3.2 (Co$^{2+}$, S=1/2). It appears that illumination at $\lambda_{exc}$=365 nm is more efficient compared to $\lambda_{exc}$=530 nm as it yields more intense EPR signals [95 and 96]. Moreover, from these preliminary tests it also emerged that a concentration of 200 μM is not high enough to give a measurable EPR signal (figure 3.31d).
Figure 3.31 CW-EPR spectra of MeCbl measured at 12 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000 G, number of points 2048, microwave power 25 dB: a) 400 μM MeCbl before and after irradiation with LED at 365 nm; b) 1 mM MeCbl before and after irradiation with LED at 365 nm; c) 2 mM MeCbl after irradiation with LED at 365 nm; d) Comparison of different concentrations (0.2, 0.4, 1 and 2 mM) of MeCbl after 1 minute irradiation with LED at 365 nm.
Figure 3.32 CW-EPR spectra of MeCbl measured at 12 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000 G, number of points 2048, microwave power 25 dB: a) 400 μM MeCbl before and after irradiation with LED at 530 nm; b) 1 mM MeCbl before and after irradiation with LED at 530 nm; c) 2 mM MeCbl after irradiation with LED at 530 nm.

Based on these findings, the experiments via the RFQ device were carried out using 2 mM MeCbl in both driving syringes and upon mixing were irradiated in the cell with an LED at 365 nm using different ageing times. Some samples were collected directly into black Eppendorf tubes and then transferred into EPR tubes (figure 3.33a), whereas others were freeze-quenched onto the rotating plates and packed into EPR tubes as previously described (figure 3.33b). However, from the EPR spectra no signals could actually be detected. A plausible reason for the lack of an EPR signal could be that the high concentration of MeCbl used in the experiment may prevent light penetration through the optical cell. Another possibility is that just a small portion of MeCbl gets photolysed and in the presence of oxygen as in a) forms a diamagnetic cobalt(III) species, while in the absence of oxygen as in b) regenerates MeCbl.
Figure 3.33 CW-EPR spectra of MeCbl measured at 12 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000 G, number of points 2048, microwave power 25 dB: a) 2 mM MeCbl was irradiated via LED at 365 nm in the quartz cell of the RFQ instrument and sprayed into black Eppendorf tubes; b) 2 mM MeCbl was irradiated via LED at 365 nm in the quartz cell of the RFQ instrument, frozen onto the rotating plates and packed into EPR tubes.

In order to determine the suitability of the system the UV-vis absorbance spectra were recorded (Cary Varian 50 spectrophotometer) under different illumination conditions. MeCbl samples were mixed through the optical cell of the RFQ device and illuminated with an LED at 365 nm prior to recording the absorbance spectra. Small changes in the absorbance spectra prior to and after illumination in the RFQ are visible (figure 3.34). MeCbl exhibits a peak at 530 nm, whereas after 5 seconds of irradiation with an LED (365 nm) inside the optical cell of the RFQ apparatus MeCbl forms cob(II)alamin with a signal at 420 nm. However, the differences between non-illuminated MeCbl and MeCbl irradiated in the ms range are not conclusive enough to prove that the principle behind the flash part of the instrument works.
Figure 3.34 UV-vis absorbance spectra of 320 μM MeCbl: a) MeCbl before (black trace) and after illumination inside the optical cell of the RFQ device (LED 365 nm, loop 1, ms, red and blue traces) and after 5 seconds of illumination inside the optical cell of the RFQ device (LED 365 nm, magenta trace); b) MeCbl before illumination (black trace) and difference spectrum of MeCbl after illumination inside the optical cell of the RFQ device (LED 365 nm, loop 1, ms, blue trace) and after 5 seconds of illumination inside the optical cell of the RFQ device (LED 365 nm, magenta trace).

3.3. Summary and conclusion

In this chapter a novel RFQ device combined with flashing capabilities has been described, which should allow studies of both light-activated and thermally-activated biological reactions. To the best of our knowledge this is the first report of an instrument that is capable of combining photoexcitation with rapid mixing/ freezing approaches for analysis by EPR spectroscopy. Other RFQ instruments have been described in the literature. One of these devices consists of two oxygen-free copper wheels arranged in a side-by-side fashion and temperature-equilibrated in liquid nitrogen at 77 K. The driving wheel is attached to a motor and drives the other one through frictional contact. As a result, the two wheels rotate in opposite directions but at the same speed. The mixed solution instantaneously freezes on the surface of the wheels and is crushed into a powder by the friction of the rotating wheels. The frozen powder is then collected into an EPR tube through a collecting funnel that is placed in the liquid nitrogen directly below the interface of the wheels [12 and 15]. In another design, the rotating wheels were replaced by a rotating plate that is in contact with liquid nitrogen. The plate, made of aluminium for its high thermal conductivity (150 W/(mK)) and characterised by a conical shape, was cooled to 77 K by immersing the bottom into a liquid nitrogen bath. In this apparatus, in order to reduce the freezing time, the plate rotates around its centre at a speed.
correlated to the flow speed of the solution in the microfluidic channel (about 0.05 rad/s for a 500 mm/s flow) [21]. The device presented in this chapter employs a novel rotating system and incorporates the flashing capability. In addition, the freezing-time of 5.2 ± 3.8 ms obtained with this device is in perfect agreement with the times of the RFQ devices described in the literature which show values between 5 and 7 ms [19 to 21].

However, some difficulties have arisen during the development of the instrument, leading to several changes in the apparatus design. In particular, these have included altering the rotating plate that is used to freeze the sample and improvements in packing the frozen samples into the EPR tubes. However, despite these modifications, it should still be possible to improve the instrument further. For instance, in order to avoid the rotating plate from becoming unbalanced, the polyurethane Dewar could be replaced with one made of stainless steel in order to have fixed holes in which the gas nitrogen lines can be put. In this case, their alignment would not need any adjustment before and during the spraying. Another issue common to all the RFQ instruments is the amount of sample required for running an experiment. The substantial improvement in the packing strategy combining the collection of frozen particles into an EPR tube by pumping through a filter placed at the bottom of the tube and the use of packing rods guarantees a reduction of the amount of reactants and a more reproducible packing. However, when a sample is less abundant, it may be beneficial to couple the RFQ to high field EPR, which requires much less sample. Both high field EPR and ENDOR have better spectral resolution than X-band, although it is likely that high field RFQ-EPR would be very challenging due to difficulty in handling the small capillaries that are used [20]. Previous developments in sample preparation have allowed the combination of the RFQ technique with a wider selection of spectroscopic techniques. These have included resonance Raman [98], magnetic circular dichroism [99], Mossbauer spectroscopy [100] and X-ray absorption spectroscopy which can allow the identification of EPR silent species [101]. It is possible that the RFQ device described here could also be modified in the future to allow the application of similar techniques.

In conclusion, a novel RFQ apparatus capable of producing EPR spectra of known test reactions with a reasonable signal-to-noise ratio has been developed and characterised. The instrument has the potential to study short-lived radical species that are formed in
other enzyme systems and will be utilised in subsequent chapters to study the coenzyme B_{12}-dependent enzyme ethanolamine ammonia lyase.
4. Application of the rapid freeze quench technique to the analysis of ethanolamine ammonia lyase (EAL)

4.1. Introduction

Ethanolamine ammonia lyase (EAL) has been an important model system to understand the catalytic mechanism of coenzyme B$_{12}$–dependent enzymes in general and it can be easily produced in *E. coli*. Several techniques have been used to investigate the relationship between catalysis and enzyme–substrate complex geometry and the properties of the active site, such as UV-vis spectroscopy [63], X-ray crystallography [64], kinetic isotope effect measurements [65], magnetic field continuous wave photolysis and EPR spectroscopy [66,67]. Despite all of these studies a full understanding of the catalytic mechanism and the contribution of the protein during catalysis has not been clarified yet [68]. The reaction is dependent on coenzyme B$_{12}$ (or 5′-deoxyadenosylcobalamin, AdoCbl, figure 4.1) and involves highly reactive radical intermediates (figure 4.2).

![Figure 4.1](image)

**Figure 4.1** Structure of cobalamin. The structure consists of a corrin ring, containing four pyrrolic units, three methylene bridges and a central cobalt ion. In 5′-deoxyadenosylcobalamin the Co$^{3+}$ is bound to an adenosyl group.
In the course of the reaction, AdoCbl is believed to be a free radical reservoir. Homolytic cleavage of the Co-carbon bond to the Ado group leads to a pair of paramagnetic species, a low-spin Co$^{2+}$ bound to the corrin ring of the cofactor fragment and a 5’-deoxyadenosylcobalamin radical. The latter intermediate promotes the abstraction of a hydrogen atom from the substrate [102]. Recent ultrafast photolysis studies have provided experimental evidence for the existence of the 5’-deoxyadenosylcobalamin radical in the active sites of the related enzymes glutamate mutase (GM) [103] and EAL [104]. Computational methods have provided further evidence for the existence of this radical by calculating free energy profiles of the species involved in the catalytic cycles [102]. However, information about the Ado radical lifetime, its interaction with the enzyme and the detailed role of the protein during Co-C homolysis are still missing. Hence, the application of the RFQ device described in Chapter 3 to study the EAL reaction in the presence of different substrates by EPR spectroscopy could be a good approach to address these questions. In this chapter initial RFQ studies on EAL to try to trap the adenosyl or substrate/product radical are described, together with supporting biochemistry and kinetic data.

Figure 4.2 General mechanism of AdoCbl-dependent isomerases. Substrate binding initiates homolysis and Ado radical generates substrate radical by H-abstraction.
4.2. Results and discussion

4.2.1. Ethanolamine ammonia lyase (EAL) expression and purification

In the current work, the EAL samples employed for analysis were overexpressed and purified using the protocol described in the Materials and Methods section. The plasmid pET sEAL, containing genes encoding both the 31 kDa β and 50 kDa α subunits, was transformed and the protein expressed in *E. coli* competent cells. To determine the purity of the final EAL sample SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of all retained samples was used and confirmed production of the α and β subunits (figure 4.3). The concentration of pure EAL (25 μM) was checked spectrophotometrically via a Cary 50 spectrophotometer upon binding to AdoCbl (figure 4.4).

