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Research paper

The effect of charge mutations on the stability and aggregation of a human single chain Fv fragment

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ABSTRACT

The aggregation propensities for a series of single-chain variable fragment (scFv) mutant proteins containing supercharged sequences, salt bridges and lysine/arginine-enriched motifs were characterised as a function of pH and ionic strength to isolate the electrostatic contributions. Recent improvements in aggregation predictors rely on using knowledge of native-state protein-protein interactions. Consistent with previous findings, electrostatic contributions to native protein-protein interactions correlate with aggregate growth pathway and rates. However, strong reversible self-association observed for selected mutants under native conditions did not correlate with aggregate growth, indicating 'sticky' surfaces that are exposed in the native monomeric state are inaccessible when aggregates grow. We find that even though similar native-state protein-protein interactions occur for the arginine and lysine-enriched mutants, aggregation propensity is increased for the former and decreased for the latter, providing evidence that lysine suppresses interactions between partially folded states under these conditions. The supercharged mutants follow the behaviour observed for basic proteins under acidic conditions; where excess net charge decreases conformational stability and increases nucleation rates, but conversely reduces aggregate growth rates due to increased intermolecular electrostatic repulsion. The results highlight the limitations of using conformational stability and native-state protein-protein interactions as predictors for aggregation propensity and provide guidance on how to engineer stabilizing charged mutations.

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1. Introduction

Biopharmaceuticals are an important part of the drug portfolio of most major pharmaceutical companies. Biologic drug candidates are used to treat metabolic, cardiovascular, cancer, autoimmune and infectious diseases, amongst others. Proteinaceous products include peptides, enzymes, monoclonal antibodies (mAbs), antibody-like proteins and other scaffolds and fusions [91,92]; all of which may suffer physical and chemical instability. Broadly,

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physical instability involves adsorption, unfolding and aggregation, all of which may occur cooperatively rather than in isolation [86]. The long term loss of monomer is generally predicted through quantitative monitoring of aggregation under accelerated and stress conditions over weeks to months. Recent progress has been made in: (i) the development of detailed kinetic models [2,4,5,34,42,49,60,96]; (ii) correlating aggregation kinetics with protein structure and folding [11,15,16,32,35,52,53,54,67,88]; (iii) and with native-state protein-protein interaction measurements [36,46,57,74,75,79,82].

Aggregation can be described through models incorporating nucleation and subsequent growth steps such as Lumry-Eyring nucleation polymerization models [1,2,9,50,96]. Nucleation generally refers to the steps prior to formation of the smallest net

irreversible aggregate, which can include partial unfolding, reversible association of partially unfolded intermediates, and conformational rearrangements. Once formed, the aggregates can grow through different mechanisms such as via monomer addition (referred to as chain polymerization), by aggregate–aggregate coalescence or condensation, and possibly phase separation or precipitation. Predicting aggregation is not always possible due to the multitude of possible pathways and difficulties in isolating key partially folded intermediates and characterizing their intermolecular interactions [68,70,94,97].

Many mutational approaches for improving aggregation resistance rely on manipulating protein electrostatic properties through charged mutations. Aggregation resistance has been increased through the method of supercharging proteins by engineering in an excess number of acidic [25,45,85] or basic residues [58] or alternatively protein net charge can be increased by covalently attaching charged amino acid tags [83,87]. Similarly, heat resistant antibody V_H domains isolated from a combinatorial library of mutations generated by phage display generally had a disproportionate number of acidic groups [3,26,39]. These studies indicate that the controlling factor is the protein net charge. However other work, based on phage display [27] and rational mutagenesis [25,65,47], indicates that the spatial location of charged mutations controls the protein stability. However, care must be taken when engineering in charged mutations. If the protein net charge is close to zero, an anisotropic charge distribution can cause protein self association [13,14,71,99], which has been correlated with increased aggregation propensity [55,80]. To avoid increasing protein charge anisotropy, charged mutations should carry the same sign as the net charge on the corresponding protein scaffold [31,100]. Deconvoluting between these competing charge effects has led to confusion over whether negatively charged mutations are more effective than positively charged mutations [44,89]. Further, solubilizing effects of charged mutations are also specific to the chemical nature of the residue concerned. An increased abundance of lysine over arginine has been correlated with higher protein solubility through an informatics analysis [95].

Improving upon rational mutational strategies requires a better understanding of how the changes impact on the rate determining steps in aggregation pathways. Most rational design strategies such as Rosetta choose mutations that minimize the free energy of the native state to avoid increasing the formation of aggregation-prone partially folded or unfolded states [25,58]. The more challenging problem is identifying partially-folded regions on a protein that expose hot spots buried in nucleation or aggregate growth steps. A recently developed approach, the spatial aggregation predictor (SAP) identifies aggregation prone segments as hydrophobic regions with high dynamic exposure [17,18]. Whether or not SAP accurately predicts aggregation prone regions is not known since only aggregation rate data has been predicted, rather than the actual location of known aggregation hot spots. Indeed very limited experimental data exists for aggregation hot spots due to difficulties in identifying the key intermediate in aggregation pathways [69].

Further insight to the association steps in aggregation pathways can be gained by rationalizing why aggregation is correlated, in some instances, with native-state protein–protein interactions. For instance, recent work has established a strong correlation between the electrostatic contribution to native protein–protein interactions and the type of aggregate growth pathways as well as aggregate growth rates [9,43,63,71,75]. Electrostatic interactions correlate with aggregate growth because charged groups remain exposed in the native state and on growing aggregates. Conversely, it is not clear whether surfaces exposed on natively-folded proteins are buried during aggregate growth and nucleation. One distinct possibility is that hot spots exposed by partially folded

regions of the protein are buried in the nucleation step, while aggregate growth occurs at least in part by burying native surfaces.

In this work, the effect of mutations on the aggregation properties of a recombinant human single-chain variable fragment (scFv) [28] was studied. Mutants with surface patches of positive or negative charge, with engineered salt bridges, and with lysine/arginine swaps in corresponding sequence-rich regions were generated. Experiments were carried out at low and moderate ionic strength and for two pH values to delineate the effects of electrostatic interactions. Thermally-accelerated aggregation studies were complemented with measurements of native protein–protein interactions obtained from dynamic light scattering (DLS), and with measurements of conformational stability quantified from equilibrium chemical denaturation and differential scanning fluorimetry measurements. The main aims of the study were first to further elucidate the relationship between steps in aggregation pathways and native-state protein–protein interactions and secondly to understand better the molecular basis for how mutations alter aggregation behaviour.

2. Materials and methods

2.1. Mutant design

Modelling of the surface properties for the scFv variants was performed with a comparative model generated from the closest available Protein Data Bank structure (www.rcsb.org; 2GHW:B), using the pairwise sequence alignment and a procedure to optimise side-chain placement [20]. A depiction of the wild type polarity surface distribution is given in Fig. 1A, where red and blue correspond to non-polar and polar regions, respectively. A large non-polar region on the solvent exposed surface was identified for introducing mutations and generating variants (all sequences are shown in Supplementary Information (SI) Fig. S1). Over-

Fig. 1. (A) The molecular surface of modelled scFv is colour-coded according to polarity, with red denoting most non-polar, blue most polar, and white intermediate. This calculation is patch based, leading to the smooth transition between regions. The largest non-polar red-patch is highlighted for wild-type scFv. (B) The colour-coding of panel (A) is maintained in this focus on the region immediately surrounding the non-polar patch. Framework scFv is shown, with sidechains for the 6 residues that are mutated to form the 3SB mutant, and the TWA segment (which is mutated to DSV). Modelled pairings for the introduced salt-bridges are shown. (C) The equivalent surface to panel A is shown, but for the 3SB mutant, showing ablation of the non-polar patch. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

charged mutants were generated by placing into the hydrophobic patch either five lysine residues (5K), five arginine residues (5R), or five glutamic acid residues (5E). A hydrophobic TWA sequence was replaced with DSV, which is a common replacement sequence found in other scFvs, including the 2GHW:B template [37]. Pairs of lysine and glutamic acid residues were introduced to create mutants with one, two, or three salt bridges, labelled as 1SB, 2SB, or 3SB, respectively. The surface locations for the three proposed salt bridges and the mutated TWA sequence are shown in Fig. 1B. Fig. 1C depicting an updated surface polarity distribution for 3SB shows how the three salt bridges reduce the non-polar surface area. Mutants were also created in which 4 lysine residues were substituted for 4 arginine residues (4RK) or 7 arginine residues were substituted for 7 lysine residues (7KR), with the sites being located more extensively over the protein surface.

