Resolution Method of Two Sets of Binding Sites for the Cationic Surfactant–Urease Interaction

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(Received March 15, 1996)

The binding of a homologous series of cationic surfactants with jack-bean urease has been studied by equilibrium dialysis at pH = 10 and 27 °C. The unusual concavity of a Scatchard plot has been interpreted by using the binding capacity concept. The relation between the binding capacity and the criterion of cooperativity (Hill coefficient) was obtained, and in the base of this relation a new method has been introduced for the resolution and characterization of two sets of binding sites in these systems, which is supported by microcalorimetric and spectroscopic data.

Urease, the first enzyme to be crystallized[1] and the first to have been shown to include nickel,[2] is found in a variety of plants and a broad range of bacterial species. Urease catalyzes the hydrolysis of urea to carbonic acid and two molecules of ammonia.[3] Despite the availability of crystals for nearly 70 years, the structure of jack-bean urease has not been determined. The relative molecular mass of the urease subunit, calculated from the amino acid sequence (excluding the two nickel ions per its only active site) is 90770.[4] Hence, a value of 545340 (including two nickel ions per subunit) for the hexamer has been reported.[4] The subunits of urease from microorganisms appear to be smaller than jack-bean urease in both size and number.[5—7]

The binding of ionic surfactants to water-soluble proteins has been studied extensively.[8,9] With respect to the amphipathic native of surfactants and proteins, it is well established that there are two kinds of interactions in the binding of ionic surfactants to proteins.[10] The binding of the ionic head group of surfactants to sites with opposite charge at the surface of the protein initially occur, followed by more extensive hydrophobic binding to hydrophobic residues in the protein.[11,12] Due to this fact, it is expected that these exist at least two sets of binding sites in the protein.

A common method which has been used for evaluating the binding of surfactants to proteins is the Scatchard plot.[13] In spite of the existence of at least two sets of binding sites, this plot was previously interpreted as representing a system with one set of binding sites.[14,15] Due to the similarities which may exist in the general shape of the Scatchard plot for systems with one set and systems with two sets of binding sites, some misinterpretation of binding data is possible.[16] However, there have been a few reports that the unusual shape of the Scatchard plot can be interpreted by considering the two sets of binding sites.[17,18]

In the present investigation, the binding of a homologous series of cationic surfactants with jack-bean urease was studied by equilibrium dialysis. Unusual Scatchard plots for these systems were observed. The existence of two sets of binding sites in these systems has been shown by a new method involving the application of the binding-capacity concept. Further information has also been obtained by the application of this novel method.

Experimental

Materials. Jack-bean urease, dodecyltrimethylammonium bromide (DTAB), tetradecyltrimethylammonium bromide (TTAB), and hexadecyltrimethylammonium bromide (HTAB) were obtained from Sigma Chemical Co. Visking membrane dialysis tubing (MW cut-off 10000—14000) was obtained from Scientific Instrument Center Ltd. (SIC, Eastleigh, Hampshire, UK). All other materials and reagents were of analytical grades, and solutions were made in double-distilled water. Glycine solution (50 mM, M = mol dm^{-3}) plus sodium hydroxide (pH 10), which contain 0.02% (w/v) sodium azide, has been used as a buffer.

Methods. Equilibrium Dialysis: Experiments were carried out at 300 K using a urease solution with a concentration of 0.05% (w/v), of which 2 ml aliquots were placed in dialysis bags and equilibrated with 2 ml of the surfactant solution, covering the required concentrations range for over 96 h. The free DTAB, TTAB, and HTAB concentrations in equilibrium with complexes were assayed by the orange-II dye method.[19] The molecular weight of jack-bean urease was taken to be 545340.[4]

Spectroscopy: Spectroscopy experiments were performed using a recording spectrophotometer (UV-3100 Shimadzu model, Japan). The sample cell contained 2ml of a jack-bean urease solution at a concentration of 0.026% (w/v) with different fixed concentrations of the surfactant from 0.00 to 2.00 mM. The reference cell was excluded of urease. Both of the cells were set at a constant temperature. The absorbtion of the sample cells was recorded at 278 nm wavelength versus the reference cells.

