THE JOURNAL OF PHYSICAL CHEMISTRY B

Article

pubs.acs.org/JPCB

Cold-Induced Precipitation of a Monoclonal IgM: A Negative Activation Enthalpy Reaction

Stefano C. Meliga,[†] William Farrugia,[§] Paul A. Ramsland,*[,§,∥,⊥] and Robert J. Falconer*[,‡]

[†]Australian Institute for Bioengineering and Nanotechnology, University of Queensland, St. Lucia, Qld 4072, Australia

[‡]Department of Chemical & Biological Engineering, ChELSI Institute, University of Sheffield, Sheffield S1 3JD, England

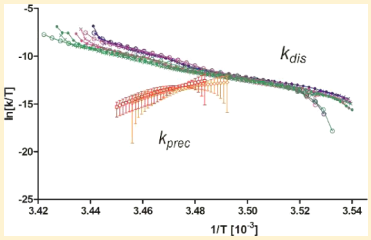
[§]Centre for Immunology, Burnet Institute, Melbourne, Vic 3004, Australia

[∥]Department of Surgery Austin Health, University of Melbourne, Heidelberg, Vic 3084, Australia

[⊥]Department of Immunology, Monash University, Alfred Medical Research and Education Precinct, Melbourne, Vic 3004, Australia

Supporting Information

ABSTRACT: Cold-induced precipitation of a monoclonal IgM cryoglobulin isolated from a patient with Waldenström's macroglobulinemia was observed to have a negative activation enthalpy. The rate of the reaction increased, as the temperature decreased. Differential scanning calorimetry of the monoclonal IgM showed precipitation as an inverted peak during a downward temperature scan. The transition temperature was between 14 and 15 °C and was possibly concentration dependent. At temperatures below the transition the precipitation was best described by second-order kinetics. The difference in change in enthalpy between precipitation and disassociation suggests that cold-induced precipitation had a fast precipitation stage followed by a slower consolidation reaction. Negligible curvature of the Eyring plot suggested the precipitation reaction was dominated by van der Waal forces and hydrogen bonding. Conversely, during an upward temperature scan, disassociation was observed as a positive enthalpy peak. This reaction had two stages, a reaction undoing consolidation followed by heat-induced disassociation that had first-order kinetics.



INTRODUCTION

The existence of cryoglobulins or cold-insoluble serum proteins was first described in detail in the 1940s in connection with a patient experiencing purpura and cold sensitivity.[1] Subsequent studies of multiple patients demonstrated that the cryoglobulins were mostly immunoglobulins and were associated with a range of disease states.[2,3] There are three categories of cryoglobulins; Type I where an individual monoclonal antibody is present, Type II where a monoclonal immunoglobulin interacts with polyclonal IgGs, and Type III where there are a mixture of polyclonal IgMs and IgGs. The Type I cryoglobulins are usually IgMs or IgGs and in rare circumstances IgAs. Cryoglobulins are of special clinical interest as they are reversibly insoluble at temperatures lower than 37 °C.[2,3] Cryoglobulins can precipitate, gelate, or in rare circumstances crystallize when the blood temperature falls. This in turn causes disruption of the blood circulation in the periphery of patients at cold temperatures. In this paper we studied two Type I cryoglobulins. Both IgMs used in this research were isolated from patients with Waldenström's macroglobulinemia, but they differed in their response to cooling, one (Yvo IgM) formed a stable gel and the other (Pot IgM) formed a fine precipitate.