![Figure 4.3 SDS-PAGE gel of EAL purification fractions. S4 is the final pure sample containing both α and β subunits. In the current figure S stands for supernatant, P stands for pellet and M for marker.](image-url)
To determine the activity of the purified EAL enzyme a well-established steady-state assay was performed. EAL bound to AdoCbl catalyses the conversion of 2-aminoethanol (2-AE) to acetaldehyde and ammonia. This reaction is coupled to yeast alcohol dehydrogenase (YADH), an enzyme that employs NADH as an electron donor to convert acetaldehyde to ethanol forming NAD$^+$ as a by-product (figure 4.5). Therefore, EAL forms the substrate for the reaction catalysed by YADH.

**Figure 4.5** EAL catalyses the conversion of 2-aminoethanol to acetaldehyde which is the substrate for the reaction catalysed by YADH. The final products are ethanol and NAD$^+$.

Since NADH gives a strong absorption signal in the UV-vis at 340 nm, its conversion to NAD$^+$ during catalysis can be followed spectroscopically. Hence, the absorbance decrease at 340 nm is directly related to the EAL rate of reaction. Consequently, the change in
absorbance at 340 nm was monitored (as described in the Materials and Methods section) using a Cary 50 spectrophotometer (figure 4.6). The decrease in absorbance at 340 nm was fitted with a linear fit to obtain an initial rate of reaction. Based on the assumption that a saturating concentration of ethanolamine was used in the assay this was used to estimate a \( k_{\text{cat}} = 25.6 \pm 0.08 \, \text{s}^{-1} \), which is similar to the published value [106].

![Figure 4.6](image.png)

**Figure 4.6** Kinetic trace of EAL (100 nM) steady-state assay monitoring NADH (150 nM) consumption at 340 nm in the presence of ethanolamine (1 mM) and YADH (100 units).

### 4.2.2. Rapid mixing experiments using 2-aminoethanol and (S)-2-aminopropanol as substrates

Initially, stopped-flow experiments have been carried out to confirm the timescales over which the reaction chemistry occurs and to define the best conditions to carry out the RFQ experiments. Therefore, the kinetic traces have not been studied quantitatively as detailed analyses of the stopped-flow data has been carried out previously [105]. RFQ experiments were performed by using the device described in Chapter 3 coupled to EPR analysis. The EAL-B$_{12}$ catalytic cycle involves highly reactive radicals: a low-spin Co$^{2+}$ bound to the corrin ring of the cofactor fragment and a 5'-deoxyadenosylcobalamin radical. In the current work, 2-aminoethanol (2-AE) and (S)-2-aminopropanol (2-AP) were used as substrates in the RFQ tests. 2-aminoethanol and (S)-2-aminopropanol were chosen because the former is the natural substrate for EAL, whereas the latter is a slower substrate and may provide a better opportunity to observe any short-live radical species.
Prior to running the RFQ experiments, EAL was concentrated down in order to reduce the signal-to-noise ratio while doing EPR measurements.

4.2.2.1. Stopped-flow of EAL with 2-aminoethanol

As explained previously, EAL catalyses deamination of vicinal amino alcohols by promoting the formation of a double bond [6 and 63]. When the substrate is 2-aminoethanol (2-AE), the reaction gives as final products acetaldehyde and ammonia (figure 4.7) and the single turnover kinetics can be measured spectroscopically by following the disappearance of the Co$^{3+}$ signal at 525 nm.

![Figure 4.7 Schematic representation of the EAL reaction mechanism. Substrate binding initiates homolysis and the Ado radical promotes the formation of the substrate radical by H-abstraction, followed by rearrangement to the product radical, which then dissociates to acetaldehyde and ammonia.](image)

EAL (holoenzyme) was mixed with its natural substrate 2-AE in the stopped-flow (Applied Photophysics) to yield typical kinetic transients shown in figure 4.8 [67 and 105]. The data shows the conversion of cob(III)alamin to cob(II)alamin over a few milliseconds. Stopped-flow experiments conducted at room temperature have previously shown that the majority of the signal disappears within the instrument dead time [67]. However, the obtained transient of EAL (15 μM) with its natural substrate 2-AE (2.5 mM) at 5°C shows that cob(II)alamin formation is slowed down sufficiently at lower temperatures to be studied by rapid mixing techniques (figure 4.8). The data were consistent in all repetitions.
and in agreement with the literature, which suggested that first-order kinetics for Co-C bond homolysis is an accurate way to describe this system [67 and 105].

![Graph](image)

**Figure 4.8** Stopped-flow transients of EAL holoenzyme (15 μM) mixed against 2-AE (2.5 mM) recorded at 525 nm at 5°C. Data are the average of three kinetic transients. The data were fitted in Origin with the following equation: $y = A_1 \exp(-x/t_1) + y_0$.

### 4.2.2.2. Stopped-flow of EAL with (S)-2-aminopropanol as a substrate

Aminopropanol (2-AP) exists as two enantiomers which are both possible substrates for EAL. It was previously demonstrated that the $S$ enantiomer has a higher $V_{\text{max}}$ and a lower $K_M$ than the $R$ one, proving that the former is the preferable substrate [107]. From isotope effects studies on (R)-2-aminopropanol, it emerged that there are two rate-limiting steps, which are the transfer of hydrogen from the substrate to the cofactor and the migration of the amino group respectively [107]. On the contrary, for (S)-2-aminopropanol it appeared that there is only one rate-limiting step, which is the same for 2-aminoethanol, namely the transfer of hydrogen from the cofactor to the product [107]. In this work, only experiments involving (S)-2-aminopropanol were performed. The reaction of EAL (holoenzyme) in the presence of (S)-2-aminopropanol leads to propionaldehyde and ammonia (figure 4.9).
Figure 4.9 Schematic representation of the EAL reaction mechanism for (S)-2-aminopropanol. Substrate binding initiates homolysis and the Ado radical promotes the formation of the substrate radical by H-abstraction followed by rearrangement to the product radical, which then dissociates to propionaldehyde and ammonia.

Stopped-flow experiments of EAL mixed against (S)-2-aminopropanol confirms that this is a slower substrate compared to 2-AE (figure 4.10) [67]. The data show that the conversion of cob(III)alamin to cob(II)alamin occurs over approximately a second at 5°C. As previously reported for the transient of EAL (holoenzyme) against 2-AE, the kinetic trace obtained with aminopropanol suggests a more complex kinetic mechanism, which is similar to the data obtained for Ado-Cbl glutamate mutase [103]. These previous studies have proven that C-Co bond homolysis is kinetically coupled to subsequent H-abstraction from the substrate, and that this step is reversible causing the multiphasic nature of the transients [103].
Figure 4.10 Stopped-flow transients of EAL holoenzyme (15 μM) mixed against 2AP a) 0.5 mM, b) 1 mM and c) 2.5 mM) recorded at 525 nm at 5°C. The data are the average of three kinetic transients. The data were fitted in Origin with the following equation:

\[ y = A_1 \exp(-x/t_1) + A_2 \exp(-x/t_2) + y_0. \]

4.2.3. RFQ studies coupled to EPR analysis of EAL against 2-aminoethanol and (S)-2-aminopropanol

The RFQ experiments coupled to EPR analysis of EAL-B_{12} against its substrates have proven that it is possible to trap radical intermediates with the device described in chapter 3. The samples were collected via the RFQ device by mixing EAL (holoenzyme) 46 μM with its substrates in large excess (10 mM). The frozen samples were analysed by CW-EPR (Bruker) at 20 K using the following parameters: centre field 2600 G, modulation amplitude 10 G, sweep width 5000 G, number of points 2048, microwave power 30 dB. These parameters were chosen because they allow the measurement of spectra with a good signal-to-noise ratio. For both substrates RFQ measurements were carried out using loop 1 with the following flow-rates: flow-rate 7, 11.1 ms; flow-rate 4, 14.0 ms; flow-rate 2, 20.7 ms. For the experiments of EAL mixed with 2-AP other time-points were also collected: loop 2, flow-rate 2, 28.2 ms and loop 3 flow-rate 3, 55.2 ms. However,
difficulties have arisen in obtaining consistent data sets with significant differences observed in the EPR spectra of the various samples (figures 4.11a and b, figure 4.12a and b). Usually, if the intensity of the EPR signal varies between two/three different samples, it could be accounted for various amounts of sample in the EPR quartz tubes, but in this case the EPR signal changes completely. This issue is mostly due to the instrument design which had to be implemented (see Chapter 3) and affects the cooling rate. Therefore, it is hard to understand which EPR features appear first during the reaction between EAL-B₁₂ against aminoethanol or 2-aminopropanol. In addition, both substrates showed a similar behaviour even if they have different rates of reaction (figure 4.11).

Figure 4.11 CW-EPR spectra of EAL-B₁₂ (46 μM) measured at 20 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000 G, number of points 2048, microwave power 30 dB: a) EAL-B₁₂ by itself (black trace), EAL-B₁₂ against 2-aminoethanol (10 mM, red trace, loop 1, flow-rate 7, 11.1 ms), EAL-B₁₂ against 2-aminoethanol (blue trace, loop 1, flow-rate 4, 14.0 ms), EAL-B₁₂ against 2-aminoethanol (pink trace, loop 1, flow-rate 4, 14.0 ms), EAL-B₁₂ against 2-aminoethanol (green trace, loop 1, flow-rate 2, 20.7 ms); b) EAL-B₁₂ by itself (black trace), EAL-B₁₂ against 2-aminopropanol (10 mM, red trace, loop 1, flow-rate 7, 11.1 ms), EAL-B₁₂ against 2-aminopropanol (red trace, loop 1, flow-rate 2, 20.7 ms).

By looking at the EPR characteristic signals in the magnetic field range of 2500 G - 4500 G, two different paramagnetic signals are observed. The EPR signal around 2850 G has been already reported in the literature and is due to the Co²⁺ spin, whereas the doublet structure between 3200-3600 G is due to the radical-signal splitting as a consequence of the spin-spin exchange coupling (figures: 4.11a and b, 4.12a and b) [108,109,110]. The signal around 2850 G is more consistent in the data obtained by mixing of EAL (holoenzyme) with 2-AE, whereas the doublet structure between 3200-3600 G is more
visible in the spectra obtained by mixing of EAL (holoenzyme) with 2AP. With both substrates, the EPR line shapes suggest that the multiplet structure observed arises from the formation of adenosyl radical and Co\(^{2+}\), and then this species decays into the doublet structure due to Co\(^{2+}\) and substrate radical spin-spin interaction (figures: 4.13a and b, 4.14a, b, c, d).

