

Detection and characterization of hemoglobin dissociation and aggregation using microcalorimetry

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Abstract Using micro-differential scanning calorimetry (μ DSC) to probe hemoglobin structural changes, previously unreported large exothermic events have been repeatedly detected at temperatures below the tertiary-to-secondary structural transition temperature (ca. 72 °C), which could be important in understanding protein subunit association. Given the importance of protein–protein interactions and protein solution stability, this event was characterized with respect to concentration and scan rate. This detailed analysis revealed the formation of aggregates that differed in size, appearance, and settling time. While repeat μ DSC scans confirmed that this aggregation event was irreversible when samples were heated beyond 55 °C, it was also found that aggregation could be prevented through the addition 0.02 M urea. All evidence points to a significant occurrence of a kinetic pathway that leads to an intermediate aggregation event that is believed to be formed from dissociated hemoglobin subunits.

Keywords Microcalorimetry · Transition · Hemoglobin · Association · Thermal transition

Introduction

Large proteinaceous molecules made up of multiple polypeptides or proteins, for example viral capsids [1], have the

potential of being used as drug carriers, slow release drug systems, or even scaffolds for nano-material synthesis. To fully exploit these systems, however, the ability to control their assembly and disassembly is of great importance. Equally as important is to understand the propensity of these particles to aggregate. Multiple polypeptide chains can assemble into a larger functional protein molecule through weak interaction forces or disulfide bridges. Dissociation of the larger structure can occur through the disruption of these forces, which has been documented using various techniques including light scattering [2]. Through the partial dissociation of these larger molecules, it may be possible that smaller subunits can become entangled into a larger complex without significant disruption of the overall conformation of the smaller subunits.

Hemoglobin is often referred to as a model molecule having quaternary structure, and as a result can be used to probe conditions to manipulate protein and polypeptide assembly. Hemoglobin is a heteromeric protein composed of 2 α (leucyl) and 2 β (methionyl) subunit chains [3, 4]. Its quaternary (4°) structure ($\alpha_2\beta_2$) is held together by non-covalent intersubunit bonds. Detailed analysis on hemoglobin structure in solution using NMR revealed that hemoglobin exists in a dynamic intermediate state of tetramers [5]. The dissociation of the 4° structure to the individual subunits requires the disruption of hydrophobic forces, hydrogen bonds, and ionic bridges [2]. 6 M urea causes the $\alpha_2\beta_2$ form of hemoglobin (bovine) to exist in equilibrium with its asymmetrical dissociation products ($\alpha_2 + \beta_2$) [4]. Experiments that detect the heat of very dilute solutions, however, have shown that the $\alpha_2\beta_2$ form of hemoglobin (bovine) can exist in equilibrium with its symmetrical dissociation product ($2\alpha\beta$) [6]. Other types of possible forms in which hemoglobin can exist include $2\alpha_2\beta\beta^* \leftrightarrow 2\alpha\beta + 2\alpha\beta^* \leftrightarrow 4\alpha + 2\beta + 2\beta^* \leftrightarrow \alpha_2\beta_2 +$

This work is being submitted posthumously for the first author YeongHo Suh.

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$\alpha_2\beta_2^*$ (* for clarification of subunit interaction) [7]. The dissociation of hemoglobin has been further studied through other methods, such as spectroscopy, light scattering, and time-resolved photoacoustic calorimetry [8–11]. Calorimetry has also been previously used to study how hemoglobin subunits associate into dimers, from which it was determined that this exothermic event releases $\sim 29.3 \text{ kJ mol}^{-1}$ of dimer formed [12]. Although hemoglobin dissociation has been studied, few if any discuss the tendency of hemoglobin and its subunits to aggregate. Aggregation can be caused by many factors, of which macromolecular crowding is one such process [13]. With respect to protein solutions, aggregation can also occur with the addition of heat [14, 15]. In general, aggregation of proteins is observed after the tertiary structure of the protein denatures and goes through a morphological change [16–18]. Along with intramolecular effects, changes in the surrounding environment also contribute to aggregation [8, 19].

Infrared spectra measurements of a $735 \text{ }\mu\text{M}$ hemoglobin solution revealed that only partial hemoglobin unfolding is needed for aggregation [20]. Exposing the protein solution to rising temperatures ($0.1 \text{ }^\circ\text{C min}^{-1}$) resulted in a single aggregation event, mediated by distinct unstable helical regions becoming solvent-exposed. However, aggregation is not expected to result as a single event. Spectrometric experiments performed on 3 and $150 \text{ }\mu\text{M}$ globin solutions (hemoglobin molecules without the heme group) at an increasing scan rate of up to $2 \text{ }^\circ\text{C min}^{-1}$, revealed the formation of soluble aggregates and insoluble gels made through entanglement of fibrous structures, which were further supported by electron micrographs [18]. More recent studies suggest the possibility that hemoglobin molecules can aggregate at temperatures slightly above body temperature, at around $40 \text{ }^\circ\text{C}$, although the mechanism behind the aggregation is not clear [10, 21, 22]. This latter phenomenon is in contrast to the generally observed protein aggregation at higher temperatures [15, 16, 18, 23–26], which occurs around $72 \text{ }^\circ\text{C}$ for hemoglobin, a temperature where the tertiary-to-secondary structure transition is known to occur [20, 27].

DSC can be used to study both the thermodynamic [17] and kinetic [27] aspects of protein transitions at different temperatures for varying energy input rates [28]. Previous studies using different scan rates [16, 26, 29] have been useful in providing insight on the kinetic dependence of irreversible transitions.

To date, a thorough investigation on the effect of solution concentration or scan-rate dependence when evaluating hemoglobin by DSC has not been done. Although a theoretical basis exists for describing the transitions of multi-meric proteins using the DSC [30], certain phenomena have

not been properly documented. Our work suggests there can be a significant amount of “intermediate aggregation,” an aggregation event happening before the tertiary-to-secondary structure transition that has not been previously reported. This type of subunit association cannot be observed for monomeric proteins. The different behaviors of multi-subunit proteins are a current research topic that is of significant interest for which an extensive review on such interactions exists [31].