2.2. Calculation of protein surface properties

The surface polarity of the modelled scFv was calculated with a patch-based scheme [95] in which each surface patch was evaluated as a ratio of non-polar to polar atom surface area; this property was included in the B-factor field of a standard coordinate file to allow plotting with a colour ramp (Fig. 1A). The theoretical net charge for each protein shown in Table 1 was calculated from its amino acid sequence, using analysis software written for a previous study [95].

2.3. Protein production

The scFv gene [28] was cloned into the pET22b vector (Novagen) using the NdeI/XhoI restriction sites (New England Biolabs). The protein sequence is shown in the supplementary information. All mutants were generated using ThermoFisher GeneArt, with codon usage optimised for *E. coli*, except DSV and 1SB which were generated by site directed mutagenesis using Q5 high fidelity DNA polymerase (New England Biolabs) according to manufacturer's instructions. Forward and reverse primers for these are presented in the supplementary information. The scFv-pET22b vector was transformed into T7 Shuffle Express cells (New England Biolabs) and a single colony grown in 50 mL 2xYT media (Formedium) containing 100 µg/mL ampicillin (Sigma) at 30 °C for 4 h, then transferred to 600 mL flasks of the same media with a starting OD of 0.075 and grown to an OD of 0.8 before induction with IPTG (Gibco) to a concentration of 0.25 mM and incubated overnight at 16 °C. After pelleting at 10,000g for 20 min, 10 g of cells were resuspended in 50 mL 50 mM Tris 25 mM NaCl pH 8.5 (pH 7.5 for 5K, 5R and 5E) with 50 µL DNase and 1x Complete protease inhibitor tablet (Roche Applied Science) and lysed by sonication for 5 min with the cellular debris removed by centrifugation at 15,000g for 30 min and supernatant filtered through a 0.4 µm filter (Millipore). Samples

were loaded onto a 25 mL protein A Sepharose column (Sigma), washed with 25 mM TRIS 25 mM NaCl pH 7.5 and eluted with 100 mM pH 3.5 citrate buffer. 1.5 M TRIS was used to pH the eluent to pH 7, and the proteins were dialysed into 20 mM NaPO₄ 150 mM NaCl pH 6.9 (pH 7.5 for 5K and 5R, pH 8 for DSV and pH 7 for 5E). The protein was concentrated and loaded onto a Superdex HiLoad 16/600 S 75 column (GE Healthcare) and the monomer fraction collected and verified by mass spectrometry. The eluted samples at a protein concentration of 10 g/L were then frozen at −80 °C until further use.

2.4. Buffers

All experiments have been carried out using either a pH 5 buffer containing sodium acetate at an ionic strength of 25 mM (containing 39.3 mM acetate ion) or a pH 7 buffer containing sodium phosphate at an ionic strength of 25 mM (containing 11.6 mM phosphate ion). Varying amounts of sodium chloride have been added to the buffers.

2.5. Sample preparation

Before each measurement, 0.5 mL of a protein sample was prepared by dialyzing two times against 1 L of the appropriate buffer using GeBAflex-Midi Dialysis tubes (3.5 kDa MWCO). A vacuum filtration unit with Millipore 0.2 µm membrane was used to remove dust from all dialysis buffers. The first and second dialysis runs were carried out for two hours and overnight, respectively. After dialysis, the sample was always filtered through a 0.02 µm syringe top filter (Whatman). Protein samples were diluted to the desired concentration using the second dialysis buffer.

2.6. Circular dichroism

Experiments were undertaken on the Chirascan spectrophotometer (Applied Photophysics). Protein solutions of 50 µL volume at 1 g/L were placed in 1 mm pathlength quartz Hellma cell with Peltier temperature controlled stage at 25 °C. Spectra were recorded at 190–260 nm with an acquisition time of 5 s at 0.5 nm increments and normalised by subtraction with a corresponding buffer blank.

2.7. Dynamic light scattering (DLS)

The diffusion coefficient and radius of hydration of each sample was determined using the Wyatt Dynapro system and DYAMICS software, using the laser wavelength of 830 nm with a scattering angle of 158°. 30 µL sample volume was used in 384 well plates (Nalge NUNC international) and capped with silicon oil. Acquisition time was set at 5 s and 10 collections taken of each, with each sample run in triplicate. Correlation functions were determined by the

Table 1
Experimental and calculated conformational properties of mutants. From left to right: theoretical isoelectric pH (pI), theoretical charge Z at pH 5 and at pH 7, tryptophan fluorescence peak maxima λ_{max} at pH 5 and at pH 7, infinite-dilution hydrodynamic radius R_{H0} at pH 5, GdnHCl midpoint unfolding concentration C_{mid} , and thermal unfolding temperature T_{DSF} .

Mutant	pI	Z(e) pH 5	Z(e) pH 7	λ_{max} (nm) pH 5	λ_{max} (nm) pH 7	R_{H0} (nm)	C_{mid} (M)	T_{DSF} (°C)
WT	7.8	5.62	1.35	334.6 ± 0.1	335.6 ± 0.1	2.41 ± 0.01	1.49 ± 0.04	44.9 ± 0.5
DSV	6.9	4.71	0.35	333.4 ± 0.3	335.6 ± 0.1	2.35 ± 0.01	1.78 ± 0.06	46.0 ± 0.2
5K	9.0	10.60	6.35	334.6 ± 0.1	336.6 ± 0.5	2.39 ± 0.01	1.25 ± 0.08	38.5 ± 0.1
5E	5.3	1.62	−3.64	334.6 ± 0.1	335.6 ± 0.1	2.60 ± 0.02	1.25 ± 0.11	43.3 ± 0.7
5R	9.0	10.62	6.35	334.6 ± 0.1	335.1 ± 0.3	2.52 ± 0.04	1.27 ± 0.10	37.2 ± 0.1
3SB	7.8	6.22	1.35	334.1 ± 0.1	335.6 ± 0.1	2.40 ± 0.01	1.47 ± 0.06	46.6 ± 2.3
2SB	7.8	6.02	1.35	334.6 ± 0.1	335.6 ± 0.1	2.39 ± 0.01	1.51 ± 0.03	46.3 ± 1.0
1SB	7.8	5.82	1.35	334.9 ± 0.2	335.6 ± 0.1	2.40 ± 0.01	1.30 ± 0.08	46.6 ± 0.7
7KR	7.8	6.62	2.35	334.5 ± 0.1	335.6 ± 0.1	2.37 ± 0.01	1.55 ± 0.04	46.1 ± 0.5
4RK	7.8	5.62	1.35	335.2 ± 0.1	335.6 ± 0.1	2.43 ± 0.01	1.25 ± 0.09	46.4 ± 0.5

DYNAMICS software. Fits to the correlation function were performed between 1.5 and $6 \times 10^4 \mu\text{s}$, using a cumulant analysis and a regularization analysis as implemented in the DYNAMICS software. The regularization fitting uses the Dynals algorithm from Alango, Ltd [33]. The cumulant analysis was used to determine the z-average diffusion coefficient D and the polydispersity P_d defined as the width of the diffusion coefficient normalized by D .

Measurements were carried out at 5 , 10 , 15 and 20°C , and then in further 1°C increments. The sample chamber was purged with nitrogen at 8 bar in conditions below 15°C to negate water vapour condensation in the instrument. Each temperature programme experiment included 18 samples with 6 different protein concentrations ranging between 0.5 and 10 g/L measured in triplicate. A typical temperature scan took 35 min including 1 min to allow for temperature equilibration.

The measurements at temperatures of 25°C and below were used to determine the interaction parameter k_D and infinite dilution protein hydrodynamic radius $R_{H,0}$. The diffusion coefficient D calculated using the cumulant analysis can be used to calculate k_D

$$D = D_0[1 + k_D c] \quad (1)$$

where D_0 is the infinite dilution of the diffusion coefficient. $R_{H,0}$ was calculated using the Stokes-Einstein relation

$$R_{H,0} = \frac{k_B T}{6\pi\mu D_0} \quad (2)$$

where μ is solvent viscosity, T is temperature, and k_B is Boltzmann's constant.