Figure 4.12 CW-EPR spectra of EAL-B\(_{12}\) (46 µM) measured at 20 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000 G, number of points 2048, microwave power 30 dB: a) EAL-B\(_{12}\) against 2-aminopropanol (10 mM, loop 2, flow-rate 2, 28.2 ms). Black and green traces show the reported doublet due to Co\(^{2+}\) and substrate radical spin-spin interaction, whereas the red trace shows a multiplet structure; b) offset data set shown in a).
Figure 4.13 CW-EPR spectra of EAL-B_{12} (46 μM) measured at 20 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000 G, number of points 2048, microwave power 30 dB: a) EAL-B_{12} against 2-aminoethanol multiplet structure (10 mM, black trace, loop 1, flow-rate 7, 11.1 ms), EAL-B_{12} against 2-aminoethanol doublet structure (black trace, loop 1, flow-rate 7, 11.1 ms); b) EAL-B_{12} against 2-aminoethanol multiplet structure (10 mM, black trace, loop 1, flow-rate 7, 11.1 ms), EAL-B_{12} against 2-aminoethanol doublet structure (black trace, loop 1, flow-rate 7, 11.1 ms). The double headed arrows show the decay and conversion from one paramagnetic species to Co$^{2+}$ and substrate radical spin-spin interaction; c) EAL-B_{12} against 2-aminopropanol multiplet structure (10 mM, red trace, loop 1, flow-rate 2, 20.7 ms), EAL-B_{12} against 2-aminopropanol doublet structure (black trace, loop 1, flow-rate 2, 20.7 ms); d) EAL-B_{12} against 2-aminopropanol multiplet structure (10 mM, black trace, loop 1, flow-rate 2, 20.7 ms), EAL-B_{12} against 2-aminopropanol doublet structure (black trace, loop 1, flow-rate 2, 20.7 ms). The double headed arrows show the decay and conversion from one paramagnetic species to Co$^{2+}$ and substrate radical spin-spin interaction.

In figure 4.14, the super-hyperfine structure (leading to six lines in the range between 3000-3600 G) observed in the red trace indicates that it arise from a paramagnetic species, whereas in the black spectrum it has completely decayed and converted into the Co$^{2+}$ and substrate radical spin-spin. Residuals of this feature are however, still present (see double headed arrows) in the black trace.
Figure 4.14 CW-EPR spectra of EAL-B_{12} (46 μM) measured at 20 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000 G, number of points 2048, microwave power 30 dB: a) EAL-B_{12} against 2-aminoethanol (10 mM, loop 1, flow-rate 4, 6.8 ms); b) EAL-B_{12} against 2-aminoethanol (10 mM, loop 1, flow-rate 4, 14.0 ms), same data sets as in a); c) EAL-B_{12} against 2-aminopropanol (10 mM, loop 1, flow-rate 4, 14.0 ms); d) EAL-B_{12} against 2-aminopropanol (10 mM, loop 1, flow-rate 4, 14.0 ms), same data sets as in c). In both cases, residual signals from the formation of adenosyl radical and Co^{2+} are present in all the traces.

The multiplet structure observed suggests the formation of a radical not reported in the literature, which might be the adenosyl radical interacting with Co^{2+}, but it shows mainly 6-lines indicated by the numbers from 1-6, rather than 8 corresponding to Co^{2+} ion (I = 7/2), approximately separated by 90-95 G (figure 4.15). The deviation from 8 line patterns normally observed for the Co^{2+} ion might be caused by strong spin-spin interaction of the Co^{2+} with the adenosyl radical. However, additional studies are needed to identify the origin of the super-hyperfine coupling and the complex EPR spectrum observed in figure 4.15.
Figure 4.15 CW-EPR spectra of EAL-B_{12} (46 μM) measured at 20 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000 G, number of points 2048, microwave power 30 dB: EAL-B_{12} against 2-aminopropanol (loop 3, flow-rate 3, 55.2 ms).

4.3. Summary and conclusion

In recent years, the RFQ technology coupled to EPR or other spectroscopy techniques has been widely employed for studying enzyme reactions [13, 17, 18, 19, 20, 21]. In the current work, the RFQ device described in Chapter 3 was applied to the EAL system in order to trap and identify radical intermediates in this B_{12}-dependent reaction. Particular interest has previously focussed on the 5'-deoxyadenosylcobalamin radical lifetime, its interaction with the enzyme and the detailed role of the protein during Co-C homolysis, features which have not been clarified yet. As previously described, the EAL-B_{12} catalytic cycle involves highly reactive radicals, involving a low-spin Co^{2+} bound to the corrin ring of the cofactor and a 5'-deoxyadenosyl radical. The latter intermediate promotes the abstraction of a hydrogen atom from the substrate [102]. Some EPR experiments on EAL-B_{12} have already been conducted at low temperatures in order to characterise the kinetics and thermodynamics of Co^{2+}-substrate radical pair formation [108 and 109]. In the current work, 2-AE and 2-AP were used as substrates in the RFQ tests.

2-AE and (S)-2-AP were chosen because the former is the natural substrate for EAL, whereas the latter is a slower substrate.
Unfortunately, the RFQ experiments did not produce consistent sets of data. The main reason for this is due to the device itself, which needs some improvements as fully described in Chapter 3 (3.3 Summary and conclusion). However, it has been possible to detect a multiplet structure in the EPR spectra of the EAL reaction with AP, which might be due to the formation of the adenosyl radical and Co$^{2+}$. The signal shows mainly 6-lines rather than 8 corresponding to Co$^{2+}$ ion ($I = 7/2$), approximately separated by 90-95 G. The deviation from the 8 line patterns for the Co$^{2+}$ ion might be caused by the strong spin-spin interaction of Co$^{2+}$ and adenosyl radical, but further studies are needed to identify the origin of the super-hyperfine coupling and the complex EPR spectrum observed in figure 4.15. The EPR signal around 2850 G has been already reported in the literature and is due to the Co$^{2+}$ spin, whereas the doublet structure between 3200-3600 G is due to the radical-signal splitting as a consequence of the spin-spin exchange coupling [108 to 110].

A critical parameter during trapping is the freezing time which allows the accumulation of the paramagnetic species. When the freezing time is faster than the rate of substrate radical reaction, the radical intermediate is trapped. On the contrary, when the freezing time and the reaction rate are similar part of the Co$^{2+}$-substrate radical pair will keep reacting. In the case of EAL, the reaction evolves to an EPR-silent diamagnetic species. To avoid the loss of radical intermediate during trapping the reaction has to be slowed down, thus the experiments were carried out at 5°C. Other research groups have studied the EAL system at cryogenic temperatures in order to cryo-trap the 5’-deoxyadenosyl radical [108 to 110]. A recent strategy involved the use of a 41% v/v DMSO/water cryo-solvent to measure EAL at low-temperatures by granting the following criteria: a) good mixing of EAL-B$_{12}$ with substrates under conditions of kinetic arrest of the reaction; b) continuous acquisition of EPR spectra over the course of the reaction [108]. It was proven that the enzyme functional and structural properties were maintained in this cryo-solvent [108]. Other approaches have used rapid freeze-quench devices with isopentane and isotopically labelled forms of the substrates [109 and 110]. Despite using different approaches the 5’-deoxyadenosyl radical has not been isolated yet. However, well-resolved EPR spectra of the substrate-radical have been obtained. Hence, the work described in this chapter may have provided the first direct experimental evidence for the
existence of the adenosyl radical during the course of the EAL reaction. However, the further RFQ experiments will be required to conclusively identify the adenosyl radical and to measure the rate of its formation / decay.

In the future it may be useful to also apply the RFQ-EPR approach to other two EAL substrates, $^2$H$_4$-2-AE and ethylene glycol. The reaction of EAL (holoenzyme) with $^2$H$_4$-2-AE has been extensively studied to investigate the Co-C bond breaking and H-transfer steps during catalysis [111]. When one of the atoms in the reactants is replaced by one of its isotopes, the kinetic isotope effect (KIE) can be examined. Stopped-flow investigations at 5°C of EAL-B$_{12}$ against $^2$H$_4$-2-AE revealed that, although homolysis was expected to be a first-order kinetic process, the transient has a multiphasic nature [67]. Ethylene glycol is a di-hydroxyalcohol which is considered a quasi-substrate for EAL [112]. The reaction mechanism of EAL (holoenzyme) in the presence of ethylene glycol is still under investigation. It has been shown that this reaction leads to the cleavage of the Co-C bond and then the adenosyl radical abstracts a hydrogen from the substrate. The enzyme forms a new corrinoid which coordinates a different upper axial ligand to the Co than the usual 5'-deoxyadenosyl residue. After the reaction is over, the new corrinoid is gradually converted to hydroxocobalamin. As a result, the enzyme activity is considerably reduced [112]. It would be interesting to run experiments with these substrates by using the RFQ device to trap any intermediate species in the reaction.

As the RFQ analysis of the EAL reaction did not provide fully conclusive results, a caging strategy applied to the ethanolamine substrate as a tool for triggering EAL catalysis by light will be described. This approach has the potential to elucidate some catalytic steps and is described in the following chapter.
5. The use of ‘caged’ ethanolamine to probe the mechanism of ethanolamine ammonia lyase (EAL)

5.1. Introduction

EAL has become an important model system to understand the catalytic mechanism of coenzyme B$_{12}$–dependent enzymes in general. Several techniques have been applied to study the relationship between catalysis and enzyme–substrate complex geometry, and the properties of the active site [6, 63 and 64]. EPR studies, in conjunction with UV-vis spectroscopy, have been used to follow the oxidation state of the cobalt during the reaction and to propose a catalytic mechanism for EAL. Stopped-flow measurements of EAL in the presence of different substrates were performed along with kinetic isotope effect (KIE) studies and showed that Co-C homolysis is coupled to subsequent hydrogen abstraction from the substrate [65]. Magnetic field effect (MFE) continuous wave photolysis of free EAL and EAL bound to AdoCbl revealed that the AdoCbl-binding site acts as a cage favouring geminate recombination [66 and 67]. In recent years, ultrafast photolysis studies have provided experimental evidence for the existence of the 5’-deoxyadenosylcobalamin radical in the active sites of the related enzyme glutamate mutase (GM) [103] and EAL [104]. Computational methods have provided further evidence for the existence of this radical by calculating free energy profiles of the species involved in the catalytic cycles [102]. However, key information about the Ado radical lifetime, its interaction with the enzyme and the detailed role of the protein during Co-C homolysis are still missing. In Chapter 4, rapid freeze-quench experiments followed by EPR analysis were performed in an attempt to address these questions. However, further studies are necessary and hence, in the present chapter a caging strategy has been applied to the ethanolamine substrate as a tool for triggering EAL catalysis by light. The caging technique is based on the idea that a biologically active substance can be rendered temporarily inactive (or “caged”) by chemical modification with a photolabile protecting group [3, 5, 27 and 28]. A caged compound is the inert precursor of an active molecule and the original bioactivity is restored upon photolysis [29 and 30]. The caging strategy and the subsequent uncaging present several advantages. For instance, uncaging can be intracellular, rapid, controlled in a spatiotemporal manner, and quantitatively controlled. Release only occurs where light is incident and this is a relevant feature of this
technology. In addition, only light is required to cleave the photo-removable protecting groups. Reactants are not necessary and that aspect allows handling of very sensitive molecules, which are incompatible (e.g. with acids or bases). As photo-release is independent of the normal biological source, the timing of uncaging is strictly dictated by irradiation. Hence, the caging strategy gives a precise ‘time zero’ and provides an opportunity to initiate a biological response with high temporal resolution.