Fundamental studies into cold-induced precipitation of cryoglobulins have the capacity to provide a better understanding of the physics behind this phenomenon, which may in turn guide the treatment of cryoglobulinemia. Early studies showed that cryoglobulin concentration played a critical role in cold-induced precipitation.[2] Protein concentration is invariably important for precipitation where two components have to come together for the reaction to occur. At higher cryoglobulin concentrations precipitation occurs more readily and can occur at higher temperatures.[2] pH plays a part in cryoglobulin precipitation, demonstrating that electrostatic interactions between protein molecules can have a role in cryoprecipitation.[2] At low ionic strengths (less than 0.2M) the addition of salt enhances precipitation for a range of cryoglobulins, which suggests that electrostatic charge shielding can overcome electrostatic repulsion and enable cold-induced precipitation.[4,5] The role of sugars, chaotropic agents, and nonchaotropic salts had a minor effect on the precipitation of the IgM cryoglobulin McE, which was used to argue that the intermolecular interactions for this cryoglobulin were due to a combination of hydrogen-bonding and weaker dipole−dipole forces (van der Waal forces).[4] The kinetics of IgM and IgG cryoprecipitation has been studied by measuring turbidity.[6−8] Several studies suggested that nucleation was a necessary first step for the precipitation of some cryoglobulins.[6−8] This has to be treated with caution as turbidity measurement can be insensitive to the early stages of a precipitation reaction and be misinterpreted as a lag-phase common to nucleation-initiated reactions. In the case of the IgG cryoglobulin Cac, the addition of a chemically linked dimer promoted the initiation of precipitation providing

Received: September 13, 2012
Revised: November 18, 2012
Published: December 14, 2012

dx.doi.org/10.1021/jp309109k | J. Phys. Chem. B 2013, 117, 490−494

strong evidence for nucleation for this individual cryoglobulin.[7] Secondary structural change was not observed by Raman spectroscopy during the nucleation of the IgG cryoglobulin Web, and it was postulated that nucleation was due to perturbation of a local site in the cryoglobulin molecule.[8] Research on the thermodynamics of polyethylene glycol-induced precipitation supported the theory that cryoglobulins are normal immunoglobulins at the insoluble end of a normal distribution[9] and that in cases of cryoglobulinemia the cryoglobulin concentration increased to a point where cold-induced precipitation could occur in the extremities of patients.

Differential scanning calorimetry (DSC) is widely used to study the kinetics of protein unfolding but its potential for studying the kinetics of cold-induced precipitation has not been exploited. DSC has previously been used to confirm the van't Hoff enthalpies of cryoglobulins determined using polyethylene glycol-induced precipitation.[9,10] In this investigation we used DSC to study cold-induced precipitation and heat-induced disassociation of Pot IgM to better understand the kinetics and thermodynamics of the cold-induced precipitation process.

EXPERIMENTAL SECTION

The cryoglobulins Pot IgM and Yvo IgM were isolated from patients with Waldenström's macroglobulinemia following the method in Vallas et al.[11] Purified IgM samples were dialyzed against phosphate-buffered saline (PBS) composed of 137 mM sodium chloride, 2.7 mM potassium chloride, and 10 mM phosphate buffer, pH 7.2. DSC was undertaken with a VP-DSC (MicroCal, Northampton, MA, USA) at a heating rate of 0.5–1.5 °C/min from 5 to 35 °C in forward scans and 35 to 5 °C in reverse scans. The instrument was held at a constant temperature for 15 min between each scan. Data evaluation used the software provided by the manufacturer (Origin, version 7.0). Buffer–buffer baselines were subtracted from sample data. The relative heat capacity for the reverse scan is calculated from the energy to heat the reference cell minus the sample cell.

RESULTS

The two cryoglobulins (Yvo IgM and Pot IgM) originating from patients with Waldenström's macroglobulinemia behaved quite differently as the temperature dropped. The obvious macroscopic difference is Yvo IgM forms a hydrogel whereas Pot IgM precipitates observed as an increase in turbidity. Previous dynamic light scattering (DLS) experiments had shown that Yvo IgM has a less clearly defined transition between the gel and soluble forms whereas Pot IgM has a clear transition between the solid and soluble phases.[11] DSC analysis for Yvo IgM shown in Figure 1 did not detect a measurable transition between the gel and soluble phases and was unable to contribute to analysis of this process. Yvo IgM gelation is not amenable to analysis by DSC and was better suited to investigation using DLS.[11] DSC analysis of Pot IgM did show a clear transition between the solid and soluble phases and was used to study the kinetics and thermodynamics of this process. This was clearly observable for both forward (increasing) and reverse (dropping) temperature scans.