Microcalorimetry: Calorimetric experiments were performed with a 4-channel commercial microcalorimetric system (Thermal Activity Monitor 2277; Thermometric, Sweden). Each channel is a twin heat-conduction calorimeter, where the heat-flow sensor

is semiconducting thermopile (multi-junction thermocouple plates) positioned between the vessel holders and the surrounding heat sink. The insertion vessel was made from stainless steel. The surfactant solution (40 mM) was injected using a Hamilton syringe into a calorimetric-stirred titration vessel, which contained 2 ml of the urease solution, 0.06% (w/v), including glycine buffer (50 mM), pH = 10.0. Thin (0.15 mm inner diameter) stainless-steel hypodermic needless, permanently fixed to the syringe, reached directly into the calorimeter vessel. Injection of the surfactant into the perfusion vessel was repeated 17 times, and each injection included 10 μl of the reagent. The calorimetric signal was measured by a digital voltmeter that was part of a computerized recording system. The heat of each injection was calculated by the "Digitam computer program". The heat of dilution and demicelization of the surfactant solutions were measured as described above, except that the enzyme was excluded. The enthalpy of dilution and demicelization for surfactant micelles was substracted from the enthalpy of the urease-surfactant interaction. The enthalpy of dilution of the urease was also negligible. The microcalorimeter was frequently calibrated electrically during the course of the study.

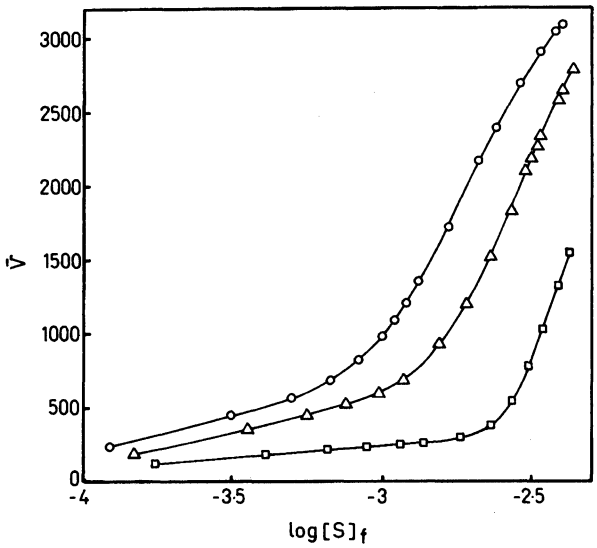

Fig. 1. Binding isotherms for binding jack bean urease with DTAB(□), TTAB (△), and HTAB (○) at pH = 10 and 27 °C.

Results and Discussion

The binding isotherms have been plotted as the average number of bound surfactants to one macromolecule, ($\bar{\nu}$) vs. log $[S]_f$, where $[S]_f$ is the free surfactant concentration, as shown in Fig. 1. It shows the binding isotherms for the binding of DTAB, TTAB, and HTAB with jack-bean urease at pH = 10 and 27°C.

The Scatchard plots are shown in Fig. 2. Scatchard in his original paper pointed out that the curvature may indicate different intrinsic constants or deviations from independent probabilities.[13] For systems with one set of binding sites and positive and negative cooperativity in binding, this curve should be downward and upward, respectively. However, these mentioned cases have not been observed in the binding of cationic surfactants on urease; however, they are upward with concavity. These unusual cases may be due to the nature of the interactions between ionic surfactants and proteins which have an amphipathic property. Thus this system may be considered with two sets of binding sites, including an electrostatic interaction and a hydrophobic interaction. The shape of the Scatchard plot is related to the difference between the strength of these two interactions, the number of binding sites and the criterion of cooperativity in each binding set.[16] Therefore, since the evaluation of these Scatchard plots has become very difficult, a new theory should be defined for analyzing such systems.

For evaluating such a system it may be possible to use the concept of the binding capacity (θ). It is the homotropic second derivative of the binding potential with respect to the chemical potential of the ligand (μ_i), and provides a measure of the steepness of the binding isotherm.[20] It represents the change in the number of moles of the ligand per mole of the macromolecule that accompanies a change in the chemical potential of that ligand. It is equal to