The most widely used caging groups are 2-nitrobenzyl or 2-nitrophenyl derivatives due to their relative robustness and their commercial availability. These groups are connected to the biologically active molecule by bonding to a hetero-atom (usually O, S or N) as an ether, thioether, ester (including phosphate or thiophosphate esters), amine or similar functional group. Usually, caged compounds are made via organic synthesis and direct caging. Both the structure of the nitrobenzylic/phenylic compound and the atom to which it is linked affect the efficiency and wavelength required for photo-removal [1, 27 and 28].

In the current work, 2-nitrophenylpropylchloroformate (NPPOC-Cl) was used to cage ethanolamine. In this chapter, the quantum efficiency of uncaging and steady-state activity of EAL under different illumination conditions are also described. This has allowed subsequent time-resolved and cryogenic absorbance measurements, as well as preliminary EPR experiments to be carried out, with the aim of developing a strategy for the identification and characterisation of reaction intermediates in the EAL-B_{12} catalytic cycle.

5.2. Results and discussion

5.2.1. Synthesis of caged ethanolamine and caged deuterated ethanolamine

The caging strategy applied to ethanolamine and its deuterated form involved the use of 2-nitrophenylpropylchloroformate (NPPOC-Cl) as a photo-removable protecting group. In recent years, NPPOC-Cl has proven to be an efficient protecting group for amino acids and amines. It was demonstrated that the light-activated uncaging of NPPOC-caged amino derivatives occurs at a rate at least twice as fast compared to other protecting groups [113]. In the current work, NPPOC-Cl was employed to protect the amino portion of the EAL substrates by using a protocol fully described in the Materials and Methods (section
2.4.1. The caged substrates were made via organic synthesis and direct caging according to the reaction scheme shown in figure 5.1.

![Figure 5.1](image-url) 

**Figure 5.1** Organic synthesis of caged 2-aminoethanol: 1) Nucleophilic addition to the carbonyl group; 2) Proton transfer between nitrogen and oxygen; 3) Chloride ion elimination; 4) Proton loss from oxygen and final product formation.

The final product was characterised by proton nuclear magnetic resonance (¹H-NMR) spectroscopy after purification via a silica column (figures 5.2 and 5.3). The proton NMR spectrum of NPPOC-caged-2-AE (Bruker 400 MHz, CDCl₃) is reported in figure 5.2 and its interpretation is described in table 5.1.

A similar approach was used to cage the selectively deuterated form of ethanolamine and the subsequent proton NMR spectrum of NPPOC-caged-²H₄-AE (Bruker 400MHz, CDCl₃) is reported in figure 5.3 and its interpretation is described in table 5.2.
Figure 5.2 $^1$H-NMR spectrum of NPPOC-caged-2-AE in deuterated chloroform processed with Mestrenova. The product analysed was obtained after purification via a silica column using ethyl acetate and hexane (1:1).
Table 5.1 NMR peaks assignment of caged-2-AE.
Figure 5.3 $^1$H-NMR spectrum of NPPO-caged-$^2$H$_4$-AE in deuterated chloroform processed with Mestrenova. The product analysed was obtained after purification via a silica column using ethyl acetate and hexane (1:1).
Table 5.2 NMR peaks assignment of deuterated caged-$^2$H$_4$-AE.

<table>
<thead>
<tr>
<th>Position</th>
<th>Type</th>
<th>Coupling constant (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>1.27 ppm</td>
<td>doublet</td>
</tr>
<tr>
<td>b</td>
<td>7.4 ppm</td>
<td>doublet 7 Hz</td>
</tr>
<tr>
<td>c</td>
<td>7.3 ppm</td>
<td>triplet 7 Hz</td>
</tr>
<tr>
<td>d</td>
<td>7.5 ppm</td>
<td>triplet 7 Hz</td>
</tr>
<tr>
<td>e</td>
<td>7.6 ppm</td>
<td>doublet 7 Hz</td>
</tr>
<tr>
<td>f</td>
<td>3.6 ppm</td>
<td>multiplet</td>
</tr>
<tr>
<td>g</td>
<td>4.0 ppm</td>
<td>triplet</td>
</tr>
<tr>
<td></td>
<td>4.04 ppm</td>
<td>quartet</td>
</tr>
<tr>
<td>NH</td>
<td>4.9 ppm</td>
<td>broad singlet</td>
</tr>
<tr>
<td>CDCl$_3$</td>
<td>7.2 ppm</td>
<td>singlet</td>
</tr>
<tr>
<td>AcOEt(CH$_2$CH$_3$)</td>
<td>1.2 ppm</td>
<td>triplet</td>
</tr>
<tr>
<td>AcOEt (CH$_3$CO)</td>
<td>2.0 ppm</td>
<td>singlet</td>
</tr>
<tr>
<td>AcOEt (CH$_2$CH$_3$)</td>
<td>4.2 ppm</td>
<td>quartet</td>
</tr>
</tbody>
</table>

The NMR analysis confirmed that both syntheses were successful and the desired caged compounds were obtained with a yield = 80-84% after purification. One of the challenges in handling caged compounds is their solubility in the targeted media (usually water and aqueous buffers). Solubility tests were carried out and 5% ethanol (v/v) in water was found to be a suitable medium to dissolve the desired compound and was also
compatible with EAL. In order to confirm that the NOPPC-caged-2-AE was not active as a substrate for EAL, steady-state assays were conducted as described in Chapter 4. EAL bound to AdoCbl catalyses the conversion of 2-aminoethanol (2-AE) to acetaldehyde and ammonia. This reaction is coupled to yeast alcohol dehydrogenase (YADH), an enzyme that employs NADH as an electron donor to convert acetaldehyde to ethanol forming NAD$^+$ as a by-product (Chapter 4, figure 4.5). Therefore, EAL forms the substrate for the reaction catalysed by YADH. Since NADH gives a strong absorption signal in the UV-vis at 340 nm, its conversion to NAD$^+$ during catalysis can be followed spectroscopically. Hence, the absorbance decrease at 340 nm is directly related to the EAL rate of reaction. Consequently, the change in absorbance at 340 nm was monitored (as described in the Materials and Methods section 2.2.3) using a Cary 50 spectrophotometer (figure 5.4). As expected EAL-B$_{12}$ in the presence of caged-ethanolamine did not form acetaldehyde, therefore no NADH consumption was detected (figure 5.4). This assay was repeated using different concentration of caged-substrate (1 mM, 2 mM, 5 mM) and the same behaviour as shown in figure 5.4 was recorded proving the inactivity of the system.

![Figure 5.4](image)

**Figure 5.4** Kinetic trace of EAL steady-state assay monitoring NADH consumption at 340 nm. The amount of reagents involved in the assay was determined spectrophotometrically in order to have 100 units of YADH, NADH 150 nM, EAL-B$_{12}$ 100 nM (HEPES 20 mM pH 7.5).
5.2.2. Extinction coefficient and reaction quantum yield determination

The first step of the photoreaction is the absorption by the caged compound of a photon at the required energy. After absorbing the photon, the caged molecule is promoted to an excited state, forming highly reactive species that can proceed to stable products. Only the light absorbed by the caged molecule acts as a trigger and, therefore, the higher the proportion of molecules absorbing the photons, the more product will be obtained. In solution, the extinction coefficient (ε) of the caged compound should be high at the wavelengths that trigger the reaction. The efficiency of photolysis is not only dependent on ε, but it is also determined by the quantum yield φ.

\[ \phi = \frac{\text{product molecules formed}}{\text{photons absorbed}} \]

The quantum yield is a measure of the effectiveness that an absorbed photon converts a caged compound to its uncaged counterpart. For caged compounds with a high extinction coefficient and high quantum yield, less light would be required to promote the photo-release of the bioactive molecules. Photolysis of the majority of available caged compounds occurs at wavelengths in the near-UV and visible region [2, 27, 28, 29 and 30]. The efficiency of the photoreaction, the bioactivity of the precursor and the formation of the by-products are influenced by the nature of the molecule being caged and by varying the substituents on the caging group. It is not possible to predict how all of these properties will be affected [27].

In this work, the extinction coefficient of the caged compound was measured by recording UV-vis absorbance spectra (Cary Varian 50 spectrophotometer) of three known concentrations of caged-2-AE (figure 5.5). For the calculation of the extinction coefficient the Beer-Lambert law was used: \( \varepsilon = \frac{A}{cl} \), where A is the absorbance, c is the concentration and l is the path length [26]. An extinction coefficient of \( 3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1} \) was obtained by linear fitting from the plot of the different concentrations of caged-2-AE against absorbance (figure 5.5 b).
Figure 5.5 UV-vis spectra of caged-2-AE (stock solutions in water 5% EtOH (v/v) and then diluted in HEPES 20 mM pH 7.5) recorded at $\lambda_{exc}= 365$ nm: a) caged-2-AE before illumination at different concentrations: 273 μM (black trace), 173 μM (red trace) and 68μM (blue trace); b) extinction coefficient determination by plotting concentration (M) against absorbance at 258 nm for the three samples (68 μM, 137 μM, 273 μM). After linear fitting: $\varepsilon= 3 \times 10^3$ M$^{-1}$ cm$^{-1}$.