Materials and methods

Materials

Lyophilized hemoglobin from bovine blood in powder form, suitable for protease assays, was purchased from Sigma Aldrich (Missouri, USA) and stored at $4 \text{ }^\circ\text{C}$. Urea was of analytical reagent grade (Malinkrodt, Kentucky, USA) and was stored at room temperature. Samples were analyzed using a micro-differential scanning calorimeter, VP-DSC (MicroCal, LLC, Massachusetts, USA). Aggregation events were further analyzed using a light scattering system, 90 plus—particle size analyzer (PSA) (Brookhaven Instruments Corporation, New York, USA). The operating temperature range of the PSA is $5\text{--}74 \text{ }^\circ\text{C}$.

Methods

Preparation of samples

Hemoglobin solutions of different concentrations were prepared using deionized water with an electrical impedance of $18.2 \text{ M}\Omega$ at $25 \text{ }^\circ\text{C}$ generated from a MilliQ apparatus (Millipore Corporation, Molsheim, France).

Conductivity and pH were measured using a FiveEasy pH meter (Mettler Toledo, Schwerzenbach, Switzerland) and a 712 conductometer (Metrohm AG, Herisau, Switzerland). Due to the fact that ionic strength [8] and pH [19] both play a role in the dissociation and conformation of hemoglobin tetramers, we ensured that both of these parameters were the same for all experiments.

Hemoglobin solutions were kept at $4 \text{ }^\circ\text{C}$ for not more than 2 weeks to detect the suspected phenomena, unless used in stability experiments where samples were kept longer. Before each experiment, all samples were sonicated using an ultrasonic bath (Branson Ultrasonics Corp., Connecticut, USA) at room temperature for 5 min. All samples were degassed by vacuum for 15 min using a ThermoVac vacuum pump (MicroCal, LLC, Massachusetts, USA).

DSC experimental condition

All experiments were done under adiabatic and isobaric conditions (25 p.s.i.). Hemoglobin concentrations ranged from 10 to 100 μM . The rate of temperature increase ranged between 0.17 and 1.5 $^{\circ}\text{C min}^{-1}$. For all experimental runs, the initial temperature was 25 $^{\circ}\text{C}$ and the final temperature was 90 $^{\circ}\text{C}$. The reference cell contained degassed deionized water. Almost flat experimental baselines were obtained using deionized water in both the reference and the sample cells for different scan rates. The baseline experiments were done twice for each condition and found to be repeatable. The experimental baselines were subtracted from all the raw data obtained according to each scan-rate condition in order to obtain the correct thermal profiles of the samples. Due to the innate limits of the DSC at higher scan rates, even though samples were heated from 25 $^{\circ}\text{C}$, the signals were detected starting at a slightly higher temperature as can be observed in Fig. 1.

Experiment reproducibility

Aggregation is a phenomenon which concerns the association of particles in a many-body system through interactions such as collision. Therefore, a number of factors can affect results obtained using DSC and reliability of observed phenomena is essential. To ensure that the observed phenomena were reproducible, multiple runs of different solutions chosen from a wide range of conditions were performed. Nearly identical results were obtained. For thermal profiles having a single peak, experiments were repeated at least three times to assess the statistical significance of the extracted thermodynamic parameters. The reproducibility of the experimental baseline for each scan rate was also checked. To ensure sample consistency, the stability of three 100 μM stock solutions was monitored over time. Solutions used within 2 weeks of preparation showed negligible difference in DSC profiles.

Process reversibility

To assess the reversible nature of the thermal events observed in the μDSC scans, samples were equilibrated to a predetermined temperature and held for 5 min at that temperature prior to performing a full scan (from 25 to 90 $^{\circ}\text{C}$). Process reversibility experiments were done at both 0.17 and 1.5 $^{\circ}\text{C min}^{-1}$ scan rates. Samples were held for 10 min at the end of the initial run (temperature of interest below 90 $^{\circ}\text{C}$) before being cooled down.

Visual identification of aggregation

100 μM hemoglobin solutions were heated using a 1.5 $^{\circ}\text{C min}^{-1}$ scan rate to different temperatures. After reaching a desired temperature, samples were removed from the DSC and diluted to one sixth of their original concentration using deionized water. After sealing the vial, aggregates were left standing for more than 2 h to allow the aggregates to settle to the bottom of the vial.

Light scattering analysis

All samples were sonicated for 5 min and passed through a 100 nm filter (Acrodisc[®] Syringe Filter, 0.1 μm Supor[®] Membrane, Low Protein Binding and Non-Pyrogenic, Pall Corporation, Michigan, USA) before measurement. A 15 mW solid state laser of 635 nm wavelength was used at a scattering angle of 90 $^{\circ}$. The refractive index of the solution was 1.33. Samples were heated within the PSA from 25 $^{\circ}\text{C}$ to a desired temperature. The heating rate for the PSA was estimated to be between 5 and 7 $^{\circ}\text{C min}^{-1}$. Once the desired temperature was reached, the effective diameter and polydispersity were measured every 3 min for a period of 45 min. The correlation functions obtained from the time-dependent intensity fluctuations were analyzed using an inverse Laplace transformation software (CONTIN) to obtain the distribution function, and the diffusion coefficients were determined and converted to hydrodynamic radii using the Stokes–Einstein equation.

Results

Concentration dependence

To examine the effect of concentration on the ability to decouple quaternary to tertiary dissociation from complete denaturation of the protein, experiments with concentrations starting from 10 μM (below which changes in heat capacity were not reliably detected) to 100 μM were conducted. The effect of concentration with respect to the rate at which the temperature of the system was increased was examined as an additional factor that could lead to insight on how rapidly equilibrium between species is reached. Multiple scan rates between 0.17 and 1.5 $^{\circ}\text{C min}^{-1}$ were used, with 1.5 $^{\circ}\text{C min}^{-1}$ being the upper limit of the instrument used.

A small transition in C_p at around 40 $^{\circ}\text{C}$ was observed at the low scan rate for concentrations up to 75 μM (Fig. 1a). The lower the scan rate or concentration, the more prominent was the transition around 40 $^{\circ}\text{C}$ when the graphs were normalized according to their nominal concentration.