2.8. Temperature ramped static light scattering (SLS)

An Optim 1000 instrument (Unchained Labs) was used to record static light scattering signals during a temperature ramp using laser excited light at a wavelength of 473 nm. Changes in light scattering intensity reflect changes in the weight average molecular weight due to aggregation. $9 \mu\text{L}$ samples at a protein concentration of 1 g/L were heated in 0.5°C increments from 25 to 80°C . The heating rate between temperature intervals was set to $1^\circ\text{C}/\text{min}$. A typical temperature scan of 48 samples took two and a half minutes including 30 s for thermal equilibration. All measurements were done in duplicate.

The temperature dependent light scattering profiles were fit to a two-parameter empirical equation given by

$$\frac{dI}{dT} = \exp \left[-E_a \left(\frac{1}{T} - \frac{1}{T_{\text{SLS}}} \right) \right] \quad (3)$$

where I is absolute light scattering intensity, T is temperature, and T_{SLS} and E_a are fitting parameters. Eq. (3) provided an accurate fit to the data over a temperature range when the light scattering signal remained below $35,000$ kcps. This range was used in all the fitting unless otherwise noted. The fitting was carried out using the leastsq minimization routine of a python script.

2.9. Extrinsic fluorescence

Temperature-ramp fluorescence measurements were carried in the Optim1000 instrument. The SYPRO® orange dye was supplied in DMSO at 100 times the recommended working concentration and added to each sample with protein concentration of 1 g/L immediately prior to loading into the $9 \mu\text{L}$ sample cuvette. The Optim1000 uses a 473 nm laser for excitation and records the fluorescence emission spectrum at wavelengths between 500 and 700 nm. The temperature programme from the static light scattering experiment was followed.

The lowest unfolding temperature T_{DSF} was determined from fitting the fluorescence intensity as a function of temperature to the Boltzmann Equation [62], which assumes a two-state unfolding transition. The T_{DSF} value corresponds to the mid-point or inflection point of the transition. The fitting was carried out using a python script and the leastsq algorithm.

2.10. Gdn HCl unfolding

A Cary Eclipse fluorescence spectrophotometer (Agilent) was used to carry out chemical equilibrium denaturation experiments on proteins samples at a concentration of 1 g/L. The instrument uses an excitation wavelength of 290 nm with a 2.5 mm slit width, and emission at 335 nm with a 5 mm slit width. Decreasing concentrations of GdnHCl were used from 6 M to 3 M in 1 M steps and 2.5 to 0 M in 0.1 M decrements. Samples were allowed to equilibrate for 1 min before acquisition, with three readings taken to ensure the sample was in equilibrium.

2.11. NMR

Protein samples for nuclear magnetic resonance (NMR) experiments were prepared in 25 mM acetate buffer pH 5 with addition of 5% D2O. ^1H NMR experiments were performed on a Bruker Avance 800 -MHZ spectrometer equipped with cryoprobe. We used the standard Bruker Topspin 3.1 pulse sequence *zgpg30*. For all constructs spectra were recorded at increasing temperatures from 5 to 70°C in 5°C increments. The sample was kept for 180 s at each given temperature before starting the experiment. Chemical shifts were referenced according to the water chemical shift dependence on temperature. Signals were integrated between -0.3 and -1.7 ppm which corresponds to the folded protein region. Integrated areas were normalised against the maximum integral value for each mutant and corrected for the change in dynamic viscosity of the buffer as a function of temperature.

3. Results

3.1. Spectroscopic studies indicate mutations do not change native conformation

The effect of mutation on protein conformation was assessed by using circular dichroism (CD) in solutions at pH 5 without sodium chloride. Spectra for all mutants and the wild type are shown in Fig. 2. The positions for the minima and maxima of all spectra vary by less than 1 nm. The close agreement indicates a similar secondary structure for all mutants, with only a small difference observed in the CD spectra of the DSV mutant versus the wild type (WT). A similar fold between all the mutants was also confirmed from intrinsic fluorescence emission spectra data taken for samples at pH 5 and at pH 7 . The location of the emission maxima (listed in Table 1) for all mutants at pH 5 are within 0.6 nm of the wild type position located at 334.6 nm. A red-shift of ca. ~ 1 nm in the peak maxima occurs with a pH shift from 5 to 7 indicating that the aromatic residues become less solvent-exposed [84] possibly because the protein fold is 'more compact' at pH 7 . There is also very little variation of the peak position between all mutants at pH 7 .

3.2. Overcharged mutants exhibit lower conformational stabilities

The relative stabilities of the mutants were compared by monitoring intrinsic fluorescence as a function of guanidium hydrochloride concentration. A 2-state denaturation model, which assumes an equilibrium between folded and denatured forms, did not provide adequate agreement with the data. This is consistent

Fig. 2. Circular dichroism spectra between 260 and 190 nm for wild type (Wt) and each mutant at pH 5.

with other studies on scFv proteins, which indicate multiple unfolding transitions [98]. The free energy of unfolding cannot therefore be determined from the fluorescence profiles; instead we report the denaturant concentration at the midpoint of the fluorescence change (referred to as C_{mid}) in Table 1 to provide a measure of the relative stabilities. A lower midpoint should reflect a lower conformational stability because less denaturant is required to unfold the protein. The mutants with the lowest midpoint concentrations correspond to the patch-charged mutants (5K, 5E, 5R) and the arginine-lysine swap mutant 4RK.

Differential scanning fluorimetry (DSF) data using the extrinsic fluorophore SYPRO® Orange was used to further characterise the thermal stability of the mutants. In a DSF experiment, the fluorescence spectrum of the dye molecule is monitored as a function of temperature. The fluorescence depends sensitively on the hydrophobic environment of the dye. In free solution, there is negligible fluorescence from the dye, while the fluorescence increases when the dye binds to hydrophobic regions of the protein that are exposed upon unfolding. The thermal unfolding temperature (T_{DSF}) corresponds to the temperature midpoint of the lowest temperature unfolding transition. The results of the measurements in solutions at pH 5 are tabulated in Table 1. The T_{DSF} is equal to 45.0 °C for the wild type protein. The basic mutants 5R and 5K exhibit significantly lower thermal unfolding transitions (T_{DSF} equal to 38.5 and 37.2 °C, respectively), while that of 5E is only slightly lower (T_{DSF} equal to 43.3 °C). The thermal transitions for all other mutants are similar to or approximately 1 °C greater than the wild type.

The mutants 5K and 5R exhibit lower chemical and thermal stability than the wild type. The decrease in conformational stability is likely due to intra-molecular charge repulsion arising from the relative proximal locations of the mutated basic groups. A similar charge repulsion is also expected for the 5E mutant as theoretical calculations indicate the acidic groups should be fully deprotonated at pH 5. The chemical stability of 5E is indeed similar to 5K and 5R, but the thermal stability is intermediate of 5K or 5R and the wild type. Because mutations have been introduced on a positively charged template at pH 5, the intramolecular charge repulsion is less for the negatively charged patch variant versus the positive variants, which might explain why 5E appears more stable than 5K or 5R.

In the Supplementary Information we provide results from differential scanning calorimetry of the wild type in solutions at pH 5, which was also used to assess the thermal folding stability. The thermal scan shown in Fig. S2 indicates a large endothermic tran-

sition at a temperature of 69.5 °C, but no peak is detected near the transition temperature expected from the DSF experiment (T_{DSF} equal to 45.0 °C for the wild type). The large difference in temperatures indicates multiple unfolding transitions. The large thermal signature of the high temperature transition indicates this corresponds to global unfolding, while the low temperature transition likely corresponds to unfolding of a localized region on the protein. The low temperature transition creates the aggregation prone states as aggregation of the wild type begins to occur at 35 °C.