To estimate the reaction quantum yield of the uncaging process experiments were carried out by coupling an LED (ThorLabs) to an oscilloscope (Tektronix TDS 3032C) in order to obtain precise illumination times. The spectra were recorded after illumination with three different excitation wavelengths (265, 280 and 365 nm) and show the conversion of the caged species, which gives a single absorbance peak with its maximum at 258 nm, to its free form. When the uncaging occurs it is possible to observe a decrease in absorbance at 258 nm and upon illumination and a double peak signal (around 280 and 325 nm) arising over time (figure 5.6).
Figure 5.6 UV-vis spectra of caged-2-AE (stock solution in water 5% EtOH (v/v) and then diluted in HEPES 20 mM pH 7.5) illuminated with LEDs at 365 nm (a), 280 nm (b) and 265 nm (c). The illumination steps were regulated via oscilloscope as follows: ten pulses of 100 ms each, twenty-one pulses of 500 ms each and 17 pulses of 1 s each.

The concentration of caged-2-AE was measured at 258 nm and then calculated using the extinction coefficient determined above. By considering the change in its concentration over time the quantum yield for monochromatic excitation could be determined as follows:

$$-\frac{d[caged - 2 - AE]}{dt} = \phi \cdot I_{abs}$$

Where $I_{abs}$ is the intensity of light absorbed by the caged compound as follow:

$$I_{abs} = I_\lambda \cdot [caged - 2 - AE] \cdot \varepsilon \cdot \frac{1 - 10^{-A}}{A}$$

Where $I_\lambda$ is the irradiation intensity, $A$ is the absorbance of the caged species and $\varepsilon$ is the extinction coefficient. In order to quantify the amount of photons used to excite the sample initially, the irradiation intensity of the 365 nm LED was determined by using a light-meter (LI-250A LI-COR Bioscience) giving a light intensity of 88000 pmol cm$^{-2}$·s$^{-1}$. The
power meter (Ophir 3A thermal sensor with Nova II power meter) also was used to measure the light intensities for the UV LEDs yielding values of 2950 pmol cm\(^{-2}\cdot s^{-1}\) for the 280 nm LED and 500 pmol cm\(^{-2}\cdot s^{-1}\) for the 265 nm LED. The quantum yield ($\phi=8\%$) was determined as the slope from the plot of the uncaged compound amount (expressed in pmoles) against pmoles of photons absorbed for the 365 nm LED (figure 5.7). This calculation was made assuming that the loss of caged compound at 258 nm is equal to the amount of uncaged product formed. The pmoles of photons absorbed were calculated based on the light intensity of the LED (88,000 pmol cm\(^{-2}\cdot s^{-1}\)) and the absorbance of the sample at 365 nm (Abs 0.06), using the following equation: Abs = ln (I\(_0\) /I\(_t\)).

![Figure 5.7](image)  

**Figure 5.7** Quantum yield determination from the slope of the caged compound loss expressed in pmoles against pmoles of photons for 365 nm LED. The data were fitted in Origin with linear fitting (red line).

The changes in absorbance at 258 and 325 nm for the three LEDs proved that the conversion of the caged compound to the free species is less efficient when recorded at 265 nm and 280 nm due to the lower number of photons (figure 5.8).
Figure 5.8 Changes in absorbance of caged-2-AE (20-23 μM) over time upon illumination with different LEDs (365, 280, 265 nm) at 258 nm (a) and at 325 nm (b).
5.2.3. Initial photolysis experiments to probe uncaging

Initial photolysis experiments were carried out by recording the UV-vis absorbance spectra (Cary Varian 50 spectrophotometer) after photolysis of the caged AE upon LED illumination in order to determine the caged compound behaviour. The uncaging has previously been shown to occur in a mechanism that involves two routes, leading to two different by-products which do not react back with ethanolamine (figure 5.9) [115].

![Diagram of uncaging mechanisms](image)

**Figure 5.9** Schematic representations of proposed uncaging mechanisms of NPOC-caged-2-AE. Both routes lead to free 2-aminoethanol [115].

The desired product, 2-aminoethanol, does not give any UV-vis signal whereas both photolysis by-products are chromophores. Therefore, the UV spectra are an indirect measurement of the free substrate. However, it is difficult to establish which path and by-product are preferential. From recent studies it was suggested that the nitroso product gives a double peak signal (around 280 and 325 nm respectively) which is present in most of the spectra reported in this work [114].

Preliminary photolysis experiments were conducted using the oscilloscope (Tektronix TDS 3032C) coupled to a 365 nm LED (Thorlabs) and the spectrophotometer (Cary Varian 50)
in order to have precise illumination times. The 365 nm LED (ThorLabs) was employed due to the higher number of photons than the UV LEDs as illustrated in the previous paragraph (5.3). Both NPPOC-caged-2-AE and NPPOC-caged-2-\( \text{H}_4 \)-AE showed the same behaviour with an initial single peak at 258 nm decreasing upon illumination and a double peak signal (around 280 and 325 nm) arising over time (figure 5.10), which corresponds to the nitroso by-product [114].

![Figure 5.10](image)

**Figure 5.10** UV-vis spectra of caged-2-AE (a) and caged-2-\( \text{H}_4 \)-AE (b) (stock solutions in water 5% EtOH (v/v) and then diluted in HEPES 20 mM pH 7.5 to a final concentration of 90 μM) illuminated with LED 365 nm. The illumination steps were regulated via oscilloscope as follows: ten pulses of 100 ms each, thirty pulses of 500 ms each and twenty-one pulses of 1 s each.

### 5.2.4. Laser photolysis experiments

Laser flash-photolysis is a UV-vis spectroscopic technique commonly used to probe very fast photochemical reactions at specific wavelengths [116]. This method allows the perturbation of a system by a short light pulse and the consequent changes can be monitored spectroscopically. In this way it is possible to investigate reaction kinetics within different time windows. However, a drawback is that signal traces can only be acquired at a single wavelength for each measurement. In this work, the light pulse is provided by a neodymium-doped yttrium aluminium garnet (Nd:YAG) laser which generates an output beam in the IR region of the spectrum at 1064 nm. It is possible to convert the IR light into the visible and UV regions by doubling, tripling or quadrupling the photon frequency with harmonics or with an optical parametric oscillator (OPO). The probe beam is provided by a 150 W Xenon Arc flash lamp and adjusted to the probing wavelength with a monochromator. After exciting the sample a second monochromator is
required to remove any laser scatter and then, the absorbance change is measured using a photomultiplier tube detector (PMT) and recorded by a digital oscilloscope (Applied Photophysics) (figure 5.11).

**Figure 5.11** Schematic representation of a laser flash-photolysis set-up. The monochromatic light beam is generated by a xenon arc lamp and the transmitted photons counted at a photomultiplier (PMT). The sample is contained into a sample cuvette and excited with a Nd:YAG laser at a selected wavelength.

### 5.2.4.1. Time-resolved laser experiments

Flow-cell experiments were carried out in order to obtain additional kinetic information about the timescale of the uncaging mechanism and the subsequent spectral changes. Therefore, the spectral changes were measured at multiple wavelengths after exciting with a laser pulse. However, since each laser pulse uncages a certain portion of the compound it is necessary to ensure that every laser pulse is exciting the same sample composition. Hence, flowing the sample guarantees fresh sample delivery for each pulse. The flow-cell set-up involved the use of a quartz cuvette connected to a sample reservoir and to a waste container via a tubing system. The flow was regulated with a pump as shown in figure 5.12.
Figure 5.12  Flow-cell set-up showing the quartz cuvette connected to the pump. The sample was irradiated with laser (Quantel Brilliant B) at both 266 and 355 nm excitation wavelengths laser over a range of wavelengths from 600 to 270 nm with 5 nm steps.

Time-resolved transients were recorded over a range of wavelengths from 600 to 270 nm with 5 nm steps and by using laser excitation at both 266 and 355 nm (figure 5.13, 5.14 and 5.15). From the data sets shown in figure 5.13, 5.14 and 5.15 it was possible to observe the same kinetic behaviour for uncaging upon photoexcitation at both 266 and 355 nm. In the wavelengths range from 600 to 515 nm the sample give a low intensity signal. The largest spectral change was detected at 420 nm, whereas from 345 to 270 nm growth signals were observed with a maximum at 320 nm. Therefore, 420 nm was chosen as detection wavelength for the flash-photolysis measurements.
Figure 5.13 Flow-cell experiments on caged-2-AE (6 mM in HEPES 20 mM pH 7.5) irradiated at 266 nm (Quantel brilliant B, 30 mJ) and measured at multiple wavelengths (600 to 270 nm). a) Kinetic traces recorded at different wavelengths; b) Absorbance profiles derived from the kinetic measurements shown in a); c) 3D plot of the flow-cell experiments on caged-2-AE measured at multiple wavelengths (600 to 270).
Figure 5.14 Flow-cell experiments on caged-2-AE (6 mM in HEPES 20 mM pH 7.5) irradiated at 355 nm (Quantel brilliant B) and measured at multiple wavelengths (600 to 270 nm). a) Kinetic traces recorded at different wavelengths with a laser power of 30 mJ; b) Absorbance profiles derived from the kinetic measurements shown in a); c) 3D plot of the flow-cell experiments on caged-2-AE measured at multiple wavelengths (600 to 270).
Figure 5.15 Flow-cell experiments on caged-2-AE (6 mM in HEPES 20 mM pH 7.5) irradiated at 355 nm (Quantel brilliant B) and measured at multiple wavelengths (600 to 270 nm). a) Kinetic traces recorded at different wavelengths with a laser power of 100 mJ; b) Absorbance profiles derived from the kinetic measurements shown in a); c) 3D plot of the flow-cell experiments on caged-2-AE measured at multiple wavelengths (600 to 270).

To better visualise the results obtained at the employed excitation wavelengths 3D plots of the data sets were made (figure 5.13c, 5.14c and 5.15c). From them, it was possible to choose 355 nm as the excitation wavelength with a power of 100 mJ to perform other photolysis experiments.