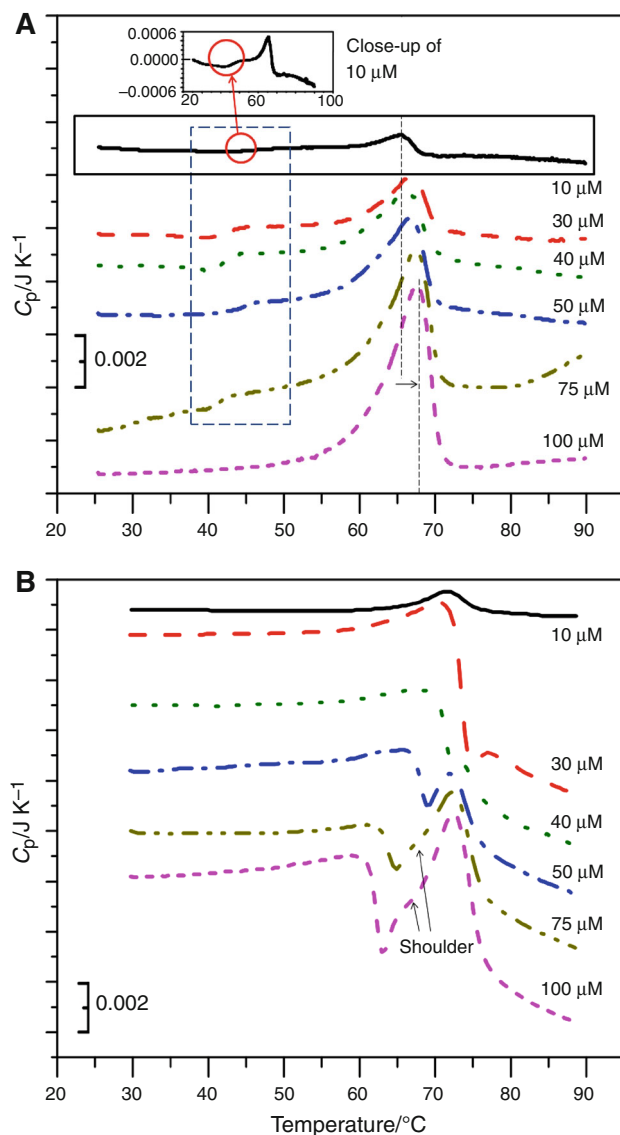


Fig. 1 Thermal profiles of bovine hemoglobin solutions at different concentrations. The experiments were done with 10, 30, 40, 50, 75, and 100 μM Hb, run at a scan rate of: **a** $0.17\text{ }^{\circ}\text{C min}^{-1}$ and **b** $1.5\text{ }^{\circ}\text{C min}^{-1}$. The result of DSC run at the 10 μM , 10 $^{\circ}\text{C}$ condition has been enlarged (**a**) to emphasize the transition around 43 $^{\circ}\text{C}$

At a scan rate of $1.5\text{ }^{\circ}\text{C min}^{-1}$, the transition seen at the low scan rate disappeared, however, multiple other events started to appear as the concentration increased (Fig. 1b). The decrease in heat capacity late in the scan was most likely due to aggregation of the denatured protein at high temperatures (above 70 $^{\circ}\text{C}$).

Scan-rate dependence

The effect of using different scan rates while maintaining a constant concentration was examined to see if the observed phenomena had a kinetic dependence (Fig. 2). The

behavior of a system having a large kinetic dependence without reaching equilibrium would rely heavily on the path the molecules take. This is in contrast to systems solely under the effect of equilibrium thermodynamics that are characterized by state functions, in which the path that the molecules take to reach the state has no effect on the state itself. The dependence observed in Figs. 2 and 3 suggests that a rapid equilibrium could not be established between hemoglobin species because the scan rate did change the overall curve. A concentration of 47 μM (Fig. 2) was chosen since multiple phenomena could be observed for different scan rates at this concentration. As can be seen in Fig. 2a, as the scan rate increased, multiple events appeared, including an exothermic event prior to the point we have ascribed to the denaturation of the protein. Similar phenomena were observed for solutions having a concentration of 75 μM (Fig. 3b).

50 % transition temperatures

The profile of the DSC curve is a cumulative account of how chemical species absorb or release energy as they change through chemical and physical means. In its simplest form, the curve can be represented as multiple Gaussian curves. The maximum point on the Gaussian represents the 50 % transition temperature (T_m). Roughly, one can estimate the 50 % transition temperature (T_m) by examining the local maxima on the curves without performing any mathematical deconvolution, as indicated by the dashed vertical lines in Figs. 1a and 2a. In Fig. 1a, it can be seen that the $3^{\circ} \rightarrow 2^{\circ}$ T_m increased slightly as concentrations increased. The $3^{\circ} \rightarrow 2^{\circ}$ T_m also increased significantly as the scan rate increased (Fig. 2a). This trend was maintained for all concentrations tested including 10, 47, 75, and 100 μM (Fig. 2b). This dependence showed a predominantly linear relationship for all concentrations with a potential transition point at scan rates between 0.67 and $1^{\circ}\text{C min}^{-1}$. This transition is most obvious at higher concentrations.

Development of exothermic events

As previously described, exothermic events can be ascribed to negative peaks in DSC curves. In Figs. 1b and 2a, a significant exothermic event was observed in the curves at temperatures below T_m , what we believe to be the $3^{\circ} \rightarrow 2^{\circ}$. This phenomenon is further exemplified in Fig. 3. The appearance of the exothermic event is concomitant to a reduction in the maximum heat capacity of the system. Furthermore, as the scan rate increases, the initial and final states of the system are quite different compared to the state of the species that were heated at a low scan rate, e.g., $0.33\text{ }^{\circ}\text{C min}^{-1}$.