The change in enthalpy (ΔH) calculated from the area under the DSC curve for Pot IgM precipitation was -36 ± 10.5 kJ mol^{-1} and disassociation was 58 ± 10.5 kJ mol^{-1} (Figure 2a,b). The difference between the two ΔH values, which should add up to zero for a complete thermal cycle suggests that there is a

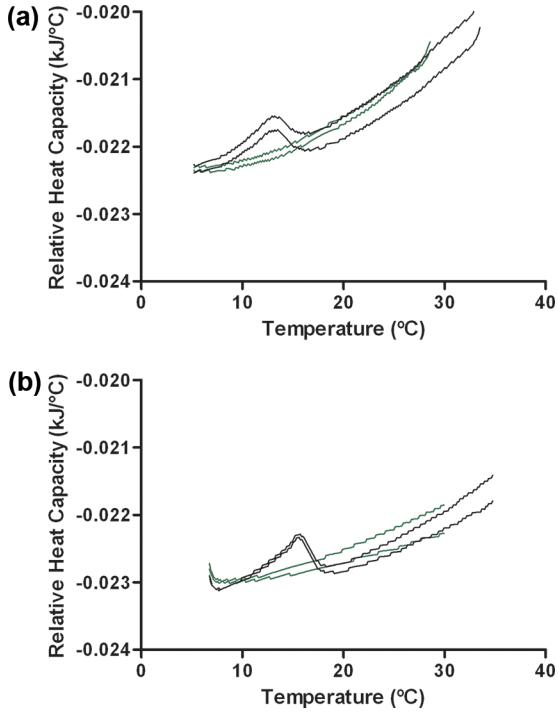


Figure 1. Differential scanning calorimetry of cryoglobulins: (a) downward thermal scan showing cold-induced precipitation (note the ΔC_p has been inverted); (b) upward thermal scan showing heat-induced disassociation. Cryoglobulin Pot IgM (black) and Yvo IgM (gray) both at 40 mg/mL with a scan rate of 0.5 °C/min. The plots are for repeated scans of the same Pot IgM and Yvo IgM samples, demonstrating the reversibility of cold-induced precipitation.

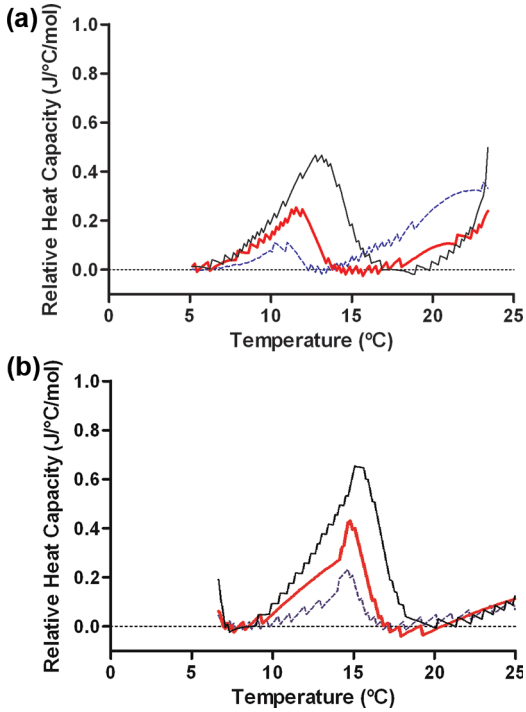


Figure 2. Differential scanning calorimetry of the cryoglobulin Pot IgM: (a) reverse thermal scan showing cold-induced precipitation (note the ΔC_p has been inverted); (b) forward thermal scan showing heat-induced disassociation 12 mg/mL (blue), 20 mg/mL (red), and 40 mg/mL (black) with a scan rate of 0.5 °C/min and after baseline correction (for ease of viewing).