5.2.4.2. Laser photolysis experiments on NPPOC-caged-2-AE and NPPOC-caged-$^2$H$_4$-AE

Kinetic traces of NPPOC-caged-2-AE and its selectively deuterated form were recorded at 420 nm after illumination with laser (355 nm, 100 mJ). At 420 nm the initial increase in absorbance is followed by a decay of the signal (figure 5.16). By fitting the traces with a single exponential after one pulse for both species the following $k_{\text{obs}}$ were found: $9.14 \pm 0.18 \text{ s}^{-1}$ for caged-2-AE and $6.24 \pm 0.12 \text{ s}^{-1}$ for the deuterated form. Both caged compounds show similar kinetic behaviour because during the photo-cleavage the
deuterium atoms are not involved in the reaction. However, they take part in the catalytic cycle of the enzyme as described below.

**Figure 5.16** Kinetic transients after 1 pulse (black), 5 pulses (blue), 10 pulses (red), 20 pulses (green), 30 pulses (magenta) of: a) caged-2-AE (1 mM in HEPES 20 mM pH 7.5) with laser (355 nm, 100 mJ) monitored at 420 nm; b) deuterated caged-2-AE (1 mM in HEPES 20 mM pH 7.5) monitored at 420 nm. All the transients are an average of three data sets.

### 5.2.4.3. Laser photolysis experiments on EAL in the presence of NPPOC-caged-2-AE and NPPOC-caged-$^2$H$_4$-AE

EAL catalyses the deamination of vicinal amino alcohols by promoting the formation of a double bond [6 and 63]. When the substrate is 2-aminoethanol (2-AE), the reaction gives acetaldehyde and ammonia as the final products and the single turnover kinetics can be measured spectroscopically by following the disappearance of the Co$^{3+}$ signal at 525 nm. By employing a caged substrate it is possible to initiate a biological response with high temporal resolution using laser excitation. As photo-release is independent of the normal biological source, the timing of uncaging is strictly dictated by irradiation. Therefore, this strategy might allow investigating further the catalytic cycle of B$_{12}$. The deuterated form of caged ethanolamine was employed in order to observe an eventual kinetic isotope effect (KIE) since it was proven that Co-C homolysis is coupled to subsequent hydrogen abstraction from the substrate [65]. Kinetic transients were monitored at 420 and 525 nm. At 420 nm both caged entities appear to have a similar kinetic behaviour as observed in the previous paragraph (figure 5.17).
Figure 5.17 Kinetic transients after 1 pulse (black), 5 pulses (blue), 10 pulses (red), 20 pulses (green), 30 pulses (magenta) of: a) caged-2-AE (1 mM in HEPES 20 mM pH 7.5) in the presence of EAL-B_{12} (15 μM) with laser (355 nm, 100 mJ) monitored at 420 nm; b) deuterated caged-2-AE (1 mM in HEPES 20 mM pH 7.5) in the presence of EAL-B_{12} (15 μM) with laser (355 nm, 100 mJ) monitored at 420 nm. All the transients are an average of three data sets.

The kinetic transients measured at 525 nm of both caged substrates in the presence of the EAL enzyme show a very small amplitude resulting in noisy data. The lack of signal from the B_{12} chromophore at 525 nm is likely to be due to the fact that very little enzyme turnover can occur in these experiments. It is possible that amount of substrate released by uncaging of the caged AE is too small due to the low quantum yield of the photo-cleavage (figure 5.18). These experiments have shown that it is necessary to optimise the efficiency of the photo-cleavage process in order to release a higher amount of substrate to obtain meaningful kinetic transients and measure an observable KIE.
Figure 5.18 Kinetic transients after 1 pulse (black), 5 pulses (blue), 10 pulses (red), 20 pulses (green), 30 pulses (magenta) of: a) caged-2-AE (1 mM in HEPES 20 mM pH 7.5) in the presence of EAL-B12 (15 μM) with laser (355 nm, 100 mJ) monitored at 525 nm; b) deuterated caged-2-AE (1 mM in HEPES 20 mM pH 7.5) in the presence of EAL-B12 (15 μM) with laser (355 nm, 100 mJ) monitored at 525 nm. All the transients are an average of three data sets.

5.2.5. Uncaging measurements at cryogenic temperatures to trap reaction intermediates

In order to have a more detailed understanding of the uncaging mechanism and to attempt to trap any reaction intermediates in the catalytic cycle of EAL illumination experiments were also carried out at low temperatures. Many biological reactions are temperature-dependent and therefore, cryogenic temperatures can be employed to slow down molecular motions of fast enzymatic processes [117]. Another advantage is that at low temperatures it may be possible to trap short-lived intermediates which can then accumulate to a detectable level. By gradually increasing the temperature, the reaction proceeds to further steps and different intermediates are formed, which can be consecutively trapped and detected. Buffer additives are usually needed to form an optical glass and to preserve the protein during the freezing process [118]. While choosing a cryo-protective agent it is necessary to consider its compatibility with the protein. In these types of experiments, organic solvents (as methanol) are commonly used, but they can affect the protein. One of the most widely used cryogens is polyethylene glycol [119] but this was seemed unsuitable for preserving EAL. As illustrated in the previous chapter EAL can react with ethylene glycol [112] and it cannot be excluded that there may be an interaction with polyethylene glycol, which can also contains some monomers. Sugars have been employed in medicine as cryo-protectants and thanks to their physiological roles they are compatible with many biological systems
In the presence of a cryo-protectant the aqueous medium, usually a buffer, changes phase from liquid to glass, thus remaining transparent and allowing spectroscopic studies [120]. The temperature at which the phase change occurs is called the glass transition temperature and is responsible for blocking the protein conformational changes [121]. In this work, measurements were carried out by coupling a UV-vis spectrophotometer (Cary Varian 50) with a cryo-chamber (OptistatDN Oxford Instrument liquid nitrogen cryostat) as the sample cell which maintained the desired temperature via a liquid nitrogen reservoir (figure 5.19).

**Figure 5.19** Cryo-chamber (OptistatDN Oxford Instrument liquid nitrogen cryostat) with a valve-controlled liquid nitrogen reservoir. The device is connected to a vacuum pump (Pfeiffer vacuum) for insulation purposes.

Additional uncaging experiments at cryogenic temperatures were also performed by heating samples in an EPR tube to a particular temperature, maintaining it at that temperature for a particular duration, and cooling it slowly back down to liquid nitrogen temperature (77 K) using a TgK Scientific cryo-bath and a TC-61 temperature controller unit. This process is called annealing. Then, the frozen samples were analysed via EPR in order to detect any cryo-trapped reaction intermediates. Also in these experiments, sucrose was employed as cryo-protectant.
5.2.5.1. Cryogenic UV-vis absorbance measurements of the uncaging of NPPOC-caged-2-AE

Cryo-uncaging measurements were performed on caged-2-AE alone by irradiating with a 365 nm LED using sucrose (100% w/v in HEPES 20 mM pH 7.5) as the cryo-protectant. All the measurements were recorded at the liquid nitrogen temperature (77 K) after illuminating the sample at 77 K or at 100 K (figure 5.20). The spectra show that it is not possible to uncage the caged-2-AE at 77 K, even with prolonged illumination times. However, illumination at 100 K revealed that an absorbance signal at 325 nm is formed over time whereas the signal at 258 nm decreases. These experiments were carried out to verify that it was possible to uncage the caged-2-AE prior to its use with the EAL enzyme.

![Figure 5.20](image_url) 77 K absorbance spectra showing the uncaging of caged-2-AE (about 500 μM in HEPES 20 mM pH 7.5) after illumination at 77 K (a) and 100 K (b). Spectra were recorded in sucrose (100% w/v in HEPES 20 mM pH 7.5) as cryo-protectant.

5.2.5.2. Cryogenic UV-vis absorbance measurements of the uncaging of NPPOC-caged-2-AE in the presence of EAL

Uncaging measurements at cryogenic temperatures were also performed on caged-2-AE in the presence of the EAL enzyme by irradiating with a 365 nm LED using sucrose (100% w/v in HEPES 20 mM pH 7.5) as the cryo-protectant. Initial experiments were carried out by irradiating the sample at 100 K (figure 5.21). The traces were compared with a sample before and after 30 minutes of continuous illumination with LED 365 nm. An increase in absorbance at approximately 320-330 nm was observed, which is in the region of the by-
product. However, no changes associated with the B_12 cofactor around 525 nm could be observed (figure 5.21 b).

**Figure 5.21**  
(a) 77 K absorbance spectra showing the uncaging of caged-2-AE (1 mM in HEPES 20 mM pH 7.5) in the presence of EAL-B_{12} (15 μM) after illumination at 100 K. Spectra were recorded in sucrose (100% w/v in HEPES 20 mM pH 7.5) as cryo-protectant; 
(b) Difference spectra of caged-2-AE in the presence of EAL-B_{12} after illumination at 100 K. The black trace corresponds to the sample prior to illumination; 
(c) Changes in absorbance at 325 nm at different times (0, 10, 20, 25 and 30 s).

Further UV-vis absorbance spectra were recorded at 77 K after warming the illuminated sample up to the stated temperature and incubating in the dark for 15 minutes (figure 5.22). In the range of temperatures from 210 to 250 K the sample went cloudy due to the melting of the sucrose solution.
Figure 5.22 77 K absorbance spectra showing the uncaging of caged-2-AE (1 mM in HEPES 20 mM pH 7.5) in the presence of EAL-B_{12} (15 μm) after illumination at 100 K. Spectra were recorded in sucrose (100% w/v in HEPES 20 mM pH 7.5) as cryo-protectant. a) blue trace recorded at 77 K after dark incubation at 150 K; b) blue trace recorded at 77 K after dark incubation at 160 K; c) blue trace recorded at 77 K after dark incubation at 170 K; d) blue trace recorded at 77 K after dark incubation at 180 K; e) blue trace recorded at 77 K after dark incubation at 190 K; f) blue trace recorded at 77 K after dark incubation at 200 K.

A bigger change was recorded after reaching 200 K at which it is possible to notice an increase in the by-product peak (figures 5.23a and b). By plotting the changes in absorbance at 325 nm against the used temperatures an exponential growth can be
observed (figure 5.23 b). However, no detectable changes in the enzyme region were recorded. Hence, similar experiments were repeated followed by EPR analysis in order to trap short-lived intermediates.