Fig. 2 **a** Scan-rate dependence of heat capacity from a 47 μM bovine hemoglobin solution. Scan-rates of 0.33, 0.66, 0.83, 1, and 1.5 $^{\circ}\text{C min}^{-1}$ are compared. **b** The T_m ($^{\circ}\text{C}$) values for denaturation plotted against scan rate ($^{\circ}\text{C min}^{-1}$). The values all show a strong increasing trend for the concentrations tested (10, 47, 75, and 100 μM)

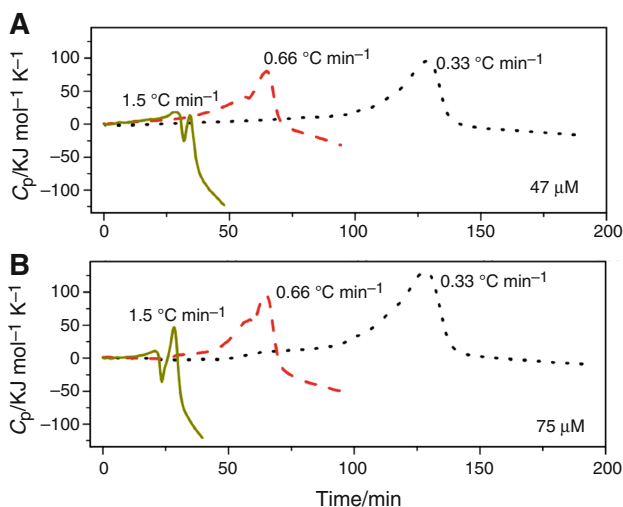
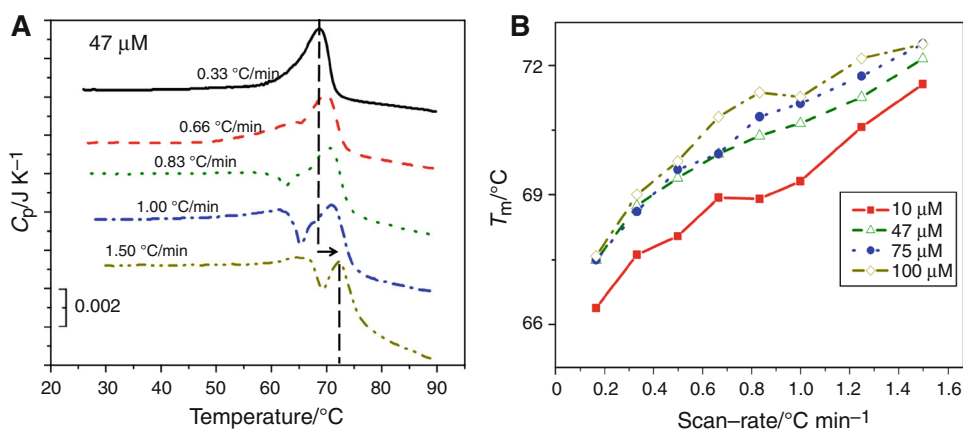


Fig. 3 The thermal profiles of bovine hemoglobin solutions having concentrations of: **a** 47 μM and **b** 75 μM at different scan rates (0.33, 0.66, and 1.5 $^{\circ}\text{C min}^{-1}$). Scans have been zeroed and normalized to the starting concentration of hemoglobin

Determining the cause of the intermediate exothermic phenomenon

Upon the appearance of this intermediate exothermic event, we investigated the state of the molecules prior to the $3^{\circ} \rightarrow 2^{\circ}$ transition. Given that exothermic events are generally associated with bond formation or physical interaction, we hypothesized that this event must have been a form of aggregation. Given that the particles should follow random Brownian motion, the concept of Rayleigh scattering can be applied. To probe if indeed aggregation was the cause of this intermediate exothermic event, light scattering measurements were conducted to gain information on the effective diameter of material in our solution. Particle sizes were measured after rapidly ramping the temperature from an initial temperature of 25 $^{\circ}\text{C}$, and the behavior of the system was monitored over time. Figure 4 summarizes the results of ramping the temperature to 35,

45, 55, 65, and 74 $^{\circ}\text{C}$ (the limit of the machine). For 10 μM Hb (Fig. 4a), the total effective diameter of the system remained much below 500 nm for all conditions tested; however, for 100 μM Hb (Fig. 4b), the values increased dramatically, especially at 55 $^{\circ}\text{C}$. At 74 $^{\circ}\text{C}$, particles around 2 μm were dominant, showing that there were still aggregates, albeit smaller than what was observed when the solution was heated to 55 $^{\circ}\text{C}$. Particles resulting from heating the solution to 65 $^{\circ}\text{C}$ had sizes that were between those seen when heating the solutions to 55 or 74 $^{\circ}\text{C}$, indicating a clear transition from one form to another. Although some of the size measurements associated with ramping the solution up to 55 and 65 $^{\circ}\text{C}$ are above the reliable measurement threshold, the particles in these solutions can still be said to be much larger than those from any other experiment. Furthermore, the trend seen for the effective diameter was found to be very reproducible.

For 10 μM Hb (Fig. 4c), there was an overall decreasing trend for polydispersity as the end point temperature increased, implying a change into a monodisperse distribution of particles, possibly the result of denaturation. For 100 μM Hb (Fig. 4d), when the solution was heated up to 55 $^{\circ}\text{C}$, a rapid increase in polydispersity was observed. For both solutions heated to 65 and 74 $^{\circ}\text{C}$, a rapid decrease in polydispersity was detected from the beginning.

As an additional confirmation that we were indeed forming aggregates, 100 μM Hb samples, heated at a scan rate of 1.5 $^{\circ}\text{C min}^{-1}$ to either 55 or 90 $^{\circ}\text{C}$, were removed from the μDSC . After verifying the presence of particulates in the samples, they were further diluted for additional investigation. Both precipitates were stable over 2 months and were quite large. These precipitates differed depending on the initial treatment (heating to 55 or 90 $^{\circ}\text{C}$). Samples heated to 55 $^{\circ}\text{C}$ possessed floc-like structures, and were lighter in color, having an orange hue. Those heated to 90 $^{\circ}\text{C}$ contained what resembled a fine powder, darker in color, which settled in a denser form at the bottom of the vial. After gently shaking the vials that contained the