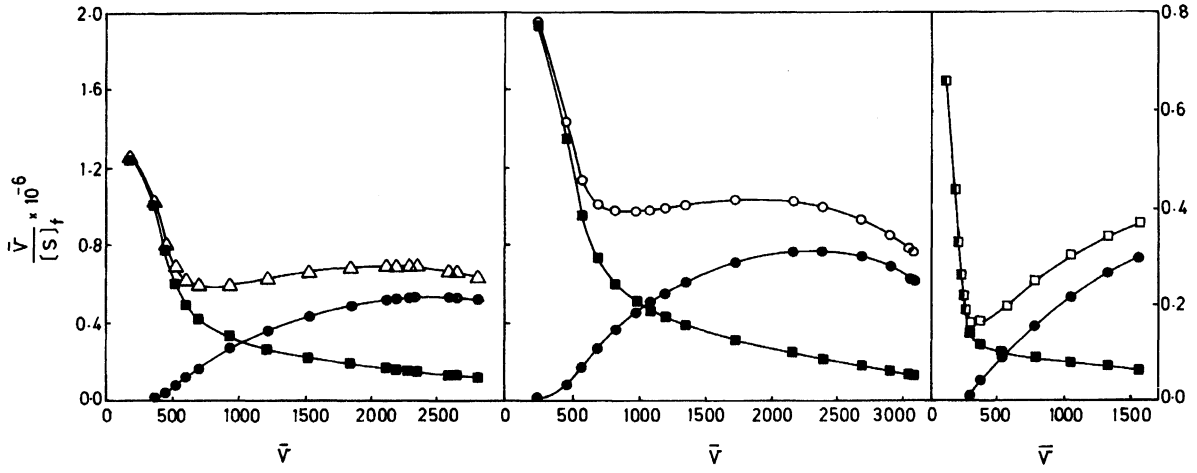

Fig. 2. The Scatchard plots for binding jack bean urease with DTAB (□), TTAB (△), and HTAB (○) at pH = 10 and 27 °C. Nonliner Scachard plot of the same data resolved into two types of binding: the first binding sites (■) and the second binding sites (●). The scale on the right ordinate-axis is only for DTAB.

$$\theta = \frac{\partial \nu_i}{\partial \mu_i} = \frac{\partial \nu_i}{RT\partial \ln [S_f]} = \frac{\partial \nu_i}{2.303RT\partial \log [S_f]}, \quad (1)$$

where R is the gas universal constant and T is the absolute temperature. By calculating the slope of the binding isotherms at any point, the values of θ at any $\overline{\nu}$ can be determined. Figure 3 shows the variation of $2.303RT\theta$ with respect to $\log [S]_f$ for the related interaction. It should be a Gaussian hyperbola curve for a system with one set of binding sites.[18] However, Fig. 3 is not like a Gaussian curve, and contains two different regions with a large difference in the slope. Due to this fact, that the binding capacity can be a measure of cooperativity, as expressed by the Hill coefficient,[20] the large difference in the slope of the different regions in Fig. 3 may be due to a large change in the amount of cooperativity during binding of the surfactant ions. It is possible to find a relation between the Hill coefficient (n, the criterion of cooperativity) and the binding capacity with respect to the definition of the Hill coefficient, as follows:[21,22]

$$n = \frac{d\log(\frac{y}{1-y})}{d\log [S_f]} = \frac{1}{y(1-y)} \frac{dy}{2.303d\log [S_f]}$$

$$= \frac{1}{gy(1-y)} \frac{d\overline{\nu}}{2.303d\log [S]_f}. \quad (2)$$

Here, y is the amount of saturation of the macromolecule at a specified free concentration of the surfactant ($[S_f]$) and is equal to

$$y = \frac{\overline{\nu}}{g}, \quad (3)$$

where g is the number of binding sites at saturation. With respect to the definition of the binding capacity (Eq. 1) since

$$n = \frac{1}{gy(1-y)}(RT\theta) \quad (4)$$

$$\theta = \frac{n\overline{\nu}}{RT}(1-y), \quad (5)$$

$$b = \frac{2.303RT\theta}{\overline{\nu}} = 2.303n(1-y). \quad (6)$$

This is a better function with respect to the binding capacity for representing the Hill coefficient.

Figure 4 shows the variation of b with respect to $\log [S_f]$ for the binding of DTAB, TTAB, and HTAB with urease at pH=10 and 27°C. It shows a large change for a specific amount of $\overline{\nu}$, which could be due to a large change in the Hill coefficient. Thus these curves prove the existence of two sets of binding sites in the binding of DTAB, TTAB, and HTAB with urease.