dx.doi.org/10.1021/jp309109k | J. Phys. Chem. B 2013, 117, 490–494

slow component of the precipitation reaction that is not detected by the DSC that accounts for the missing ΔH. Neither the Pot IgM precipitation nor the disassociation reactions showed any dependence on scan rate and the temperature of maximum change in heat capacity (T_m) which was unchanged for scan rates of 30, 40, and 60 °C/min, as shown in Figure 1 in the Supporting Information. The two reactions, however, differed in their response to Pot IgM concentration. Disassociation showed slight concentration dependence with T_m values of 15.1 ± 0.4 °C at 20 mg/mL, 15.5 ± 0.4 °C at 30 mg/mL, and 15.8 ± 0.1 °C at 40 mg/mL. Precipitation had a marked concentration dependence (with transition occurring at higher temperature at higher Pot IgM concentrations, with T_m values of 11.7 ± 0.1 °C at 20 mg/mL, 12.9 ± 0.1 °C at 30 mg/mL, and 13.4 ± 0.2 °C at 40 mg/mL

Precipitation of a cryoglobulin is a complex reaction that may involve multiple amino acid side chains and the displacement of numerous solvent molecules. Eyring's transitional-state theory[13] was applied to this analysis. This describes the reaction kinetics in terms of an equilibrium between the native soluble state and an activated complex with an activation enthalpy ($\Delta H^{\ddagger}$). In the case of cryoglobulin precipitation, the "activated complex" may represent the cryoglobulins with sufficient solvent molecules displaced from its hydration layer to enable intermolecular bonds to form between the two cryoglobulin molecules. The disassociation activated complex may represent the cryoglobulins with the intermolecular bonding broken before the hydration layer between the cryoglobulin molecules is reformed.

For the sake of our analysis we assumed the disassociation reaction was first order and the slight change in T_m values at different concentrations was trivial. The calculations for the kinetic constant for disassociation (k_{dis}) used the equation proposed by Sanchez-Ruiz et al 1988.[12]

$$vC_p = k_{dis}(Q_t - Q) \quad (1)$$

where v is the scan rate, C_p is the excess heat capacity at a given temperature, Q_t is the total heat, and Q is the accumulated heat at a given temperature. This equation assumes the reaction to be first-order and irreversible and was originally proposed to study protein unfolding. The heat-induced disassociation rate constant k_{dis} was calculated for three Pot IgM concentrations and three scan rates and was displayed on an Eyring plot (Figure 3). $\Delta H^{\ddagger}$ was calculated from the slope of the line in the Eyring plot ($\Delta H^{\ddagger} = -\text{slope} \times R$). There are two distinct stages; the first below 14 °C and the second above 15 °C. Each stage is quite linear for a range of Pot IgM concentrations and scan rates. The transition between the two stages is between 14 and 15 °C and may be concentration dependent though the analysis here is inconclusive. The estimate of the $\Delta H^{\ddagger}$ for disassociation is 400−500 kJ/mol.

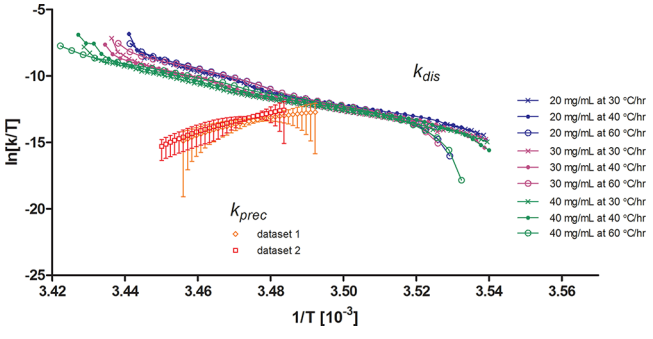


Figure 3. An Eyring plot of DSC thermal scans of cryoglobulin Pot IgM showing cold-induced precipitation and heat-induced disassociation. Note that the second order rate constant k_{prec} was multiplied by a reference concentration (20 mg/mL) to allow the comparison with the first-order k_{dis} by consistent use of the units s^{-1}.