3.3. Thermal ramped NMR provides relative measure of monomer loss

NMR was used to provide insight into the temperature-ramped kinetic behaviour of the mutants in the pH 5 buffer. Estimates of monomer concentration can be determined from integrating the peak intensities obtained from a 1D 1H NMR scan. Only monomers contribute to the 1H NMR signal, as signals from aggregates over 100 kDa broaden to the point where they are not detected [12]. Fig. 3 shows a plot of the normalized peak area with increasing temperature, in 5 °C increments between 20 and 70 °C. The relative monomer-loss rates are reflected by T_{NMR} (shown in Table 2), which corresponds to the temperature where approximately one half of the protein is aggregated. Monomer loss occurs at the lowest temperatures for the over-charged mutants, while the wild-type, 4RK, and 7KR mutants exhibit the slowest monomer loss.

3.4. Protein-protein interactions characterized in terms of k_D

Characterizing native-state protein-protein interactions from diffusion coefficient measurements in terms of k_D values requires carrying out measurements on solutions without any detectable levels of aggregates. To check how the dynamic light scattering analysis changed with aggregate formation, we carried out extended isothermal runs for the wild type at temperatures of 29 °C and 33 °C. At 29 °C, the radius of hydration (R_H) and the polydispersity (P_d) remained constant for a period of 3 h. At 33 °C, after 10–30 min, the onset of aggregation was reflected by a gradual increase in R_H and a dramatic increase in the P_d from 10% to values much greater than 20%, or in most cases a multimodal population formed. As such, we only report k_D values for conditions corresponding to a monomodal size population when P_d values remained constant on the time scale of the experiments (~3 h). In most instances P_d values were less than 10%, indicating a monodispersed sample. However, for a small number of samples noted below, we observed P_d values greater than 10% but less than 20%, indicating the presence of small oligomers.

Fig. 3. Fractional integrated 1H NMR peak intensities for samples at pH 5 as a function of temperature for (a) WT, 1SB, 2SB, 3SB, and DSV and (b) WT, 4RK, 5E, 5K, 5R, and 7KR. All spectra were taken at a protein concentration of 2.85 mg/mL. Lines are drawn as a guide to the eye.

Table 2

Measured aggregation and protein-protein interaction parameters. k_D values at 20 °C at pH 5 without NaCl (denoted by *) and with 125 mM NaCl (denoted by **), temperature onset of aggregation T_{DLS} at pH 5 without NaCl (denoted by *) and with 125 mM NaCl (denoted by **), temperature mid-point of monomer loss by NMR T_{NMR} at pH 5.

Mutant	k_D (mL/g) 20 °C *	k_D (mL/g) 20 °C **	T_{DLS} (°C) *	T_{DLS} (°C) **	T_{NMR} (°C)
WT	0.9 ± 0.5	−2.6 ± 0.5	37	26–28	55.7 ± 0.4
DSV	0.4 ± 0.2	−2.8 ± 0.3	36	25–26	53.9 ± 0.3
5K	11.4 ± 1.9	−13.0 ± 2.5	30	21–24	44.2 ± 0.5
5E	−9.4 ± 0.5	−5.6 ± 2.2	29	22	47.5 ± 0.2
5R	−4.3 ± 0.5	−10.3 ± 1.8	30	26–29	45.1 ± 0.5
3SB	2.4 ± 0.5	−7.4 ± 1.1	36	23–26	50.4 ± 0.2
2SB	1.7 ± 0.7	−1.4 ± 0.7	36	28	50.8 ± 0.3
1SB	−0.5 ± 0.6	−5.0 ± 1.3	37	27–28	50.0 ± 0.4
7KR	0.3 ± 0.6	−6.7 ± 0.7	35	23–26	52.2 ± 0.5
4RK	2.3 ± 1.3	−3.6 ± 1.6	>43	31	56.4 ± 0.9

Table 1 shows the infinite-dilution hydrodynamic radius $R_{H,0}$ of each mutant measured at 20 °C in the pH 5 buffer. Each mutant has a similar size to the wild type equal to 2.41 nm providing additional support that mutations have not impacted protein conformation. The exceptions are two of the charged mutants 5E and 5R with increased radii of 2.60 and 2.52 nm respectively. The slight increase in size may be due to partial protein expansion near the highly charged patch introduced by the mutations.

k_D values are shown for each of the mutants in solutions at pH 5 without any added salt in Fig. 4a and b, and with 125 mM sodium chloride in Fig. 4c and d. Values taken at 20 °C are also tabulated in Table 2. Increasingly positive values of k_D reflect stronger net protein-protein repulsion, while increasingly negative values correspond to enhancing attractive protein-protein interactions. Protein-protein interactions are insensitive to temperature, except for a noticeable increase in repulsion for all mutants at 5 °C.

Changes to the values of k_D with increasing ionic strength can be rationalized in terms of electrostatic interactions using the double layer force derived from Derjaguin-Landau-Verwey-Overbeek (DLVO) theory [6,59,71]. The double-layer force is given by a repulsive Yukawa potential that has a magnitude proportional to the protein net charge squared and follows an exponential decay with a range given by the Debye length (equal to the inverse of the Debye-Huckel parameter κ). Using the repulsive Yukawa potential, the calculated decrease in k_D when increasing sodium chloride concentration from 0 to 125 mM is 3.0 mL/g and 4.7 mL/g for a theoretical protein charge equal to 5e and 6e, respectively. The calculated change is in good agreement with the measured values of k_D

for 4RK, wild type, 1SB, 2SB and DSV proteins (see Table 2 for corresponding k_D values). There is a much larger drop in the k_D value for 3SB equal to 10 mL/g reflecting a stronger salt-induced self-association through an unknown mechanism.

The effect of charged mutations on the protein-protein interactions for the 5K mutant was manifested by a much larger k_D value when compared against the wild-type for the solutions without added salt (11.4 mL/g for 5K versus 0.9 mL/g for wild-type). A much larger electrostatic repulsion is due to the increase in net charge of 5K. A similar enhancement in protein-protein repulsion is expected for 5R. Interestingly, protein-protein interactions for 5R (k_D equal to −4.3 mL/g) are more attractive than for the wild-type appearing to indicate the absence of an electrostatic repulsion. The 5R mutant samples exhibited higher polydispersities than the other mutants (~20% compared to <10%). High polydispersities are indicative of small oligomer formation, and therefore make interpretation of k_D values in terms of simplified potential models more difficult.

On the other hand, the 5E mutant exhibited the strongest protein-protein attraction of all the variants in solution without sodium chloride (k_D equal to −9.4 mL/g), which is expected due to the low net charge of 5E at pH 5. For 5E, the salt-induced increase in k_D reflects the presence of an electrostatic attraction between proteins [48,55,71]. This behaviour occurs when proteins have near net neutral charge and large anisotropic charge distributions, which likely arises in 5E, from engineering the negative charge patch on a positively charged scaffold.

The k_D value for 5K in solutions with added sodium chloride equal to −13.0 mL/g is much lower than the corresponding value for the wild type equal to −2.6 mL/g indicating the presence of a strong short-ranged attractive interaction. Similarly, the other overcharged mutant 5R also exhibited enhanced protein-protein attractions relative to the wild type.

3.5. Thermal ramp dynamic light scattering studies

Dynamic light scattering (DLS) experiments were recorded as a function of temperature for each of the mutants in solutions at pH 5 without added salt and with 125 mM sodium chloride. The reported values of R_H are a weighted-average, which biases the measurement towards the aggregate population. Nevertheless, the temperature at which aggregates are initially detected provides a relative indicator for the monomer loss kinetics as the initial formation of aggregates will deplete the monomer population. As such, for each mutant, we define an aggregation onset temperature (T_{DLS}) as the temperature when the hydrodynamic size increases by more than 0.1 nm relative to the previous temperature. At T_{DLS} , we found a large increase in polydispersity above 20% from cumulant fitting and a transition from a monomodal to a multimodal population (a decrease in monomer peak intensity below 95%) for all mutants except 5K and 5R. The values of T_{DLS} for samples

Fig. 4. k_D values measured as a function of temperature in pH 5 solutions for WT, 1SB, 2SB, 3SB, DSV (a) without added sodium chloride, and (b) with 125 mM sodium chloride, and for 5R, 5E, 5K, 4RK, 7KR (c) without added sodium chloride, and (d) with 125 mM sodium chloride. Lines are drawn as a guide to the eye.

at a concentration of 1 g/L are reported in Table 2. For the solution conditions without any added sodium chloride, there is good agreement between the ranking of the mutants according to their T_{DLS} values and the T_{NMR} values, where the latter provides a direct measure of the monomer loss as a function of temperature. The overcharged mutants are the least stable mutants according to both measurements, while the 4RK mutant is the most stable. The only discrepancy is the wild type, which exhibits a T_{DLS} value similar to the salt bridge mutants, but the T_{NMR} value is more similar to the 4RK mutant.