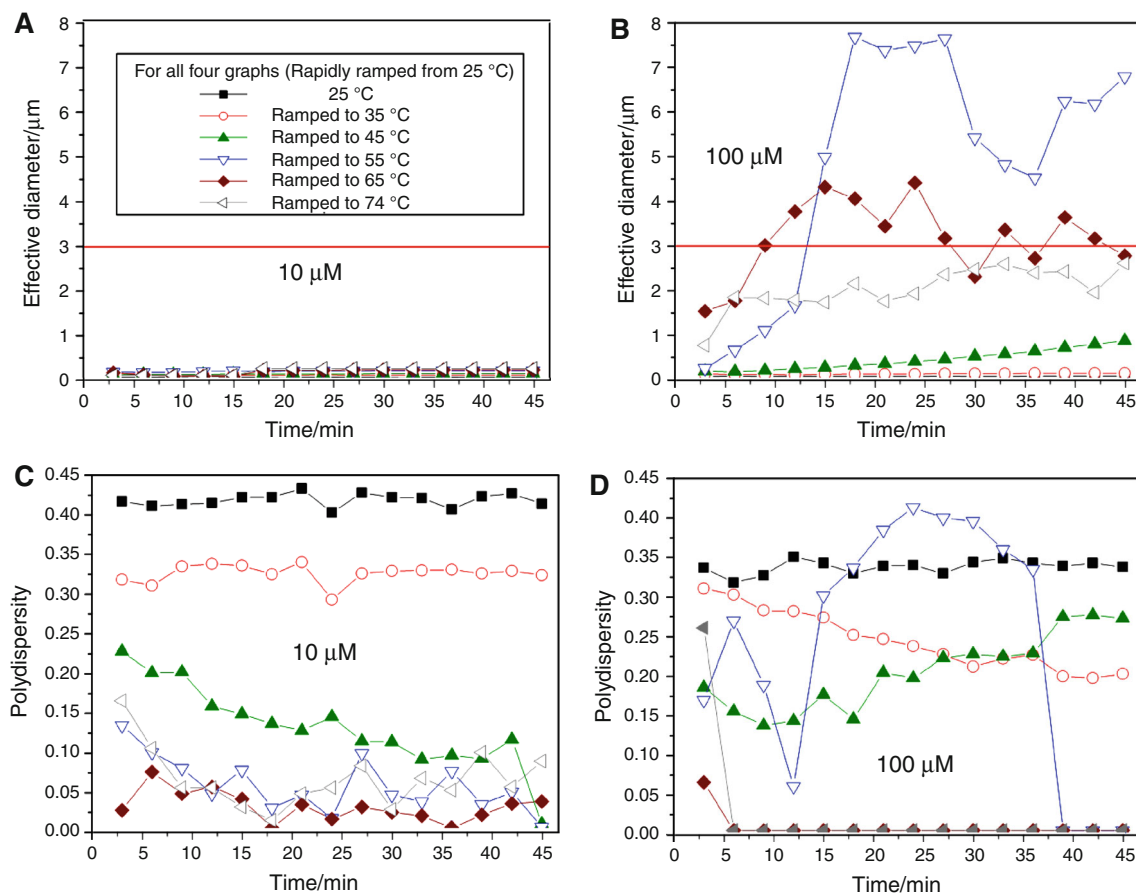


Fig. 4 The effective diameter (a, b) and polydispersity (c, d) obtained from light scattering experiments for 10 μM (a, c) and 100 μM (b, d) hemoglobin solutions heated to various temperatures

and monitored for 45 min. The thick red horizontal line (a, b) at 3 μm represents the upper size limit for reliable measurements for the instrument

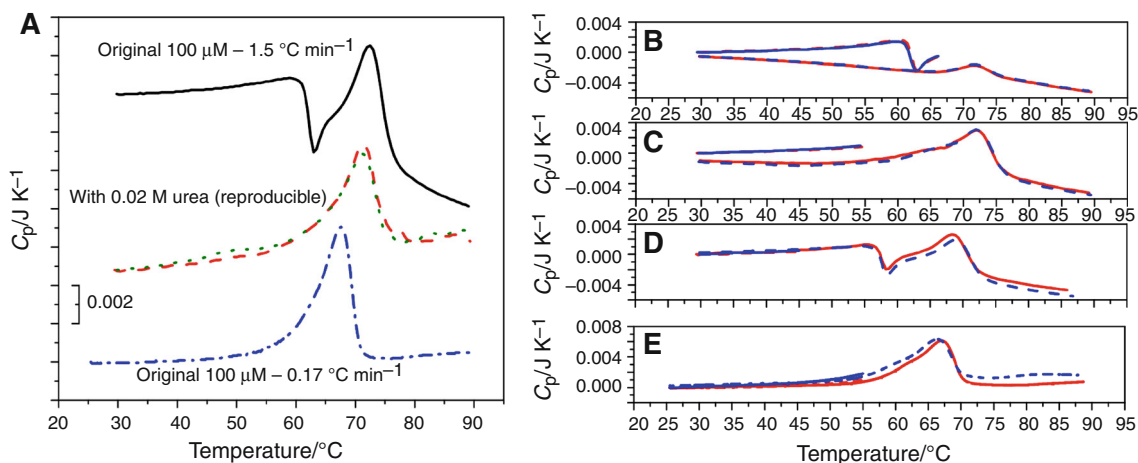


Fig. 5 DSC curves of 100 μM Hb. a Addition of very small amounts of urea (0.02 M, repeatable results shown) prevents the intermediate exothermic phenomenon, even at high concentration and high scan rate (1.5 $^{\circ}\text{C min}^{-1}$). b Samples heated to 67 $^{\circ}\text{C}$, rapidly cooled to 25 $^{\circ}\text{C}$, and reheated to 90 $^{\circ}\text{C}$ at a rate of 1.5 $^{\circ}\text{C min}^{-1}$. c Samples

heated to 55 $^{\circ}\text{C}$, rapidly cooled to 25 $^{\circ}\text{C}$, and reheated to 90 $^{\circ}\text{C}$ at a rate of 1.5 $^{\circ}\text{C min}^{-1}$. d Samples heated to 45 $^{\circ}\text{C}$, rapidly cooled to 25 $^{\circ}\text{C}$, and reheated to 90 $^{\circ}\text{C}$ at a rate of 1.5 $^{\circ}\text{C min}^{-1}$. e Samples heated to 55 $^{\circ}\text{C}$, rapidly cooled to 25 $^{\circ}\text{C}$, and reheated to 90 $^{\circ}\text{C}$ at a rate of 0.17 $^{\circ}\text{C min}^{-1}$