With respect to the nature of the binding in a protein-surfactant system, it was reported that the first binding set involved an electrostatic interaction and the second binding set involved a hydrophobic interaction.[11,12] Here, it is attempted to show that the first binding set is electrostatic, accompanied by a preliminary hydrophobic interaction, followed by a more extensive pure hydrophobic interaction at the second binding set. Nevertheless, for obtaining approximated values of binding parameter, it might be possible to fit the binding data to Hill equation with more than one term,[15,16]

$$\overline{\nu} = \frac{g_1(K_1[S_f])^{n_1}}{1+(K_1[S_f])^{n_1}} + \frac{g_2(K_2[S_f])^{n_2}}{1+(K_2[S_f])^{n_2}}, \quad (7)$$

where g_1, K_1, and n_1 are the number of binding sites, binding constant, and Hill coefficient for first binding set and g_2, K_2, and n_2 are the corresponding parameters for second binding set. The binding data for the binding of DTAB, TTAB, and HTAB to urease have been fitted to Eq. 7 using a computer program for nonlinear least-square fitting[23] (tabulated in Table 1). The large steepness in b (see Fig. 4) occurs at the concentration of the surfactant adapting to $\overline{\nu}$, which is close to g_1(the number of binding sites in the first binding set). Since the values of n_2 are more than n_1 in all cases, this can be a reason why the value of b is increased in this system.

The absorption changes of the urease solution at λ_{max}=278

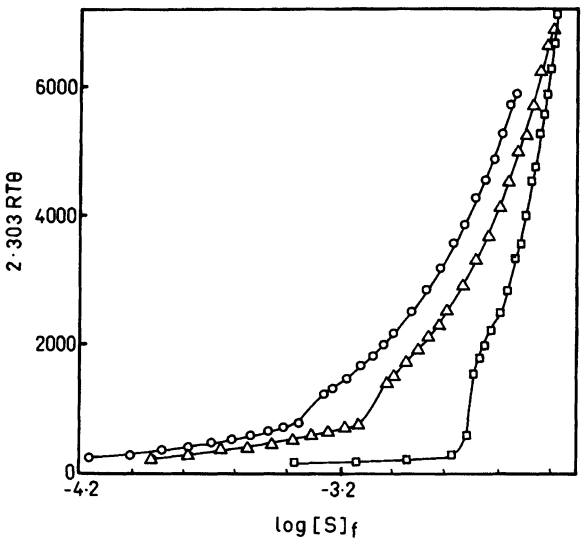

Fig. 3. The variation of 2.303 $RT\theta$ respect to $\log [S]_f$, obtained from Fig. 1, for jack bean urease with DTAB (□), TTAB (△), and HTAB (○) at pH=10 and 27 °C.

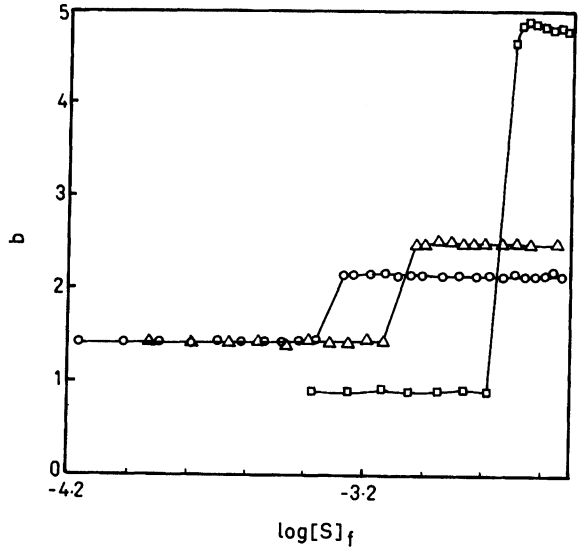

Fig. 4. The variation of b respect to $\log [S]_f$, obtained from Fig. 3, for jack bean urease with DTAB (□), TTAB (△), and HTAB (○) at pH=10 and 27 °C.

nm in the presence of different concentrations of surfactants is shown in Fig. 5. The values of $\overline{v}$ in each total concentration of the surfactant were calculated from Fig. 1, using the following equation:

$$[\text{surfactant}]_{\text{total}} = [\text{surfactant}]_{\text{free}} + \overline{v}[\text{urease}]_{\text{total}} . \quad (8)$$

The sigmoidal curves of denaturation given in Fig. 5 were obtained in the concentration range of the electrostatic interaction ($\overline{v} < g_1$). It is important to note that more than 90% unfolding of urease occurs in this concentration range.[24] This might be due to the fact that the electrostatic interaction perturbs the charge balance at the surface of the protein.

Figure 6 shows the variation in the calorimetric enthalpy per mole of protein, which has been divided to $\overline{v}$; $\Delta H_{\overline{v}} = \Delta H/\overline{v}$, with respect to the total concentration of the surfactant. The calorimetric enthalpy involves the heat of surfactant binding to the protein and the heat of protein unfolding. The interactions of DTAB and TTAB are exothermic, while HTAB is endothermic. However, the exothermicity of DTAB, especially at higher values of $\overline{v}$, is more than that of TTAB.