In Fig. 5 the measured hydrodynamic sizes are reported as a function of temperature for the wild type, the salt bridge mutants, 4RK, each at different protein concentrations in solutions at pH 5 without added salt. At every protein concentration, the initial onset of aggregation occurs consistently at 1–2 °C higher for the 1SB and wild-type when compared against the 2SB and 3SB mutants. However, a cross-over temperature is observed where the R_H values for 1SB and for the wild type become larger than for 2SB or 3SB. Above this temperature, the main contribution to the average R_H value is from growing aggregates, rather than from any new aggregates formed by nucleation. As such, the smaller dR_H/dT values indicate slower aggregate growth rates for 2SB and 3SB when compared against 1SB or the wild type. Conversely, the lower values of T_{DLS} for 2SB and 3SB give insight into the relative rates of nucleation. The aggregates detected initially by dynamic light scattering at T_{DLS} have undergone aggregate growth and nucleation as they are much larger than typically sized nuclei observed in aggregation pathways. However, because 2SB and 3SB exhibit slower aggregate growth, the earlier detection of their aggregates might be indicative of a faster nucleation step compared to 1SB and wild type.

The 4RK mutant exhibits a much slower increase in size than any of the other mutants as the aggregation onset temperature is more than 5 °C greater than the wild type at all protein concentrations. There is insufficient data to determine whether or not the decreased aggregate formation is due to slower nucleation or slower aggregate growth.

A comparison of the temperature profiles of R_H for 5E, 5K, and 5R at pH 5 are shown in Fig. 6. The initial increase in R_H was observed at significantly lower temperatures than for the other mutants. The aggregation behaviour exhibited by 5E follows a similar pattern to the wild type. At T_{DLS} , bimodal distribution of protein sizes was observed indicating the immediate formation of larger aggregates on the timescale of the experiment. This con-

Fig. 6. Hydrodynamic size R_H plotted versus temperature for solutions at pH 5 without sodium chloride at varying protein concentration for (a) 5K (open symbols) and 5R (closed symbols), and (b) 5E. Protein concentrations shown in legend have units of g/L. Lines are drawn as a guide to the eye.

trasts with the behaviour exhibited by the 5K and 5R mutants. For the 5K and 5R mutants, the measured correlation function can be fitted to a monomodal population at temperatures up to 5 degrees greater than the aggregation onset temperature. Decreased aggregate growth rates of 5R or 5K are immediately apparent from comparing the R_H values shown in Fig. 5a for the wild type and Fig. 6a for either 5R or 5K. At a protein concentration of 1 g/L, wild type aggregates are not detected until 37 °C, while aggregates of 5R or 5K are formed at 32 °C. However, at 38 °C, the R_H values are already greater for the wild type than for 5R or 5K.

The lower values of T_{DLS} for the overcharged mutants indicate faster rates of nucleation and monomer loss kinetics relative to the wild type. As mentioned previously, we do not know *a priori* if aggregates detected initially by dynamic light scattering reflect only the nucleation step or nucleation and aggregate growth. However, as 5K and 5R exhibit slower aggregate growth, the earlier onset of aggregation can only be rationalized in terms of a faster nucleation step.

A similar set of dynamic light scattering experiments was carried out for each of the mutants at pH 5 with a sodium chloride concentration of 125 mM. For all mutants, when adding 125 mM sodium chloride, there is on average a 10 °C drop in T_{DLS} as tabulated in Table 2. Qualitatively different behaviour was observed in solutions with sodium chloride than without. There was a much larger increase in R_H immediately after the initial onset of aggregation. The aggregation behaviour of all mutants appeared to be more stochastic, where T_{DLS} varied by up to 3 or 4 °C for samples at the same solution condition and protein concentration. The mutants can be broken up into three groups based on their onsets of aggregation T_{DLS} . Mutants 5E, 5K, and 3SB aggregated at the lowest temperature, whereas 4RK aggregated at the highest temperature and the other mutants aggregated at intermediate temperatures.

3.6. Temperature-ramped static light scattering experiments

Static light scattering profiles have been measured as a function of temperature for each mutant in solutions at four different sodium chloride concentrations at either pH 5 or at pH 7. By systematically varying the pH and increasing salt concentration provides insight into the role of electrostatic interactions. Representing data in terms of fitting parameters provides a manageable way for comparing behaviour across the large factorial of experimental conditions (mutants and solution conditions) studied here. Initially each of the light scattering profiles was fit to Eq. (3) to give the values of E_a and T_{SLS} shown in Table 3a and b. The light scattering profiles can only be compared with each other in terms

Fig. 5. Hydrodynamic size R_H plotted versus temperature for WT, 1SB, 2SB, 3SB, 4RK in solutions at pH 5 without sodium chloride at different protein concentrations (a) 1 g/L, (b) 2 g/L, (c) 4 g/L, (d) 9 g/L. Lines are drawn as a guide to the eye.

Table 3
Parameters determined from fitting the temperature-ramp light scattering profiles to Eq. (3) for each mutant as a function of salt concentration at pH 5 (3a) or at pH 7 (3b). $T_{SL,S}$ obtained when E_a/R is fixed at $90,000 \text{ K}^{-1}$ in 3a and fixed at $100,000 \text{ K}^{-1}$ in 3b.