The following equation was used to determine the kinetic constants for precipitation (k_{prec}) for irreversible reactions of any reaction order. Derivation of this equation is shown in the Supporting Information.

$$vC_p = nk_{prec}\frac{(Q_t - Q)^n}{Q^{n-1}}[\text{IgM}]_0^{n-1} \quad (2)$$

where n is the reaction order and $[\text{IgM}]_0$ is the concentration of the protein at time zero. Reverse scans were processed using the C_p and the Q, which were taken from the curve right to left (as opposed to left to right used for processing forward scans). The reaction was assumed to be second-order and free of a nucleation event. The cold-induced precipitation rate constant k_{prec} was calculated using two data sets of combined Pot IgM concentrations and scan rates and was displayed on an Eyring plot (Figure 3). The behavior is not consistent with the Arrhenius equation. The reaction had a single stage and a reasonably straight line on the Eyring plot between 14 and 16 °C where the precipitation reaction was taking place. The $\Delta H^{\ddagger}$ of precipitation was approximately −500 kJ/mol.

Circular dichroism (CD) spectroscopy of Pot IgM detected no change in helicity (data not shown), indicating that the secondary structure is unaffected by the transition between soluble to insoluble forms. This is consistent with previous analysis of cold-induced precipitation of cryoglobulins using CD.[21]

DISCUSSION

There is a high degree of variability in the response of cryoglobulins' at temperatures below the transition between the soluble and insoluble forms of the protein. Pot IgM forms a precipitate, Yvo IgM forms a hydrogel and crystalline forms have also been observed.[14] In each case the reduction in temperature pushed the cryoglobulin below its solubility. Of these two molecules, the cold-induced precipitation of Pot IgM was amenable to analysis using DSC, giving a measurable change in enthalpy. In this study we assumed the precipitation was a second-order reaction with each step comprising the joining of two subunits. The modeling indicated that the kinetics was best explained by a second-order irreversible reaction, suggesting disassociation was negligible and explaining its deviation from reversible reaction kinetics. When the temperature was raised beyond the transition, the disassociation reaction fitted the kinetics of an irreversible first-order reaction. There was a significant but small deviation from first-order kinetics observed as an increase in T_m with concentration. It is possible that Pot IgM aggregates at higher concentrations form denser, more cross-linked particles that require a little more kinetic energy to disassociate.

When the enthalpy of cold-induced precipitation and heat-induced disassociation are added together, it does not equal zero. The positive enthalpy of disassociation (57.5 ± 10.5 kJ mol^{-1}) is measurably greater than the negative enthalpy of precipitation (−35.7 ± 10.5 kJ mol^{-1}). This may be due to the precipitation reaction having two stages, a quick precipitation

dx.doi.org/10.1021/jp309109k | J. Phys. Chem. B 2013, 117, 490−494

reaction followed by a slow consolidation reaction. There are many examples of two-stage reactions such as papilloma virus self-assembly, where the assembly reaction is followed by consolidation or maturation reactions.[15,16] It is worth noting that the change in enthalpy for heat-induced disassociation is a fraction (approximately 0.5%) of that for heat-induced unfolding of IgM. IgG has an enthalpy of unfolding of 2700 kJ/mol.[17] Unlike protein folding where numerous bonds are involved, cold-induced precipitation involves the formation of relatively few bonds.