**Figure 5.23** a) Difference spectra of caged-2-AE (1 mM in HEPES 20 mM pH 7.5) in the presence of EAL-B₁₂ (15 μM) after dark incubation at the stated temperatures. The black trace corresponds to the sample prior to illumination; b) Changes in absorbance at 325 nm at different temperatures (150, 160, 170, 180, 190 and 200 K).

5.2.5.3. EPR measurements at cryogenic temperatures of EAL in the absence of caged-2-AE

Cryogenic temperatures experiments were performed on EAL alone by irradiating with a 365 nm LED using sucrose (100% w/v in HEPES 20 mM pH 7.5) as the cryo-protectant. EPR spectra of frozen EAL samples were recorded before and after 30 minutes of continuous illumination at room temperature with a 365 nm LED (figure 5.24). All the other spectra were collected by using a sample illuminated for 5 minutes while frozen, heating it up to the stated temperature and freezing it slowly in liquid nitrogen (figure 5.25). The EPR signal around 2850 G has already been reported in the literature and is due to the Co²⁺ spin, whereas the sharp signal at 3350 G is due to a radical and was detected after illumination[108 to 110]. By looking at the annealing data sets it is possible to observe an initial increase in the signals at 3350 G followed by a sudden decrease after 238.15 K (figures 5.25 and 5.26). The disappearance of the signal at 3350 suggests that the enzyme decays to a diamagnetic species over time.
Figure 5.24 CW-EPR spectra of EAL-B$_{12}$ (60 µM) were measured at 20 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000, number of points 2048, microwave power 30 dB before (black trace) and after illumination (red trace).
Figure 5.25 CW-EPR spectra of EAL-B_{12} (60 μM) were measured at 20 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000, number of points 2048, microwave power 30 dB: a) after annealing at 177.15K (blue trace); b) after annealing at 187.15 K (blue trace); c) after annealing at 199.15 K (blue trace); d) after annealing at 209.15 K (blue trace); e) after annealing at 218.15 K (blue trace); f) after annealing at 238.15 K (blue trace); g) after annealing at 245.15 K (blue trace); h) after annealing at 253.15 K (blue trace).
Figure 5.2 CW-EPR spectra of EAL-B$_{12}$ (60 μM) were measured at 20 K, centre field 3350 G, modulation amplitude 1 G, sweep width 400, number of points 2048, microwave power 30 dB: after annealing at 177.15K (black trace), 187.15 K (red trace), 199.15 K (green trace), 209.15 K (blue trace), 218.15 K (light blue trace), 238.15 K (pink trace), 245.15 K (yellow trace), 253.15 K (dark green trace).

5.2.5.4. EPR measurements at cryogenic temperatures of EAL in the presence of caged-2-AE

Uncaging measurements at cryogenic temperatures were performed on NPPOC-caged-2-AE in the presence of EAL by irradiating with a LED 365 nm using sucrose (100% w/v in HEPES 20 mM pH 7.5) as the cryo-protectant. EPR spectra of frozen samples of caged-2-AE in the presence of EAL were recorded before illumination and after 30 minutes of continuous illumination at room temperature with the 365 nm LED. The EPR signal around 2850 G is due to the Co$^{2+}$ spin and is present in both samples before and after illumination (figure 5.27). A radical signal with small intensity in the region 3200-3500 G is detected prior to irradiation suggesting that the enzyme can interact with the caged entity before cleavage (figure 5.27 b). All the other spectra were collected by using a sample illuminated for 5 minutes while frozen in liquid nitrogen (77 K), heating it up to the stated temperature and freezing it slowly in liquid nitrogen (figure 5.28).
Figure 5.2 CW-EPR spectra of EAL-B_{12} (60 μM) with caged-2-AE (5 mM in HEPES 20 mM pH 7.5) were measured at 20 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000, number of points 2048, microwave power 30 dB before (black trace) and after illumination (red trace).

However, the peak considerably increases after irradiation and by looking at the annealing data sets it is possible to observe an initial increase followed by a sudden decrease after 238.15 K (figures 5.28, 5.29 and 5.30). Probably over time the system decays to a diamagnetic species. Moreover, the signal in the region 3250-3400 G shows a different shape compared to the sharp radical signal for the enzyme alone and to the doublet due to Co^{2+} and substrate radical spin-spin interaction of the enzyme in the presence of 2-aminopropanol (Chapter 4).
Figure 5.28 CW-EPR spectra of caged-2-AE (5 mM) in the presence of EAL-B$_{12}$ (60 μM) in HEPES 20 mM pH 7.5 and sucrose were measured at 20 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000, number of points 2048, microwave power 30 dB: a) after annealing at 177.15 K (blue trace); b) after annealing at 187.15 K (blue trace); c) after annealing at 199.15 K (blue trace); d) after annealing at 209.15 K (blue trace); e) after annealing at 218.15 K (blue trace); f) after annealing at 238.15 K (blue trace); g) after annealing at 245.15 K (blue trace); h) after annealing at 253.15 K (blue trace); i) after annealing at 263.15 K (blue trace); l) after annealing at 253.15 K (blue trace).

EPR spectra of the region of interest (3200-3400 G) were collected at the different annealing temperatures to observe the radical peak (figure 5.29).
Figure 5.29 CW-EPR spectra of caged-2-AE (5 mM) in the presence of EAL-B12 (60 μM) in HEPES 20 mM pH 7.5 and sucrose were measured: a) at 20 K, centre field 3350 G, modulation amplitude 1 G, sweep width 400, number of points 2048, microwave power 30 dB: after annealing at 177.15 K (black trace), 187.15 K (red trace), 199.15 K (green trace), 209.15 K (blue trace), 218.15 K (light blue trace), 238.15 K (pink trace), 245.15 K (yellow trace), 253.15 K (dark green trace), 263.15 K (navy trace), 273.15 K (purple trace); b) at 20 K, centre field 3250 G, modulation amplitude 10 G, sweep width 1500, number of points 2048, microwave power 30 dB.

Figure 5.30 3D plot of CW-EPR spectra of caged-2-AE (5 mM) in the presence of EAL-B12 (60 μM) measured at 20 K, centre field 3350 G, modulation amplitude 1 G, sweep width 400, number of points 2048, microwave power 30 dB after annealing at 177.15 K, 187.15 K, 199.15 K, 209.15 K, 218.15 K, 238.15 K, 245.15 K, 253.15 K, 263.15 K, 273.15 K.

By comparing the traces of all the species involved in the reaction it is possible to notice a radical signal with small intensity is visible prior to illumination suggesting that the enzyme can interact with the caged entity before photolysis (figure 5.31).
Figure 5.31 CW-EPR spectra of EAL-B$_{12}$ (60 μM) with and without cage were measured at 20 K, centre field 2600 G, modulation amplitude 10 G, sweep width 5000, number of points 2048, microwave power 30 dB: a) before illumination; b) after illumination.

EPR spectra of frozen caged 2-AE samples were recorded before illumination and after 30 minutes of continuous illumination at room temperature with the 365 nm LED. The illuminated sample proves that the signal recorded in the 3250-3400 G region is due to the caged compound (figure 5.32). Since the uncaging reaction involves radicals formation it might be possible that they got trapped and partially covered the signals due to the interaction of EAL with the free substrate.

Figure 5.32 CW-EPR spectra of caged-2-AE (5 mM in HEPES 20 mM pH 7.5 and sucrose) were measured at 20 K, centre field 3350 G, modulation amplitude 1 G, sweep width 400, number of points 2048, microwave power 30 dB before (black trace) and after 30 minutes of illumination (red trace).
5.3. Summary and conclusion

EAL has become an important model system to understand the catalytic mechanism of coenzyme B$_{12}$-dependent enzymes in general. The EAL catalytic cycle involves highly reactive radicals, involving a low-spin Co$^{3+}$ bound to the corrin ring of the cofactor and a 5'-deoxyadenosyl radical. The latter intermediate promotes the abstraction of a hydrogen atom from the substrate [102]. Several techniques have been used to investigate the relationship between catalysis and enzyme–substrate complex geometry and the properties of the active site, such as UV-vis spectroscopy [63], X-ray crystallography [64], kinetic isotope effect measurements [65], magnetic field continuous wave photolysis and EPR spectroscopy [66 and 67]. In recent years, ultrafast photolysis studies have provided experimental evidence for the existence of the 5'-deoxyadenosylcobalamin radical in the active sites of the related enzyme glutamate mutase (GM) [103] and EAL [104]. Computational methods have provided further evidence for the existence of this radical by calculating free energy profiles of the species involved in the catalytic cycles [102]. However, further information about the Ado radical lifetime, its interaction with the enzyme and the detailed role of the protein during Co-C homolysis are still missing.

A caging strategy to study the EAL kinetic mechanism has been applied involving the use of 2-nitrophenylpropylchloroformate (NPPOC-Cl) as a photo-removable protecting group. Different types of experiments were performed in an attempt to follow any catalytic steps in the reaction and to trap short-lived intermediates. The NPPOC-caged-2-AE has an extinction coefficient of $3 \times 10^3$ M$^{-1}$ cm$^{-1}$. However, the obtained quantum yield (φ= 8 %) indicates a low reactivity of the nitro group which promotes the uncaging after illumination [115]. Both laser and EPR measurements have shown difficulties in providing information when the caged entities are studied in the presence of the EAL enzyme. Since it has been reported that EAL has a high binding affinity for ethanolamine ($K_M = 1.97 \times 10^{-3}$ mM) [106] and after a single pulse the substrate released has a concentration of 400 μM, the low quantum yield is not satisfactory to explain the absence of signals in the experiments. Further studies are necessary and other caging groups will need to be tested to optimise these measurements. In general, the o-nitrobenzyl derivatives remain the most popular photo-removable protecting groups due to their robustness and commercial availability and because they usually release amines in good yields [122].
Quantum efficiencies depend on the substituents on the aromatic ring and it was observed that better efficiencies were obtained with two o-nitro groups, which considerably increased the probability for the hydrogen atom abstraction by one of the nitro groups [122]. Despite all the improvements aimed to obtain faster release rates, better solubility, and more inert by-products, it is not currently possible to find a photo-removable group able to fulfil all these criteria. One of the main issues remains the low solubility in aqueous media. However, many of these groups have found applications in different biological fields [1, 2, 3 and 5]. An interesting approach to characterise the binding affinity of the caged-substrate and EAL is the isothermal calorimetry (ITC). This technique can prove if the caged-substrate reaches the binding site of the enzyme by providing information about stoichiometry, association constant and binding enthalpy in a single experiment.