Mutant	$C_{NaCl} = 0 \text{ mM}$				$C_{NaCl} = 25 \text{ mM}$				$C_{NaCl} = 75 \text{ mM}$				$C_{NaCl} = 125 \text{ mM}$			
	$T_{SL,S} \text{ (K)}$	$E_a/R \times 10^3 \text{ (K}^{-1}\text{)}$	$T_{SL,S}^* \text{ (K)}$	$E_a/R \times 10^3 \text{ (K}^{-1}\text{)}$	$T_{SL,S} \text{ (K)}$	$E_a/R \times 10^3 \text{ (K}^{-1}\text{)}$	$T_{SL,S}^* \text{ (K)}$	$E_a/R \times 10^3 \text{ (K}^{-1}\text{)}$	$T_{SL,S} \text{ (K)}$	$E_a/R \times 10^3 \text{ (K}^{-1}\text{)}$	$T_{SL,S}^* \text{ (K)}$	$E_a/R \times 10^3 \text{ (K}^{-1}\text{)}$	$T_{SL,S} \text{ (K)}$	$E_a/R \times 10^3 \text{ (K}^{-1}\text{)}$	$T_{SL,S}^* \text{ (K)}$	$E_a/R \times 10^3 \text{ (K}^{-1}\text{)}$
3a																
WT	303.2 ± 2	74.6 ± 3.3	305.3 ± 1	89.5 ± 3.1	301.3 ± 3	89.5 ± 3.1	301.4 ± 2	96.1 ± 4.2	298.3 ± 2	96.1 ± 4.2	297.6 ± 1	96.1 ± 4.2	296.2 ± 2	93.8 ± 3.2	295.7 ± 2	93.8 ± 3.2
DSV	305.3 ± 2	74.4 ± 1.2	307.3 ± 5	75.7 ± 1	301.7 ± 1.2	75.7 ± 1	303.4 ± 5	86.1 ± 5.7	299.0 ± 1	86.1 ± 5.7	299.4 ± 2	86.1 ± 5.7	296.6 ± 6	79.7 ± 8.6	297.3 ± 1	79.7 ± 8.6
5K	311.0 ± 2	13.4 ± 1.9	307.1 ± 1	38.2 ± 1	307.1 ± 1	38.2 ± 1	307.1 ± 1	76.6 ± 2	302.8 ± 1	76.6 ± 2	303.7 ± 2	76.6 ± 2	300.5 ± 2	84.7 ± 2.8	300.3 ± 2	84.7 ± 2.8
5E	294.5 ± 1	102 ± 2.2	293.6 ± 6	109 ± 28	293.6 ± 6	109 ± 28	293.6 ± 6	120.0 ± 12	293.5 ± 2	120.0 ± 12	299.5 ± 2	120.0 ± 12	293.2 ± 2	115.0 ± 1	297.0 ± 2	115.0 ± 1
5R	307.7 ± 2	22.5 ± 1.2	304.0 ± 1	46.8 ± 1	304.0 ± 1	46.8 ± 1	305.7 ± 1	81.9 ± 6.5	299.4 ± 2	81.9 ± 6.5	302.4 ± 2	81.9 ± 6.5	297.0 ± 1	97.9 ± 8	297.0 ± 2	97.9 ± 8
3SB	303.4 ± 3	56.3 ± 3.4	309.9 ± 2	94.1 ± 6.3	306.2 ± 1	94.1 ± 6.3	306.1 ± 1.3	86.6 ± 4.7	302.0 ± 4	86.6 ± 4.7	301.9 ± 4	86.6 ± 4.7	302.3 ± 4	108.0 ± 7	300.7 ± 3	108.0 ± 7
2SB	308 ± 6	75.7 ± 8.2	309.8 ± 1	88.7 ± 15.0	305.9 ± 1.5	88.7 ± 15.0	306.1 ± 1.3	93.0 ± 9.9	301.5 ± 9	93.0 ± 9.9	298.3 ± 4	93.0 ± 9.9	299.9 ± 9	85.0 ± 12.2	299.7 ± 4	85.0 ± 12.2
1SB	304.1 ± 3	76.0 ± 24.6	306.1 ± 2	96.9 ± 8.1	303.2 ± 6	96.9 ± 8.1	302.0 ± 1	73.8 ± 8.4	298.6 ± 7	73.8 ± 8.4	298.3 ± 4	73.8 ± 8.4	296.4 ± 1	90.0 ± 8.7	296.4 ± 2	90.0 ± 8.7
7KR	301.2 ± 3	66.4 ± 6.6	301.2 ± 3	43.3 ± 1.4	295.2 ± 1	43.3 ± 1.4	295.2 ± 1	126.0 ± 1	289.9 ± 1	126.0 ± 1	287.0 ± 3	126.0 ± 1	287.0 ± 3	130.0 ± 8	287.0 ± 3	130.0 ± 8
4RK	309.0 ± 1	55.2 ± 1.8	304.0 ± 7	86.8 ± 11.8	304.0 ± 7	86.8 ± 11.8	304.0 ± 7	101 ± 5	300.8 ± 1	101 ± 5	299.2 ± 7	101 ± 5	299.2 ± 7	105.4 ± 5.0	298.3 ± 1	105.4 ± 5.0
3b																
WT	297.7 ± 4	95.9 ± 9.1	297.9 ± 1	99.0 ± 9.3	298.1 ± 6	99.0 ± 9.3	298.2 ± 2	101 ± 5	298.3 ± 2	101 ± 5	298.2 ± 1	101 ± 5	298.7 ± 2	111 ± 10.3	298.3 ± 1	111 ± 10.3
DSV	298.8 ± 7	93.8 ± 12.2	299.4 ± 2	89.9 ± 2.8	298.3 ± 2	89.9 ± 2.8	299.3 ± 1	96.9 ± 4.2	298.5 ± 2	96.9 ± 4.2	299 ± 1	96.9 ± 4.2	299.2 ± 4	136.3 ± 1.6	298.4 ± 2	136.3 ± 1.6
5K	301.3 ± 2	109.6 ± 9.2	301.3 ± 2	128.1 ± 11.0	301.1 ± 4	128.1 ± 11.0	301.1 ± 4	126.3 ± 8.5	299.8 ± 2	126.3 ± 8.5	303.3 ± 1	126.3 ± 8.5	299.4 ± 1	106.9 ± 9.5	302.6 ± 1	106.9 ± 9.5
5E	307.1 ± 2	63.0 ± 6.7	305.4 ± 2	83.2 ± 4.7	305.4 ± 2	83.2 ± 4.7	305.4 ± 2	90.8 ± 6	303.3 ± 1	90.8 ± 6	303.3 ± 1	90.8 ± 6	302.6 ± 1	149.8 ± 4.1	302.6 ± 1	149.8 ± 4.1
5R	297.3 ± 1	125.6 ± 3.4	296.9 ± 3	130.9 ± 7.4	296.9 ± 3	130.9 ± 7.4	296.9 ± 3	137.8 ± 11.8	295.6 ± 2	137.8 ± 11.8	303.7 ± 2	137.8 ± 11.8	303.8 ± 4	99.1 ± 8.9	303.9 ± 1	99.1 ± 8.9
3SB	303.7 ± 4	109.4 ± 14.5	303 ± 3	118.6 ± 17.6	304.7 ± 5	118.6 ± 17.6	303.3 ± 2	106.2 ± 16.4	304.2 ± 5	106.2 ± 16.4	302.8 ± 1	106.2 ± 16.4	303.4 ± 1	105.9 ± 1.5	302.8 ± 1	105.9 ± 1.5
2SB	308.0 ± 6	75.7 ± 8.2	302.8 ± 6	88.3 ± 13.5	301.6 ± 1.1	88.3 ± 13.5	302.3 ± 3	96.0 ± 3.5	302.3 ± 3	96.0 ± 3.5	299.6 ± 1	96.0 ± 3.5	300.1 ± 1	106.3 ± 1.2	299.6 ± 2	106.3 ± 1.2
1SB	298.4 ± 2.3	96.4 ± 16.6	298.9 ± 1.2	99.5 ± 18.0	299.3 ± 8	99.5 ± 18.0	299.3 ± 1	107.2 ± 3.8	300.1 ± 1	107.2 ± 3.8	289.9 ± 1	107.2 ± 3.8	287.0 ± 3	134.2 ± 29.0	287.0 ± 3	134.2 ± 29.0
7KR	301.0 ± 3	66.4 ± 6.6	301.0 ± 3	43.3 ± 1.4	295.2 ± 2	43.3 ± 1.4	295.2 ± 2	73.7 ± 8.4	289.9 ± 1	73.7 ± 8.4	299.7 ± 1	73.7 ± 8.4	299.7 ± 1	118.5 ± 9.8	299.7 ± 1	118.5 ± 9.8
4RK	299.7 ± 1	114.7 ± 4.6	299.7 ± 1	133.1 ± 6.5	299.8 ± 1	133.1 ± 6.5	299.8 ± 1	128.6 ± 4.5	299.8 ± 1	128.6 ± 4.5	299.8 ± 1	128.6 ± 4.5	299.8 ± 1	128.6 ± 4.5	299.8 ± 1	128.6 ± 4.5

of $T_{SL,S}$ values obtained for the same E_a value due to the strong correlations between these parameters. As such, the data were also fitted while holding E_a fixed. For the wild-type, DSV, and salt bridge mutants, when fitting both parameters, E_a slightly increases when increasing sodium chloride concentration from 0 to 25 mM, after which it remains relatively constant at $90,000 \text{ K}^{-1}$ for the solutions at pH 5 and at $100,000 \text{ K}^{-1}$ for pH 7. These values were constrained in the fitting to determine the $T_{SL,S}^*$ values shown in Table 3, where we use the * to denote the fit value corresponds to constraining E_a . In all cases, a goodness of fit r^2 value greater than 0.99 was obtained with the exception of the 3SB ($r^2 = 0.95$) mutant at 25 mM ionic strength at pH 5. The experimental and fit temperature profiles for the wild type and the salt bridge mutants 1SB, 2SB, and 3SB at pH 5 without added salt and with 125 mM sodium chloride are compared in Fig. 7a through d. For the mutants 5R, 5K, 5E, 4RK, and 7KR, reasonable fits to the data are only obtained when varying E_a and $T_{SL,S}$, except for 5K or 5R at higher salt concentrations with pH equal to 5, when reasonable fits are obtained with E_a equal to $90,000 \text{ K}^{-1}$.

