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Role of electrostatic repulsion on colloidal stability of *Bacillus halmapalus* alpha-amylase

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ABSTRACT

The colloidal stability of charged particles in suspension is often controlled by electrostatic repulsion, which can be rationalized in a semi-quantitative way by the DLVO theory. In the current study, we investigate this approach towards understanding irreversible protein aggregation, using *Bacillus halmapalus* α-amylase (BHA) as a model protein. Repulsive forces between partly unfolded monomers were shown to strongly affect aggregation. Adding salt, increasing valence of counter ions or decreasing pH in the direction of p*I* resulted in a shift in the rate-limiting step from association to unfolding as evidenced by a change in aggregation kinetics from second to first-order in protein concentration. Charge screening effects by salts resulted in increased average size of protein aggregates but only moderately affected the secondary structure of protein within the aggregates. Salt and pH effects could be explained within the DLVO framework, indicating that partially unfolded BHA monomers can be modelled realistically as colloids with a random charge distribution.

1. Introduction

Interest in the molecular mechanisms underpinning irreversible protein self-assembly has recently been much stimulated by a number of medical and technological problems including the role of amyloid fibers in neuro-degenerative diseases [1–4] and the challenges of preventing so-called amorphous aggregates in protein pharmaceuticals [5–10]. Although the mechanisms for protein aggregation remain to be elucidated fully, critical advances have been gained. Thus, it is now clear that the kinetics of irreversible protein aggregation are dependent on both the structural stability of the native protein conformation [8,11,12] and the activation free energy for the kinetically controlled association of the non-native proteins [7,13–15].

The overall aggregation process is often rationalized in the two-step Lumry Eyring scheme [16] (Eq. (1)).

$$N \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} U \overset{k_2}{\rightarrow} Ag \qquad (1)$$

Here N represents the native conformation of the protein, in equilibrium with U, which represents a (partially) unfolded conformation— that irreversibly associates to form an aggregate (Ag). k_1, k_{-1} and k_2 represents rate constants of unfolding, refolding and association respectively. Due to the two-step process from N to Ag, strategies in protein formulation are often directed towards stabilisation of N and ensuring an appropriate repulsion between U (and N). The latter, which is the focus of the current study, is mainly controlled by adjusting pH and ionic strength of the solution [7,13,17] or by covalent modulation of protein net charge by amino acid substitutions or methylation/succinylation [18–20]. The resulting changes in electrical properties of the protein molecule are often rationalized in the framework of the DLVO theory [7,21,22].

The DLVO theory stipulates an interaction potential ($V(h)$, Eq. (2)) consisting of attractive van der Waals (V_A) and electrostatic repulsive potential energies (V_R) [23–26]. Thus if the particles possess a sufficient charge density, their mutual interaction will be dominated by electrostatic repulsion at separation distances (h) greater than h_{VMax}, the separation distance at which maximum repulsion occurs. At intermolecular distances shorter than h_{VMax}, $V(h)$ will be increasingly dominated by attractive van der Waals forces until a distance where $V(h)$ approaches zero and the two particles will associate.

$$V(h) = \pi R\left(-\frac{H_{121}}{12\pi h} + \frac{64kTC_0\Gamma_0^2}{\kappa^2} exp^{(-\kappa h)}\right) \qquad (2)$$

Here, h is the distance between the particles, T the temperature, k Boltzmann's constant, C_0 the electrolyte concentration, Γ_0 the Gouy Chapman coefficient (related to surface potential of the particle), κ the inverse Debye screening length and H_{121} the Hamaker constant and R

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the radius of the particle. If the potential energy barrier, V_{Max}, control protein aggregation, V_{Max} comprise the activation free energy ($\Delta G^{\#}$) of aggregation.

DLVO theory relies on some critical assumptions, and the question arises if the theory can be used to describe the energetics and kinetics of irreversible protein aggregation. The most important assumptions are that the protein molecules can be described as hard spheres with homogenous surface charge densities, and that electrolytes do not bind to specific sites on the proteins. If these assumptions are violated, non-uniform charge distributions can result in a substantial dipole moment of the protein molecule, resulting in directional electrostatic interactions and deviation from DLVO behaviour [27,28]. However, if the above assumptions hold, increasing salt concentrations (in 0–100 mM range) or decreasing protein net charge (e.g. by changing pH) is expected to increase the rate of aggregation due to a decrease in V_{Max}, observed in a number of experimental studies [29–31]. However, changing the magnitude of V_{Max} should also change the reaction order of aggregation. Thus, according to Eq. (1), aggregation kinetics will be first-order in protein concentration if the unfolding of N to U is slow with respect to the subsequent association of U to form Ag. Conversely, if association of U is rate-limiting, higher-order kinetics will be observed. Thus, DLVO theory suggest that conditions where V_{Max} is large (e.g. low ionic strength or at pH values far from the protein's p*I*) will tend to result in higher-order kinetics, whereas first-order kinetics are more likely when V_{Max} is smaller.

In the current study we address this hypothesis by investigating effects of solution pH, ionic strength and valance of counter ions on the kinetics of heat induced aggregation of *Bacillus halmapalus* α-amylase (BHA). Analysis of the results with respect to aggregation rates, kinetic order and aggregate size suggested that DLVO theory provides a realistic semi-quantitative framework for the colloidal stability of this protein.