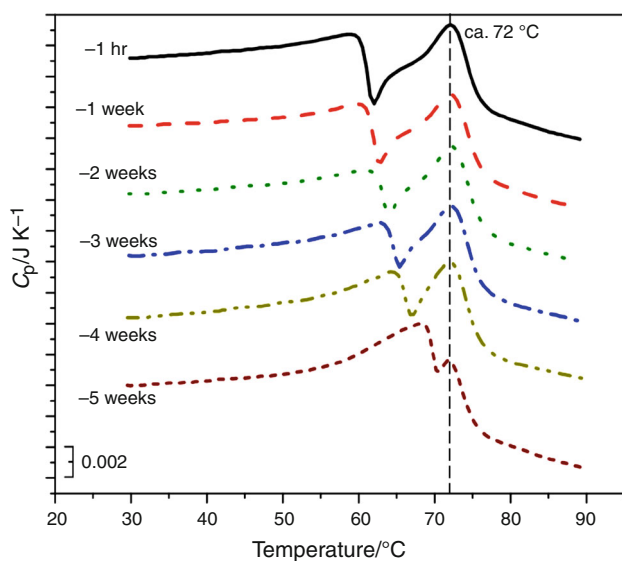


Fig. 6 The thermal profiles of a 100 μM sample kept over time at 4 $^{\circ}\text{C}$ and scanned at 1.5 $^{\circ}\text{C min}^{-1}$

samples, there was a significant difference in the settling time. Those samples heated to 55 $^{\circ}\text{C}$ settled fully in ca. 20 min while it took more than an hour for the precipitates in samples heated to 90 $^{\circ}\text{C}$.

Given that aggregation of proteins is generally driven by hydrophobic interactions, DSC scans were repeated for equilibrated samples in the presence of urea which is known to disrupt non-covalent bonds. Low concentrations of urea were chosen (0.02 M) to minimize any significant tertiary subunit structure changes in the hemoglobin. The addition of urea prevented the formation of the intermediate exothermic event at a 1.5 $^{\circ}\text{C min}^{-1}$ scan rate (Fig. 5a).

Thermal reversibility

To initially assess the reversibility of the observed phenomenon, scans (1.5 $^{\circ}\text{C min}^{-1}$) of 100 μM were stopped at various points along the scan and the system was brought back down to the initial point before allowing a complete scan over the entire temperature range. Bringing the system up to 67 $^{\circ}\text{C}$, arresting the scan, bringing the system back to its initial state and allowing a complete scan to proceed caused an irreversible change to the system (Fig. 5b). This was characterized by a change in the heat capacity of the system as well as a loss of a clear intermediate exothermic event, which was evident when initially heating the sample up to 67 $^{\circ}\text{C}$ (Fig. 5b). Even heating the sample to a point prior to the exothermic event (55 $^{\circ}\text{C}$) seen in Fig. 5b resulted in an irreversible change to the system, albeit less profound as seen by a more significant endothermic event (Fig. 5c). Not allowing the temperature of the system to go

over 45 $^{\circ}\text{C}$ allowed the system to return to its native state (Fig. 5d), a sign of reversibility. On the other hand, reversibility was also influenced by the scan rate. Although at a scan rate of 1.5 $^{\circ}\text{C min}^{-1}$, the system was altered irreversibly when heated to 55 $^{\circ}\text{C}$, a solution with the same concentration but scanned at 0.17 $^{\circ}\text{C min}^{-1}$ did not show any change (Fig. 5e).

Solution stability and the effect on thermal events

Given that high concentrations (100 μM) and high scan rates (1.5 $^{\circ}\text{C min}^{-1}$) promoted the appearance of the intermediate thermal event under investigation, these conditions were used to assess the stability of the hemoglobin solutions stored at 4 $^{\circ}\text{C}$ over time. Solutions tested within a 2 weeks period yielded nearly identical heat capacity profiles (Fig. 6). However, beyond 4 weeks, the appearance of an endothermic event resulted in a shift to the appearance of the intermediate exothermic event under investigation. These shifts in thermal events did not influence what we had ascribed as the 3 $^{\circ}$ \rightarrow 2 $^{\circ}$ transition, which was maintained at 72 $^{\circ}\text{C}$.

Discussion

Thermal profiles

The simplest thermal profiles occurred at low scan rates (Figs. 1a, 7a). The asymmetric nature of the major peak in the curves may be indicative of the occurrence of at least two events presumed to be the 4 $^{\circ}$ \rightarrow 3 $^{\circ}$ and 3 $^{\circ}$ \rightarrow 2 $^{\circ}$ transitions for hemoglobin in aqueous solutions (Fig. 7a). A second feature of the thermal profiles obtained at low scan rates (0.17 $^{\circ}\text{C min}^{-1}$) was a small hump detected at around 43 $^{\circ}\text{C}$ for sample concentrations below 75 μM (Fig. 1a). This temperature is slightly below the point at which higher concentration samples underwent irreversible changes (Fig. 5). It is possible that this was the point at which the 4 $^{\circ}$ \rightarrow 3 $^{\circ}$ dissociation started (Fig. 7a). This transition was more prominent at lower scan rate and lower concentration conditions. Attributing this to dissociation, it is hypothesized that a greater extent of the reaction can be reached for each temperature at a low scan rate, thus evolving more energy at lower temperatures. However, when the scan rate is increased, the energy related to dissociation is distributed over a greater temperature range thus becoming convoluted in the 3 $^{\circ}$ \rightarrow 2 $^{\circ}$ transition. Furthermore, it is thought that dissociation was better visualized at lower concentration because of a smaller number of collisions between molecules resulting in an environment where there is enough room for subunits to freely separate as long as enough energy is provided.