The enthalpy curves of DTAB and TTAB have a distinic minimum, which could be due to an endothermic process during an exothermic process. These minima points occur at $\overline{v}$ equal to 300 and 170 for DTAB and TTAB, respectively. In addition, these appearance are along with predominate unfolding of the protein according to the spectroscopic

Table 1. The Values of Binding Parameters for Cationic Surfactants Interaction with Jack Bean Urease at pH=10 and 27°C

Surfactants	g_1	K_1/M^{-1}	n_1	g_2	K_2/M^{-1}	n_2
DTAB	310	3499.7	1.03	1890	268.2	5.79
TTAB	540	4403.6	1.54	2935	348.5	2.90
HTAB	540	7015.4	1.65	2935	520.2	2.56

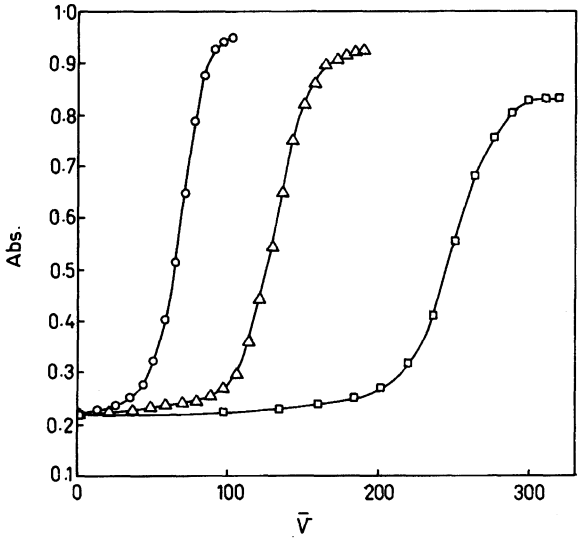

Fig. 5. The variation of absorption respect to $\overline{v}$ for jack bean urease (0.026% w/v) with DTAB (□), TTAB (△), and HTAB (○) at pH=10, T=27°C and λ_{max}=278 nm.

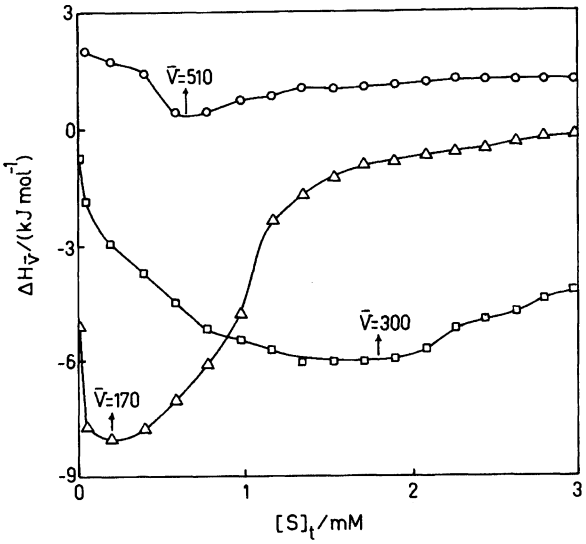

Fig. 6. The variation of calorimetric enthalpy per mole of protein and per mole of surfactant ($\Delta H_{\overline{v}}$) respect to total concentration of DTAB (□), TTAB (△), and HTAB (○) at pH=10 and T=27°C. The urease concentration was 0.06% (w/v).

data (Fig. 5). The unfolding of the protein (endothermic process) and the change in the kind of binding from electrostatic (exothermic process) to pure hydrophobic interactions (endothermic process) lead to a minimum in the enthalpy curves of DTAB and TTAB. The large difference in $\overline{v}$ at the minimum point of the enthalpy curve with g_1 for TTAB, with respect to DTAB, is related to the higher denaturation strength of TTAB. This fact shows the preliminary interaction due to the hydrophobic tail of the surfactant with hydrophobic patches at the surface of the protein, which accompanies an electrostatic interaction of cationic heads of the surfactant with anionic amino acids at the surface of the protein. These neutralizations perturb the force balance in protein structure and ultimately the denaturation of the protein.