2. Method

2.1. BHA aggregation

Recombinant *B.halmapalus* α-amylase (BHA) expressed in *Bacillus licheniformis* was purified to>95%, measured by SDS PAGE and size exclusion chromatography. BHA binds three calcium ions and the holoprotein unfolds at temperatures of 95 °C [32]. At pH 8, aggregation of 20 μM BHA was measured in 5 mM HEPES buffer solutions containing 0–10 mM of respectively NaCl, KCl and $NaC_2H_3O_2$. At pH 9 aggregation of 20 μM BHA was measured in solutions buffered with 5 mM sodium borate containing 0–40 mM NaCl and in 0–120 μM $[Co(NH_3)_6]Cl_3$. All chemicals were of analytical grade and purchased from Sigma, St. Louis, USA. Aggregation in samples incubated at 60 °C was initiated by the addition of EDTA in a molar ratio of 9 (EDTA/BHA) [33]. The chelator removes 1–2 calcium ions and thus destabilizes the otherwise thermo stable protein to an extent where it unfolds and aggregates within a half to a few hours at pH 8–9 depending on the ionic strength. The exchange of calcium from the protein to EDTA is fast and not rate-limiting for the subsequent unfolding and aggregation reactions [33,34]. To obtain information on aggregation kinetics, sub-samples were quenched at regular time intervals during aggregation at 60 °C in the different solutions. Quenching was done by transferring 1 ml aliquots from samples (10 ml, in plastic tubes) to eppendorf tubes on ice. Static light scattering measurements conducted at 25 °C for 1 h confirmed that the population of aggregates and monomers remained unchanged after quenching (data not shown).

2.2. Size exclusion chromatography with multi angle static light scattering and UV detection (SEC-MALLS)

Twenty to forty microliter aliquots of the quenched samples were injected on a Superose 6 column 10/300 GL (GE Healthcare, Uppsala Sweden) and eluted at 0.4 ml min^{-1} using an Agilent 1100 HPLC pump (Agilent Technologies, Waldbronn Germany). The elution buffer was 50 mM borate, pH 9, adjusted with NaOH. The eluted sample first passed through a light scattering detector (DAWN EOS, Wyatt Technology, Santa Barbara, CA) equipped with a K5 flow cell followed by a UV detector (Beckman, Fullerton, CA). Measuring both UV and static light scattering intensity at several angles (θ) allows determination of both the average molecular weight (M_{avr}) and the molar concentration of aggregates (C_n, in moles of particles per liter). The scattering intensity was measured at 16 different angles (15–163°) and the average molecular weight was estimated using Astra 5.1.9.1 by extrapolating $M_{avr}(\theta)$ to zero scattering angle (in so-called Debye plots).

2.3. Infrared spectroscopy

The secondary structure of protein contained in aggregates was assessed by IR spectroscopy. Spectra were acquired with a Bomem, MB-104 spectrometer (Quebec Canada) and analyzed using Grams-32 software. Because this method requires more than 15 mg protein/ml to get high quality spectra, the aggregates were concentrated to ~20 mg/ml by centrifugation over a 100 kD membrane (Amicon ultra, 100 kD, Millipore, Billerica, MA). Centrifugation is not expected to affect the secondary structure of aggregates since experiments at different protein concentrations resulted in similar area-normalized spectra, apart from an increase in the signal to noise ratio. In the case of 40 mM NaCl at 10 mg/ml protein, the aggregates precipitated before centrifugation, and as seen in the result section (Fig. 5), the secondary structure between soluble and insoluble aggregates were very similar. If centrifugation acted on the aggregates through increased aggregate interactions, one would expect that the soluble aggregates changed structure compared to the insoluble. Both background spectra for atmosphere and buffer were subtracted from the protein spectra, and the spectra were area-normalized for comparison [35,36]. Spectra of aggregates formed under conditions resulting in first and second-order kinetics (respectively unfolding and association-limited aggregation, see result section) were measured at pH 9 in 5 mM borate and in 5 mM borate + 40 mM NaCl, respectively.

2.4. Estimations of protein net charge, surface potential and theoretical rate constants of aggregation

To test results against DLVO theory, we numerically estimated interaction potentials. A simplified model was used where the counter ions surrounding the charged particles were described as one diffusion layer with a distribution of ions following the typical Boltzman distribution. The protein surface charge density was estimated using the software "PROPKA", which provides an empirical prediction of protein pk_a values on the basis of protein crystal structure [37]. The crystal structure of BHA [38] was used, and 2 additional negative charges were added to the calculated net charge due to stripping of one calcium ion with EDTA. The pI of BHA was calculated to 6.0 and the net charge at pH 8 and 9 to −14.2 and −20.7 respectively. Thus, average surface charge density was approximated by the net charge and an estimated radius of gyration of BHA of 2.7 nm assuming spherical shape (radius = 3.5 nm of the corresponding sphere) [39]. Surface potentials were calculated from the above charge densities using Eq. (3), on the assumption of constant surface charge density with salt concentration, thus neglecting a possible Stern layer, meaning that the resulting surface potentials represent an upper limit. Clearly the above rather crude assumptions only suggest a semi-quantitative analysis and the use of Eq. (2) is complicated by the same length scale of the diffusive double layer (κ^{-1}) and radius (R) of BHA, where κ^{-1} is larger than R at the lowest ionic strengths and smaller at the higher ionic strengths (use of an alternative equation with small values of κR is not applicable here due to the high surface potential of BHA at the lowest ionic strengths [23]($\Phi_0 = -130$ mV at 1.7 mM 1:1