Cold-induced precipitation of Pot IgM is an example of a negative enthalpy ($\Delta H^{\ddagger}$) reaction, getting faster as the temperature is reduced and having a negative slope on an Eyring plot ($\ln(k/T)$ versus $1/T$). There is a small proportion of reactions that have a negative $\Delta H^{\ddagger}$ including Diels–Alder reactions, radical reactions, and proton and electron transfer reactions (for a review see Frank et al.).[18] The closest reaction to cold-induced precipitation is protein folding. Both protein folding and cold-induced precipitation fail to conform to Arrhenius behavior and have a negative $\Delta H^{\ddagger}$.[15] Both reactions also involve the displacement of water from the surface of the protein followed by the formation of hydrogen bonds plus van der Waals attraction. Unlike protein folding where the Eyring plot showed marked curvature,[19] the line for cold-induced precipitation was nearly straight. The lack of curvature in the Eyring plot (which is due to a change in heat capacity) suggests hydrophobic interaction is minimal for this reaction, suggesting a predominance of van der Waals forces and hydrogen bonding between Pot IgM molecules, rather than the displacement of water from around apolar residues. To come together, two Pot IgM molecules would have to displace the hydration layer around the parts of the protein that eventually link together. It is possible that this water displacement is a critical part of the transition state. In the case of a normal chemical reaction the positive $\Delta H^{\ddagger}$ is due to the breaking of bonds to form the intermediate; in cold-induced precipitation the negative $\Delta H^{\ddagger}$ is probably related to the displacement of the water from the surface of the protein. Another phenomenon that occurs at low temperature that should not be confused with cold-induced precipitation is cold denaturation. The mechanism for this reaction is quite different and involves the increased stabilization of water around apolar side chains at lower temperatures, which moves the equilibrium to favor the unfolded state.[20] Although cold denaturation could lead to precipitation, the lack of discernible change to the secondary structure of Pot IgM when analyzed by CD spectroscopy suggests this did not occur. Denaturation of immunoglobulins is an irreversible process. The tertiary structure of IgMs are dominated by β-barrel structures that do not refold to their native state but form structures dominated by α-helices that are readily detected by CD spectroscopy.[17] Previous CD analysis of the IgG cryoglobulin Web at temperatures above and below the temperature where it precipitates also demonstrated insufficient change in helicity to indicate cold denaturation was taking place.[21]

The research outlined in this paper supports the theory that cryoglobulins are normal immunoglobulins at the insoluble end of a normal immunoglobulin population.[9] It is unlikely that cold-induced precipitation is limited to immunoglobulins isolated from patients suffering from cryoglobulinemia. This phenomenon can also be expected to be observed during storage of diverse proteins at high concentrations in liquid formulations at low temperatures. Clinically, this study confirms that Type I cryoglobulinemia will be most severe in patients with high immunoglobulin concentrations and that individual cases can be radically different as cold-induced gelation and precipitation are likely to result in a difference in clinical severity. The thermodynamics of cold-induced precipitation is an interesting finding, as it provides an example of a negative activation enthalpy reaction which does not conform to Arrhenius behavior and has marked similarities to the thermodynamics of protein folding.[19]

■ ASSOCIATED CONTENT

Supporting Information

Derivation of eq 1. T_m values at different concentrations and scan rates. $\Delta H^{\ddagger}$ calculated for cold-induced precipitation and heat-induced disassociation. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*R.J.F.: tel, +441142228253; fax, +441142227501; e-mail, r.j.falconer@sheffield.ac.uk. P.A.R.: tel, +6139282 2178; e-mail. pramsland@burnet.edu.au.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank Emeritus Professors Robert L. Raison and Allen B. Edmundson for providing the Pot IgM and Yvo IgM cryoglobulins, respectively, and Ms. Cherrine Chan for the DSC analysis. P.A.R. is supported by the Sir Zelman Cowen Fellowship Fund, Burnet Institute. The work was supported in part by a National Health and Medical Research Council of Australia Project Grant ID543317. The authors gratefully acknowledge the contribution to this work of the Victorian Operational Infrastructure Support Program received by the Burnet Institute.