All the experiments covered in the current work show that this approach can be employed for biological studies with some implementations.
6. Discussion and future directions

A full characterisation of an enzyme’s catalytic cycle requires determination of the rate constants and identification of the intermediates involved in the reaction. For the majority of enzymes these are generally measured via stopped-flow techniques [8]. Usually, the intermediates generated during catalysis are characterised by short lifetimes, distinctive light absorption features, and in some cases vibrational signatures too. Electron paramagnetic resonance (EPR) is a spectroscopy technique that is able to detect species with unpaired electron and provides a great deal of information about the electronic environment of such species. Hence, it is an ideal technique for the identification of radical intermediates or for studies of many metal-containing enzyme reactions. However, EPR techniques typically require relatively long measurement times and very often, low temperatures to fully characterise these short-lived species [23]. Therefore, over the last decades rapid freeze-quench devices have been developed in order to kinetically trap these transient radicals and allow their spectroscopic characterisation via EPR analysis [10 to 21].

In the current work, a novel rapid freeze quench device combined with flashing capabilities, which should allow studies of both light-activated and thermally-activated biological reactions, was developed in conjunction with TgK Scientific (Chapter 3). To the best of our knowledge this is the first report of an instrument that is capable of combining photoexcitation with rapid mixing / freezing approaches for analysis by EPR spectroscopy. Several difficulties have arisen during the development of the device and as a consequence, some technical improvements were made. One of the main issues was related to the rotation of the plates on which the samples are sprayed. Hence, a new improved rotation system was designed using a wheel made of aluminium and composed of two parts in which there are two opposing magnets with the same charge. The effect of the magnets causes the upper part to float on the base and the rotation between the two strictly depends on the flow of nitrogen gas around the upper part. By using this innovative rotating system a better rotation was obtained, although some condensation was still present, which caused friction. During the rotation the wheel can also become unbalanced due to misalignment of the nitrogen gas lines and therefore, it was necessary to carefully check their alignment before each experiment. In order to avoid the rotating
plate from becoming unbalanced it may be possible in the future to replace the polyurethane Dewar with one made of stainless steel and to have fixed holes in which the gas nitrogen lines can be placed. In this case, the alignment of the gas lines would not need any further adjustment before and during the spraying. Also, in the present work the packing set-up was modified to improve the packing efficiency of the frozen sample powder after spraying onto the rotating plate. Initially, the packing strategy was completely manual involving the scraping of the frozen sample from the plates inside a foam Dewar filled with liquid nitrogen to obtain a powder which was then transferred to a funnel attached to an EPR tube placed on top of a second foam Dewar filled with liquid nitrogen. The sample was then pushed into the EPR tube using packing rods. To improve the packing efficiency and to remove as much oxygen as possible the EPR tubes were subsequently connected to a vacuum pump via a silicon tube. The bottom of the EPR tubes were cut off and sealed with a customised filter. The pump helped to compress the sample grains more efficiently and as a result, it was possible to observe a reduction in the oxygen background noise in the spectra [18,19]. This system also enabled the collection of smaller frozen particles, which are generated at higher flow rates. As a result the freezing-time of 5.2 ± 3.8 ms obtained with this device was comparable to the freezing times of other RFQ devices described in the literature, which show values between 5 and 7 ms [19 to 21]. Another issue common to all the RFQ instruments is the amount of sample required for running an experiment. Previous developments in sample preparation have allowed the combination of the RFQ technique with a wider range of spectroscopic techniques such as resonance Raman [98], magnetic circular dichroism [99], Mossbauer spectroscopy [100] and X-ray absorption spectroscopy, which can allow the identification of EPR silent species [101]. It is possible that the RFQ device described here could also be modified in the future to allow the application of similar techniques. In conclusion, by using the instrument developed in this work it was possible to collect EPR spectra of known systems and therefore, it has the potential to study short-lived radical species that may be formed in enzymatic reactions. However, its application on the ethanolamine ammonia lyase system did not provide conclusive results (Chapter 4).

Ethanolamine ammonia lyase (EAL) is a coenzyme B_{12}^- or AdoCbl–dependent enzyme that catalyses the formation of acetaldehyde and ammonia (NH\textsubscript{3}) from 2-aminoethanol or
ethanolamine [6.63]. EAL has been extensively studied as it is considered an important model system to understand the catalytic mechanism of coenzyme B_{12}-dependent enzymes in general and it can be easily produced in *E. coli*. The EAL catalytic cycle involves highly reactive radical species that have been investigated by using a wide range of techniques such as EPR spectroscopy, UV-vis spectroscopy [63], stopped-flow measurements [64], kinetic isotope effect (KIE) measurements [65] and magnetic field effect (MFE) measurements [66 and 67]. These techniques showed that Co-C homolysis is coupled to subsequent hydrogen abstraction from the substrate and this allowed a catalytic mechanism to be proposed. In recent years, computational methods and ultrafast photolysis studies have provided further evidence for the existence of the 5’-deoxyadenosylcobalamin radical [102 to 104]. However, key information about the Ado radical lifetime, its interaction with the enzyme and the detailed role of the protein during Co-C homolysis are still missing. To explore further the EAL reaction mechanism two strategies have been applied in the current work, the rapid freeze-quench technique and the application of a caging approach to enable studies using light-activation.

Rapid freeze-quench experiments on EAL have previously been conducted at low temperatures in order to characterise the kinetics and thermodynamics of the Co^{2+}-substrate radical pair formation [108 and 109]. In the current work, ethanolamine (2-AE) and aminopropanol (2-AP) were used as substrates in the RFQ tests. Although the RFQ experiments did not produce conclusive data, due to the instrument limitations described in Chapter 3, it was possible to detect a multiplet structure in the EPR spectra of the EAL reaction with AP. The signal shows mainly 6-lines rather than 8 corresponding to Co^{2+} ion (I = 7/2), approximately separated by 90-95 G, which might be due to the formation of the adenosyl radical and Co^{2+}. The deviation from the 8 line patterns for the Co^{2+} ion might be caused by the strong spin-spin interaction of Co^{2+} and adenosyl radical, but further studies are needed to identify the origin of the super-hyperfine coupling. It would be interesting to investigate two other EAL substrates, ^2H_4-2-AE and ethylene glycol, in an attempt to trap any intermediate species formed in these reactions. The reaction of EAL (holoenzyme) with ^2H_4-2-AE has been extensively studied to elucidate the Co-C bond breaking and H-transfer steps during catalysis [111]. Stopped-flow investigations at 5°C of EAL against ^2H_4-2-AE revealed that, although homolysis was expected to be a first-order
kinetic process, the kinetic transients that were obtained had a multiphasic nature [67]. The reaction mechanism of EAL (holoenzyme) in the presence of ethylene glycol is still under investigation. It has been shown that this reaction leads to the cleavage of the Co-C bond and then the adenosyl radical abstracts a hydrogen from the substrate. The enzyme forms a new corrinoid which coordinates a different upper axial ligand to the Co than the usual 5'-deoxyadenosyl residue. After the reaction is over, the new corrinoid is gradually converted to hydroxocobalamin. As a result, the enzyme activity is considerably reduced [112].

As the freeze-quench experiments were not conclusive a caging strategy was subsequently used to follow the EAL reaction. In this thesis a caging approach was applied to the ethanolamine (and its selectively deuterated form) substrate as a tool for triggering EAL catalysis by light. In the current work, NPPOC-Cl was employed to protect the amino portion of the EAL substrates via organic synthesis. Both syntheses were successful and after silica column purification the caged products were obtained with a yield = 80-84%. The NPPOC-caged-2-AE has an extinction coefficient of $3 \times 10^3$ M$^{-1}$ cm$^{-1}$ at 258 nm. However, the obtained quantum yield ($\phi = 8\%$) indicates a low reactivity of the nitro group which promotes the uncaging after illumination [115]. Both laser and EPR measurements have shown that difficulties arise in obtaining information when the caged entities are studied in the presence of the EAL enzyme. The kinetic transients measured at 525 nm of both caged substrates in the presence of the EAL enzyme have very small amplitudes resulting in noisy data. It is possible that the amount of substrate released upon uncaging of the caged AE is too small due to the low quantum yield of the photocleavage. Hence, very little enzyme turnover can occur in these experiments leading to a lack of signal change from the B$_{12}$ chromophore at 525 nm. Further studies are necessary for this approach to work and other caging groups will need to be tested to optimise these measurements. In general, the o-nitrobenzyl derivatives remain the most popular photo-removable protecting groups and the quantum efficiency can be improved by testing substituents on the aromatic ring. It was observed that better efficiencies were obtained with two o-nitro groups, which considerably increased the probability for the hydrogen atom abstraction by one of the nitro groups [122]. An interesting approach to characterise the binding affinity of the caged-substrate and EAL is the isothermal
calorimetry (ITC). This technique can prove if the caged-substrate reaches the binding site of the enzyme by providing information about stoichiometry, association constant and binding enthalpy in a single experiment.

In conclusion, new tools to study enzymes kinetics and trap radical intermediates have been developed. The rapid freeze quench device can be applied to other enzymes containing a metal centre (e.g. haems) or to other system involving radicals such as flavins. As previously illustrated, it might be interesting to couple RFQ with other spectroscopy techniques (i.e. Raman spectroscopy). The caging strategy gives the opportunity to explore more options. In the current work this approach was applied on the enzyme substrate ethanolamine and can be improved by changing the substituents on the NPPO-Cl aromatic ring or trying other protecting groups such as 9-xanthenylmethyl[123]. Another approach is the application of the caging strategy on proteins whose activity is rendered inert by covalent modification of a vital amino acid with a photo-removable group [35 and 36]. However, this approach is very challenging and implies more variables [36]. Since many biological reactions require oxygen to take place, a cobalt-based caged oxygen molecule ((m-peroxo)(m-hydroxo)bis(bis(bipyridyl)cobalt(III)) nitrate) has been synthesised in order to deliver dioxygen in a controlled manner at cryogenic temperatures. This caged oxygen molecule is able to release dioxygen at 100 K. This approach has the potential to investigate in detail various oxygen-dependent enzymes [55]. Upon cryo-photolysis, the temperature increases and the active compound is released initiating catalysis and as a consequence the accumulation of intermediates can be observed. Also a similar approach might be considered in the future for studying different system.
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