The fitted values of $T_{SL,S}^*$ for the wild-type, salt bridge mutants, and DSV are shown graphically in Fig. 8a and b. The meaning of $T_{SL,S}^*$ is clear from comparing the values to the light scattering profiles shown in Fig. 7. For the wild type and each of the salt bridge mutants, the approximate 10°C decrease in $T_{SL,S}$ when increasing sodium chloride concentration from 0 to 125 mM corresponds to

Fig. 7. Static light scattering profiles shown as a function of temperature for wild type (WT) in solutions with varying sodium chloride concentration at (a) pH 5 and at (b) pH 7, and for WT, 1SB, 2SB, and 3SB in solutions at pH 5 (c) without sodium chloride and (d) with 125 mM sodium chloride. Solid lines are fits to Eq. (3), where fit parameters are given in Table 3a. All samples are at a protein concentration of 1 g/L.

Fig. 8. Fit values of $T_{SL,S}^*$ plotted versus ionic strength for WT, 1SB, 2SB, 3SB, DSV in solutions (a) at pH 5 and (b) at pH 7.

the shift along the temperature axis in the corresponding experimental light scattering curve.

4. Discussion

4.1. Changes in T_{SL}^* reflect relative aggregate growth rates

The time evolution of aggregate population is expected to reflect both the rate of aggregate growth and the rate of nucleation, as nucleation determines the initial concentration of seeds or nuclei. However, because changes to only one parameter T_{SL}^* are required to capture differences in the light scattering profiles, the data can be differentiated from each other based on only one characteristic rate constant. The characteristic timescale is most likely related to an aggregate growth rate as the fitting is sensitive to the part of the profile where the light scattering reading is biased towards the aggregate population. A similar observation has been made from isothermal measurements of light scattering profiles for a range of antibody molecules under different solution conditions (e.g. temperature, pH, and for various salt types). The profiles collapse on a master curve when rescaled by a characteristic aggregate growth rate constant [5,73]. This universal behaviour is predicted using population balance modelling only when the nucleation step can be neglected and when aggregate growth occurs through the same pathway dominated by aggregate-aggregate coalescence rather than by chain polymerization [5].

As such, we expect T_{SL}^* values to reflect relative growth rates; a decrease in T_{SL}^* will correlate with an increase in aggregate growth rate. The corresponding ranking of the mutants at pH 5 follows the order 1SB ~ wild type > 2SB ~ 3SB, which is remarkably similar to that observed in the dynamic light scattering experiment. Indeed, similar R_H profiles (shown in Fig. 5a) are observed for wild type and 1SB and for 2SB and 3SB, where the latter grouping exhibits the slower aggregate growth (as reflected by the lower dR_H/dT values).

The ionic strength and pH trends of T_{SL}^* reflect the expected changes in aggregate growth rates due to the impact of electrostatic interactions, which has been rationalized using DLVO theory [5,19,63]. A non-specific increase in aggregate growth rates occurs when the repulsive double-layer forces are reduced either by changing pH to reduce protein net charge or by electrostatic screening through increasing in the ionic strength [5,43,47,60,61,63,66]. The electrostatic interactions are greater for the scFv at pH 5 versus pH 7 due the larger net charge (see Table 1 for theoretical estimation of net charge). As such, the decrease in T_{SL}^* for each mutant at pH 5 with increasing sodium chloride concentration is due to screening electrostatic repulsion between growth units. At pH 7, the values of T_{SL}^* remain invariant with ionic strength indicating no electrostatic repulsion as expected since the proteins have a net charge close to 0e.

A key question is why the static light scattering profiles are insensitive to changes in nucleation rates for the systems described by the same activation energy. One possibility is that nucleation rates are similar across the corresponding set of mutants and ionic strength conditions. This seems plausible when comparing the behaviour of the mutants in solutions at pH 5 without added salt as the initial onset of aggregation (as reflected by the T_{DL}^* values shown in Table 2) across the wild type and salt bridge mutants varies only by 1 °C. However, for each mutant, when increasing sodium chloride concentration to 125 mM, there is a substantial decrease in the T_{DL}^* values. Possibly, the earlier onset of aggregate detection at high salt is due to an increase in aggregate growth rate, rather than increased nucleation. However, if this was true, the salt-induced changes to the T_{DL}^* values and T_{SL}^* values should follow similar trends. This observation holds true for all mutants

except 3SB, which has an earlier onset of aggregation, even though the aggregate growth rate appears to be slower compared to the other salt bridge mutants and wild type.

4.2. The patch-charged mutants

The dynamic light scattering and NMR studies indicated that introducing either a positive or negative charged patch on the scFv caused significant changes to both the nucleation and aggregate growth rates. The measured static light scattering profiles as a function of pH and ionic strength for the overcharged mutants 5K and 5R shown in Fig. 9 provide additional insights into how electrostatic interactions alter their aggregate growth steps. The behaviour observed for 5K and 5R at low ionic strength is consistent with the dynamic light scattering studies indicating these mutants exhibit much lower aggregate growth rates. Changing ionic strength has a dramatic effect on the light scattering profiles indicating strong repulsive electrostatic interactions between aggregating units at low ionic strength, which is due to increased net charges on 5K or 5R due to mutation.

The dramatic changes in aggregate growth behaviour observed for the 5R and 5K mutants reflect a change in growth pathways with increasing ionic strength, which has been previously observed for proteins such as α -chymotrypsinogen [51] and a handful of monoclonal antibodies [8,9,43,75]. With decreasing strength of electrostatic interactions, there is a transition from nucleation dominated growth (aggregates form but do not grow) to chain polymerization (aggregates only grow by addition of monomers or small building blocks) to aggregate-aggregate coalescence and finally to aggregate precipitation. At pH 5, for both 5R and 5K, dynamic light scattering indicates a monomodal size population with low polydispersity reflecting the presence of small oligomers with size just greater than monomer. With increasing temperature further, a bimodal population distribution develops indicating oligomers grow to form larger aggregates, but the large aggregates do not precipitate even at the highest temperature of 90 °C measured during the static light scattering experiment. This behaviour reflects conditions with strong electrostatic repulsion, where nucleation dominated growth followed by chain polymerization occur with increasing time or temperature [10,71,75]. This pattern should be contrasted with what happens when electrostatic repul-

Fig. 9. Static light scattering profiles shown as a function of temperature for WT, 5E, 5K, and 5R in solutions at (a) pH 5 without sodium chloride, (b) pH 7 without sodium chloride, (c) pH 5 with 125 mM sodium chloride, and (d) pH 7 with 125 mM sodium chloride. Solid lines are fits to Eq. (3), where fit parameters are given in Table 3a and b. All samples are at a protein concentration of 1 g/L.

sion is weakened by increasing ionic strength. For 5R and 5K with 125 mM sodium chloride, at the temperature onset of aggregation, there is a bimodal size population with high polydispersity indicating immediate formation of large aggregates. Further, aggregate precipitation occurs at higher temperatures in the static light scattering experiment. Similar characteristics are observed for the other scFv proteins (WT, 1SB, 2SB, 3SB, and DSV) at all salt concentrations. This behaviour reflects aggregation pathways governed by much weaker electrostatic interactions, where aggregate growth initially occurs through chain polymerization followed by aggregate–aggregate condensation and then precipitation either with increasing time or temperature [11,75].

5K and 5R exhibit other characteristics observed in the aggregation behaviour of highly charged proteins such as antibodies [11,43,75] and α -chymotrypsinogen [51] in acidic conditions. The increase in net positive charge at low pH causes an increase in intramolecular charge repulsion and a reduced conformational stability. This, in turn, correlates with a faster nucleation step and increased monomer loss kinetics. We also find introducing the charged patch on 5K, 5R, (and 5E) leads to a lower conformational stability as is evident from the lower denaturant midpoint concentration relative to the wild type. Conversely, the faster monomer loss kinetics have been inferred from the NMR and dynamic light scattering studies.

The 5E mutant at pH 5 exhibited the fastest aggregate growth rates as reflected by the dynamic light scattering experiment (see Fig. 6b) or according to the static light scattering profile shown in Fig. 9a and c. Part of the reason is likely due to the absence of any electrostatic repulsion as the theoretical net charge of 5E at pH 5 (equal to 1.6e) is much less than any of the other mutants. Further, introducing a negatively charged patch on a positive scaffold creates a large anisotropic charge distribution, which also causes an electrostatic-driven self association. This behaviour is consistent with the increase in the k_D value for 5E when adding sodium chloride. Previous studies have found a strong correlation between increased aggregation rates and electrostatic self-association [13,55,80,99], which might also explain the increased aggregation of 5E.