■ REFERENCES

(1) Lerner, A. B.; Watson, C. J. *Am. J. Med. Sci.* **1947**, *214*, 410–415.
(2) Meltzer, M.; Franklin, E. C. *Am. J. Med.* **1966**, *40*, 828–836.
(3) Brouet, J. C.; Clauvel, J. P.; Danon, F.; Klein, M.; Seligmann, M. *Am. J. Med.* **1974**, *57*, 775–788.
(4) Middaugh, C. R.; Litman, G. W. *J. Biol. Chem.* **1977**, *252*, 8002–8006.
(5) Litman, G. W.; Gerber-Jenson, B.; Litman, R.; Middaugh, C. R.; Scheffel, C. *Mol. Immunol.* **1980**, *17*, 337–44 1980..
(6) Scoville, C. D.; Turner, D. H.; Lippert, J. L.; Abraham, G. N. *J. Biol. Chem.* **1980**, *255*, 5847–5852.
(7) Vialtel, P.; Kells, D. I. C.; Pinteric, L.; Dorrington, K. J.; Klein, M. *J. Biol. Chem.* **1982**, *257*, 3811–3818.
(8) Lawson, E. Q.; Brandau, D. T.; Trautman, P. A.; Aziz, S. E.; Middaugh, C. R. *Mol. Immunol.* **1987**, *24*, 897–905.
(9) Middaugh, C. R.; Lawson, E. Q.; Litman, G. W.; Tisel, W. A.; Mood, D. A.; Rosenberg, A. *J. Biol. Chem.* **1980**, *25*, 6532–6534.
(10) Brandau, D. T.; Lawson, E. Q.; Trautman, P. A.; Middaugh, C. R. *Immunol. Invest.* **1987**, *16*, 21–32.
(11) Vallas, V.; Farrugia, W.; Raison, R.; Edmunson, A. B.; Ramsland, P. A. *J. Mol. Recognit.* **2007**, *20*, 90–96.
(12) Sanchez-Ruiz, J. M.; Lopez-Lacoma, J. L.; Cortijo, M.; Mateo, P. L. *Biochemistry* **1988**, *27*, 1648–1652.
(13) Eyring, H. *Chem. Rev.* **1935**, *17*, 65–77.
(14) Navazza, V.; Gabba, S.; Alfieri, A.; Giorgetti, S.; Marchese, L.; Palladini, G.; Mattevi, A.; Ascari, E.; Caporali, R.; Montecucco, C.; Merlini, G.; Perfetti, V. *Mol. Immunol.* **2008**, *45*, 1519–1524.

(15) Buck, C. B.; Thompson, C. D.; Pang, Y. Y. S.; Lowy, D. R.; Schiller, J. T. *J. Virol.* **2005**, *79*, 2839−2846.
(16) Hanslip, S. J.; Zaccai, N. R.; Middelberg, A. P. J.; Falconer, R. J. *Biochem. Biophys. Res. Commun.* **2008**, *375*, 351−355.
(17) Vermeer, A. W. P.; Norde, W. *Biophys. J.* **2000**, *78*, 394−404.
(18) Frank, R.; Greiner, G.; Rau, H. *Phys. Chem. Chem. Phys.* **1999**, *1*, 3481−3490.
(19) Oliveberg, M.; Tan, Y-J; Fersht, A. R. *Proc. Natl. Acad. Sci. U. S. A.* **1995**, *92*, 8926−8929.
(20) Graziano, G.; Catanzano, F.; Riccio, A.; Barone, G. *J. Biochem.* **1997**, *122*, 395−401.
(21) Scoville, C. D.; Abraham, G. N.; Turner, D. H. *Biochemistry* **1979**, *18*, 2610−2615.