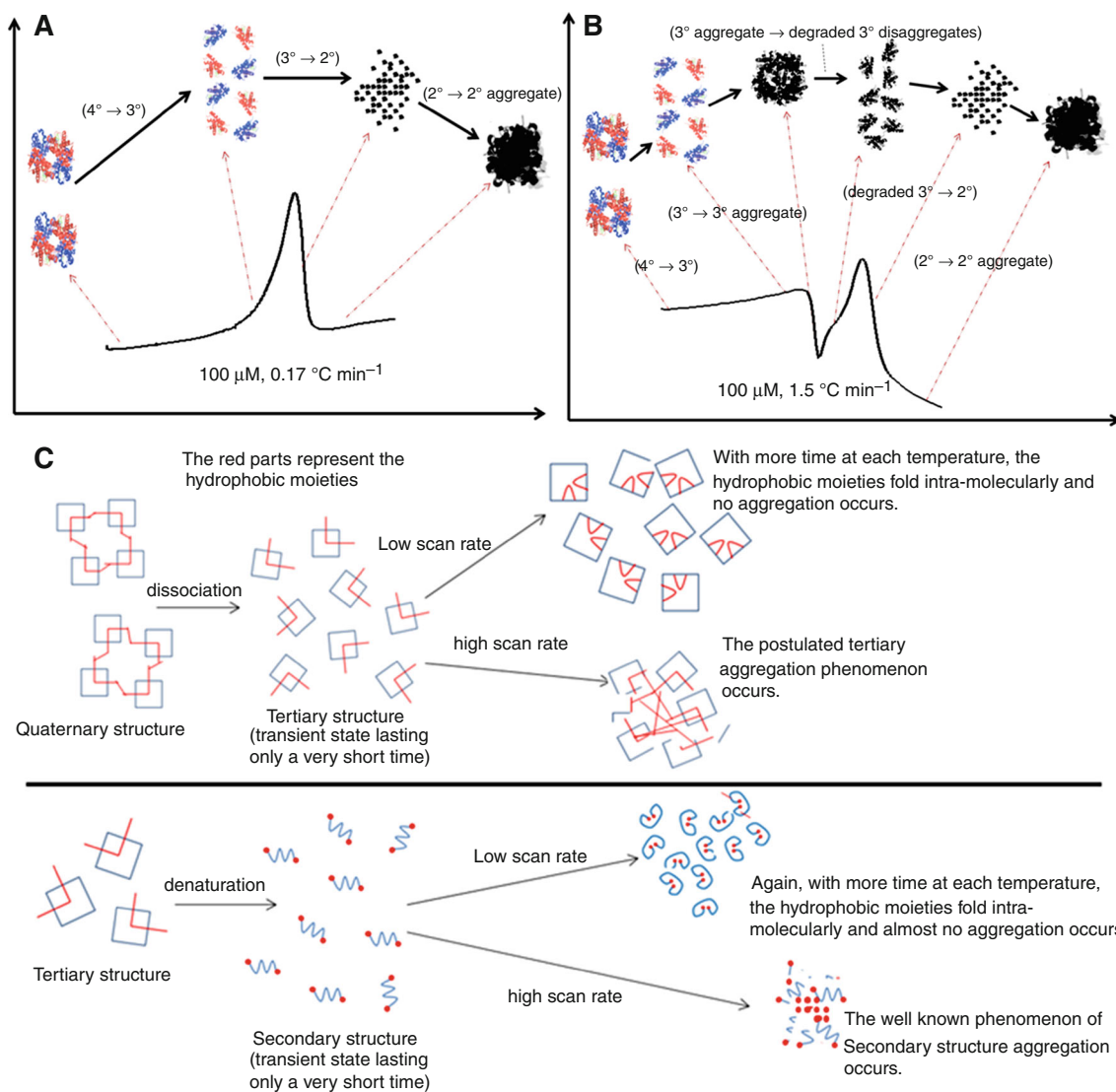


Fig. 7 Interpretation of thermal profiles. Thermal profile of 100 μM hemoglobin solution scanned at: **a** $0.17\text{ }^{\circ}\text{C min}^{-1}$ and **b** $1.5\text{ }^{\circ}\text{C min}^{-1}$. **c** Proposed mechanisms showing how the dominant protein structure is thought to be affected by the scan rate

At higher scan rates, a number of additional features appeared (Figs. 1b, 7b). Increasing the concentration from 10 to 30 μM (Fig. 1b) resulted in a sharp decrease in heat capacity when the sample was heated at $1.5\text{--}90\text{ }^{\circ}\text{C}$. The sudden development of this change within a small concentration range suggests cooperative interaction based on nucleation of particles within the system, unlike what was suggested before [21]. This sharp decrease also revealed a separation of endothermic events: one which occurred at around $71\text{ }^{\circ}\text{C}$ and another at $77\text{ }^{\circ}\text{C}$. Because the sharp decrease was not observed for the $10\text{ }^{\circ}\text{C}$ solution, it was believed that the decrease in the higher concentration systems was not entirely due to a change in heat capacity of the species in solution but was most likely due to aggregation (Fig. 7b).

The formation of new interparticle bonds in a protein aggregate may not be the sole explanation for the exothermic event. Sasahara et al. have explained this phenomena by the changing role of solvent–protein interaction as an aggregate structure is formed. Hydration patterns related to the arrangement of hydrophobic groups in a protein are also thought to influence the DSC result [32] and has been characterized by others through the accessible surface area of a protein [15, 31–33]. This parameter, which represents changes in the hydration of hydrophobic groups buried from the solvent, supports the theory that hydrophobic moieties of a protein become shielded from water through aggregation. This parameter, however, is not easily accessible.

An increased number of hydrogen-bond mediated water-cages around newly exposed surface areas as hemoglobin

dissociates could also contribute to the exothermic event. However, given that the magnitude increase in surface area would be minimal from the dissociation of a tetramer, and that light scattering data (Fig. 4) showed the presence of aggregates, this contribution is thought to be negligible.