In contrast, at pH 7, the aggregate growth rates of the 5E mutant with and without added sodium chloride are reduced relative to the 5R or 5K mutants as indicated by the reduced light scattering profiles shown by Fig. 7b and d, even though the absolute net charge calculated for 5E is less than that for either 5R or 5K (see Table 1). The results suggest that patch-charging with acidic versus basic groups is more effective when introducing charge on a near net neutral template. Negatively charged groups also appear to be more effective at preventing self-association of the native protein at moderate salt concentrations when electrostatic interactions are sufficiently screened. In solutions at pH 5 with 125 mM sodium chloride, the k_D value for 5E is much greater than either the 5K or 5R mutant (see Table 2). The increased solubility observed here of negative versus positive charged groups has also been deduced from mutation studies of Ribonuclease SA [89] and from the salting-out behaviour for a series of seven proteins [44].

4.3. Correlations between k_D and aggregation propensity

Much recent research has explored the link between protein–protein interactions, recorded through B_{22} or k_D measurements, and aggregation behaviour, usually probed under non-native or accelerated conditions. A decreasing k_D value is often correlated with increased aggregation propensity when reducing electrostatic repulsion by increasing ionic strength or using a salt or buffer ion that neutralizes protein charge [35,46,57,74,78,82,19,46,63,77,81]. Consistent with these studies, for the wild-type, SB and DSV mutants, the relative rates of aggregate growth (as reflected by

the corresponding T_{SL}^* values) and protein–protein interactions (as reflected by the k_D values) decrease non-specifically upon addition of NaCl due to screening of electrostatic interactions. Mechanistically, a reduction in growth rates occurs due to a cross-over in aggregation pathways from aggregate–aggregate coalescence to chain polymerization to nucleation dominated growth, which has also been correlated with native-state protein–protein interactions in terms of B_{22} values [9,43,75]. The overcharged mutants, 5R and 5K, both exhibit a similar ordering of aggregate growth mechanisms with reducing sodium chloride concentration at pH 5 reflecting a strong electrostatic repulsion arising from the increased protein net charge. The electrostatic repulsion should also be reflected by the native-state protein–protein interactions. Indeed the k_D value for 5K is much larger than for the wild type as expected, but the corresponding k_D value for 5R is surprisingly lower. As such, for the 5R mutant, the charged properties of the protein, rather than the value for k_D , provide a better predictor for the aggregate growth behaviour. We expect the electrostatic repulsion exists when in the native state, but is hidden by the presence of strong self-association as k_D values reflect an averaged protein–protein interaction.

It is also of interest to examine other correlations of the native state protein–protein interactions with aggregate growth rates. There is no clear relationship when correlating k_D values for the wild type and the set of mutants 1SB, 2SB, 3SB, and DSV and their corresponding T_{SL}^* values obtained at 125 mM sodium chloride. As a good example, out of this group, the slowest aggregate growth rates are observed for 3SB, but 3SB exhibits the strongest self-association (or lowest k_D value). Another example is given by comparing the 5K mutant to the wild type in solutions at pH 5 with 125 mM sodium chloride. The static light scattering profile shown in Fig. 9c for 5K is accurately fit to determine a T_{SL}^* value equal to 303.2 K (see Table 3a). This value is much greater than that for the wild type indicating a slower aggregate growth rate, even though 5K exhibits the strongest native-state association out of any mutant ($k_D = -13.0$ mL/g). These examples indicate the association steps involved in aggregate growth are determined by partially unfolded regions of the protein that do not contribute to native state protein–protein interactions [4].

4.4. Lysine protects partially unfolded regions from associating

The 5K mutant is more resistant to aggregation than the 5R mutant under all solution conditions. In solutions without added salt at pH 5, both mutants appear to exhibit nucleation-dominated growth with similar onset temperatures of aggregation. However, when comparing 5R to 5K, the sizes of soluble oligomers are larger and a population of large aggregates is detected at a much lower temperature. At moderate ionic strength, 5R forms larger aggregates than 5K over all temperatures investigated. In Fig. 10, the static light scattering profiles are shown for the arginine-lysine swap mutants and the wild type in solutions at pH 5. The increased aggregation resistance of 4RK and, conversely, the decreased resistance of 7KR provides additional support that arginine to lysine mutations can stabilize proteins against aggregation.

In all cases, swapping arginine for lysine leads to reduced aggregate formation and growth. For 7KR, the increased aggregation propensity is correlated with a slightly lower k_D value reflecting more attractive interactions between the native-state proteins. However, the increased aggregation resistance of 4RK over the wild type does not correlate with increased conformational stability or reduced native-state protein–protein attraction; the k_D values for 4RK are similar to wild type at low and high sodium chloride concentration. At high salt concentration, 5K exhibits the greatest native-state self association, but 5R exhibits faster aggregation kinetics. As such, the stabilizing effectiveness must be related to

Fig. 10. Static light scattering profile as a function of temperature for WT, 4RK and 7KR in solutions at pH 5 without sodium chloride (closed symbols) and with 125 mM sodium chloride (open symbols).

the ability of lysine groups to reduce interactions between partially folded states. We stress that the location of the mutations are especially important. Swapping arginine for lysine on the natively folded protein does not lead to any measurable differences in interactions. As such, we postulate aggregation can only be suppressed by introducing extra 'lysines' on protein surfaces when they occur next to partially unfolded regions that expose hot spots buried during nucleation or aggregate growth. The molecular basis for the increased protective ability of lysine over arginine is determined by a couple of factors. Surveys of crystal databases indicate an increased propensity for arginine to be buried in crystal contacts and functional interfaces while lysine groups occur on the rims of crystal contact interfaces [23,24,38,64]. These increased solubilizing effects of lysine have been attributed to the high conformational entropy of lysine and the preference of arginine to form cation- π interactions with aromatic groups [22,30,56].

5. Conclusions

The main aims of this study were two-fold, the first was to provide a more in-depth study of how native-state protein-protein interactions relate to aggregation rates, while the second was to understand better the structural determinants of aggregation propensity. We have largely succeeded in the first task. We have confirmed previous studies indicating that native-state electrostatic interactions correlate with aggregate growth pathways [9,43,71,75] and growth rates [5,19,63], although, we have highlighted a protein, the 5R mutant, where the native-state electrostatic interactions are not apparent from the value of k_D . As such a better indicator of electrostatic interactions might be a direct quantification of the protein electrostatic properties either by theoretical calculations or by experimental verification using electrophoretic measurements. The latter would be more preferred as some commonly-used buffers or salts neutralize protein electrostatic properties [40,41,46,72], which would not be reflected by a theoretical calculation. More importantly, we have provided examples where aggregation does not correlate with conformational stability and native-state protein-protein interactions. As such, there will be limited utility of using k_D values as a predictor for aggregate growth rates in the absence of strong electrostatic interactions [7,78].

The more demanding task is to identify structural mutations that reduce aggregation propensity and then to rationalize the

mechanism as to make the mutational strategy more broadly applicable to other systems. Comparing the behaviour of salt bridge mutants to the wild type indicates the location of the second engineered salt bridge is involved in aggregate growth. The aggregate growth rates for 1SB and wild type and for 2SB and 3SB are similar; only changing the second salt bridge has a significant impact on aggregate growth. As such, this region of the scFv provides an ideal location for studying the impact of other mutations on aggregate growth rates. For the arginine-lysine swap mutants, we have not attempted to identify what regions of the protein surface are involved in aggregation. Nevertheless, we have still gained some important insights. A key problem is to determine the structural requisites that determine the stickiness of partially unfolded regions. One approach for biopharmaceutical aggregation [93,76] is to use primary-sequenced base aggregation predictors that have been primarily developed for amyloid formation by natively unfolded proteins [21,90,29]. We note these predictors do not distinguish between the aggregation suppression effects of lysine over arginine indicating that there are additional factors that control association rates or energetics between partially folded states involved in nucleation or aggregate growth. While these findings are only preliminary, they offer a good starting point for future studies.

Conflict of interest

The authors declare no conflict of interest that would influence the submitted work.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ejpb.2017.01.019>.

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