Figure 6 shows a distinic minima for the interaction of HTAB with urease. This point occurs at $\overline{v} \cong g_1 = 510$, showing the change in the kind of binding. This has certified our binding data analysis in terms of two sets of binding sites. However, a spectroscopic denaturation study (Fig. 5) indicates that the predominate unfolding of urease occurs at $\overline{v}$=90, which is much lower than g_1. It is consistent with our above discussion concerning the role of the length of the surfactant tail in the denaturation strength. The calorimetric enthalpy curve of the HTAB-urease interaction(Fig. 6) does not show the status of the enthalpy interaction below v=90, because urease was denatured by the first injection of HTAB into the protein solution.

The spectroscopic and calorimetric studies show that the predominate unfolding occurs at a region where the first binding set has been occupied, and that the strength of denaturation of the surfactants has been increased by increasing the length of the hydrophobic tail. The increase in the denaturation strength of the surfactant with increasing the tail

length is related to preliminary hydrophobic interactions of the surfactant tail with hydrophobic patches, which are accompanied by a neutralization of the charge at the surface of the protein. The electrostatic interaction is thus accompanying by preliminary hydrophobic interactions which is occurred initially, followed by a more extensive pure hydrophobic interaction. This matter certifies our binding-data analysis by considering two sets of binding sites.

Therefore, this method is suitable for evaluating the binding process of ionic surfactants to water-soluble proteins, and can interpret the unusual shape of Scatchard plot and certify the existence of two sets of binding sites in these systems.

The financial support of the Research Council of University of Tehran is gratefully acknowledged.

References

1) J. B. Summer, *J. Biol. Chem.*, **69**, 435 (1926).
2) N. E. Dixon, C. Gazzola, R. E. Blakeley, and B. Zerner, *J. Am. Chem. Soc.*, **97**, 4131 (1975).
3) H. L. T. Mobley and R. P. Hausinger, *Microbiol. Rev.*, **53**, 85 (1989).
4) K. Takishima, T. Suga, and G. Mamiya, *Eur. J. Biochem.*, **175**, 151 (1988).
5) H. Nakano, S. Takenishi, and Y. Watanabe, *Agric. Biol. Chem.*, **48**, 1495 (1984).
6) S. Christians and H. Kaltwasser, *Arch. Microbiol.*, **145**, 51 (1986).
7) R. P. Hausinger, *J. Biol. Chem.*, **261**, 7866 (1986).
8) M. N. Jones, *Chem. Soc. Rev.*, **21**, 127 (1992).
9) M. N. Jones, "Biological Interfaces," Elsevier, Amesterdam (1975), Chap. 5.
10) G. Prieto, J. M. del Rio, M. I. Paz Andrade, F. Sarmiento, and M. N. Jones, *Int. J. Biol. Macromol.*, **15**, 343 (1993).
11) J. A. Raynold, S. Herbert, H. Polet, and J. Steinhardt, *Biochemistry*, **6**, 937 (1967).
12) E. Tipping, M. N. Jones, and H. A. Skinner, *J. Chem. Soc., Faraday Trans.*, **70**, 1306 (1974).
13) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).
14) F. E. Beyhl, *IRCS Med. Sci.*, **14**, 417 (1986).
15) M. N. Jones and P. Manley, *Int. J. Biol. Macromol.*, **4**, 201 (1982).
16) A. K. Bordbar, A. A. Saboury, and A. A. Moosavi-Movahedi, *Biochem. Educ.*, in press (1996).
17) A. A. Moosavi-Movahedi and M. R. Housaindokht, *Int. J. Biol. Macromol.*, **13**, 50 (1991).
18) A. A. Moosavi-Movahedi and M. R. Housaindokht, *Int. J. Biol. Macromol.*, **16**, 77 (1994).
19) A. V. Few and R. H. Ottewill, *J. Colloid Sci.*, **11**, 34 (1965).
20) J. Wyman, S. J. Gill, and E. D. Cera, *Proc. Natl. Acad. Sci. U. S. A.*, **85**, 449 (1988).
21) A. V. Hill, *J. Physiol.*, **40**, iV—Vii (1910).
22) A. A. Saboury and A. A. Moosavi-Movahedi, *Biochem. Educ.*, **22**, 48 (1994).
23) M. L. James, G. M. Smith, and J. C. Wolford, "Applied Numerical Methods for Digital Computer," 3rd ed, Harper and Row Publisher, New York (1985).
24) A. A. Saboury, A. K. Bordbar, and A. A. Moosavi-Movahedi, *J. Chem. Thermodyn.*, in press (1996).