It was suspected that the major species involved in forming aggregates at the lower temperature were polypeptides that formed from the dissociation of hemoglobin ($4^\circ \rightarrow 3^\circ$), because the drop occurred between two endothermic events. As the concentration increased, these unique thermal events required less energy and an overall shift was seen toward the left. Fig. 7b summarizes an overall appreciation of events that is thought to be behind the thermal profiles observed. A large basis of our initial interpretation of the thermal profiles was that dissociation, denaturation, and disaggregation could be viewed as endothermic events while aggregation is often an exothermic event. It was therefore postulated that hemoglobin sequentially goes through $4^\circ \rightarrow 3^\circ$ dissociating transition, $3^\circ \rightarrow 3^\circ$ aggregation, 3° aggregation $\rightarrow 3^\circ$ disaggregation, $3^\circ \rightarrow 2^\circ$ denaturation and finally 2° structure aggregation at conditions of high concentration and high scan rates (Fig. 7b). Given that aggregates having very different characteristics have been observed, we believe it is possible to attribute the exothermic event described in this paper to $3^\circ \rightarrow 3^\circ$ aggregation. Furthermore, it is believed that the reason aggregation and disaggregation do not occur simultaneously is due to the specific molecular mechanisms involved in these processes. Aggregation is a cooperative procedure mediated through nucleation [8, 13, 15, 34], which occurs spontaneously. There is no lag phase detected for the initiation of tertiary aggregation due to the fact that the starting nuclei are already present, i.e., the dissociated subunit. However, disaggregation is a result of a comparatively simple energy input, happening at higher temperatures, only when aggregates are present.

Kinetic dependence and mechanism of action

Along with the decrease in heat capacity, the greater number of thermal events observed as scan rate increased was attributed to the inability of the protein to adapt to its changing environment (Fig. 3). The effect of scan rate has been previously reported for the $3^\circ \rightarrow 2^\circ$ transition, which has led to the interpretation that the $3^\circ \rightarrow 2^\circ$ transition is an event that depends not only on thermodynamics but also on the kinetics of the system [35]. As a kinetic event, the transition is believed to be influenced by the collision of molecules. It is also believed that the exothermic event that occurs prior to the $3^\circ \rightarrow 2^\circ$ transition is also influenced by the kinetics of the system. Given the increased rate of energy input to the system, the observed phenomenon may be a result of both an increased frequency of collision [13]

and appropriate contacts to initiate aggregation. Changes in these molecules have been reported to include exposure of hydrophobic moieties when subjected to heat [15]. Slow overall energy input rates allow time for species to proceed through a particular kinetic pathway, giving sufficient time for the molecule to fold back into itself (intramolecular interaction dominance) decreasing the possibility of aggregation. A rapid overall energy input rate on the other hand maintained each molecule in a “transient” state causing the hydrophobic moieties to interact between different and separate subunits or domains (intermolecular interaction dominance), thus promoting aggregation. A schematic of the mechanism proposed for the transitions of hemoglobin studied in this work is shown in Fig. 7c. Previous studies investigating molecule hydrophobicity have also suggested a similar reasoning [15, 36–38]. One study in particular suggests that for some molecules, this phenomenon has a strong kinetic dependence [36]. Studies on the distribution of hydrophobic residues in bovine hemoglobin [7] further support this viewpoint.

The suggested mechanism is also valid for samples having a concentration of $30 \mu\text{M}$, and scanned at $1.5 \text{ }^\circ\text{C min}^{-1}$ (Fig. 1b). The sudden drop and second upward peak ($\sim 77 \text{ }^\circ\text{C}$) can be attributed to both pathways (Fig. 7c) occurring concurrently due to the dispersed state of molecules that can interact. This small event has been previously overlooked in other studies [21, 39]. It is possible that at $30 \mu\text{M}$ a smaller number of collisions and dissociation occur at the same time resulting in a fraction of the population interacting in the same way molecules would at high concentrations, e.g., at $100 \mu\text{M}$. It is therefore thought that tertiary aggregates form to a lesser amount, and require more time than samples having a higher concentration of hemoglobin. This, in turn, contributed to the detection of a late endothermic change around $77 \text{ }^\circ\text{C}$ followed by the $3^\circ \rightarrow 2^\circ$ denaturation. On the other hand, for the fraction of population that does not collide with each other at all before denaturing, the quaternary structures will break down without forming tertiary aggregates along the transition path. This group, however, will still form secondary aggregates around $72 \text{ }^\circ\text{C}$, having an overall exothermic effect on the system. The large drop in the $30 \mu\text{M}$ (Fig. 1b) is thought to be due to detection of overlapping exothermic events from the formation of both secondary and tertiary aggregates. The aberrantly small size of the second peak which can be generally labeled as the $3^\circ \rightarrow 2^\circ T_m$ can also be explained as partially hidden due to overlapping thermal events.

Upon investigating the effect of small quantities (0.02 M) of urea on high concentration hemoglobin solutions ($100 \mu\text{M}$) analyzed at a high scan rate ($1.5 \text{ }^\circ\text{C min}^{-1}$), it was found that the negative thermal event could be eliminated (Fig. 5a). Urea is known to be effective in disrupting non-covalent bonds by changing the configuration of hydrogen bond mediated water

cages around protein molecules. Although the exact mechanism of urea's effect on protein is still being debated, the two generally accepted modes of action include: (1) the "direct effect" of hydrogen bonds between urea and the peptide backbone; or (2) the "indirect effect" where apolar amino acid residues are susceptible to high contact frequencies with urea molecules [33, 40–42]. The latter effect is postulated to be due to the fact that urea molecules are polar, which would push the hydrophobic moieties of a tertiary structure into itself. Both the "direct" and "indirect" effects are thought to be reasons for the prevention of aggregation [25]. The fact that only minor changes in pH and conductivity were observed following urea addition also supports the notion that the major change was only in the hydrophobic force balance in the system. Similar effects on proteins are also observed with β -cyclodextrin [43].

Conclusions

The occurrence of an unreported form of aggregation having a significant kinetic dependence has been described, and is believed to be the result of protein subunit association. This study points to a rapid onset of aggregation mediated by the availability of hydrophobic moieties and triggered by the rate at which energy is added to the system. Although both concentration and the rate of energy introduced to the system play a role in the susceptibility of the protein to aggregate, the latter is dominant.

This top-down investigation approach into protein aggregation is thought to provide additional insight into other forms of higher order molecular aggregates in a system; complementing work done by others using bottom-up approaches [32, 34, 38]. These higher order aggregates include those that occur with proteins having quaternary structure, where microphase separation and macroseparation can occur as different forms, and which can lead to ordered events such as self-assembly.

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