Three Binding Sets Analysis of α-Lactalbumin by Interaction of Tetradecyl Trimethyl Ammonium Bromide

M. R. Housaindokht,* J. Chamani,† A. A. Saboury,† A. A. Moosavi-Movahedi,† and M. Bahrololoom

Department of Chemistry, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran
†Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran
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The interaction between tetradecyl trimethyl ammonium bromide (TTAB) with bovine α–lactalbumin has been investigated at pH = 9 and at 37 ºC by isothermal titration calorimetry, equilibrium dialysis and UV-Vis spectrophotometry methods. The binding data from unusual Scatchard plot have been analyzed in terms of the Hill equation for three sets of binding sites. The calorimetric data show that TTAB interacts endothermically with α-lactalbumin and causes protein unfolding below 2 mM concentration of TTAB, which is confirmed by spectrophotometric data. The unfolding of the protein would be mainly due to occupation of the second set of binding sites.

Keywords : TTAB, α-Lactalbumin, Unfolding, Scatchard plot.

Introduction

Recent advances, in the effort to unravel the mechanisms of protein unfolding, have emphasized the importance of a detailed structural description of folding intermediates.[1,2] Yet, a key question that remains unsolved concerns the relative importance of local versus long-range interactions in these early events. A better understanding of this question will require more detailed structural information on binding sites and folding intermediates.

In studies of protein folding, α-lactalbumin, α-La, has received considerable attention. α-La has an amino acid sequence and conformation comparable to that of c-type lysozyme, but contains a Ca^{2+} binding site.[3]

The study of three-state unfolding of α-La has resulted in the formulation of the molten globule concept.[4] α-La is one of the best known example where in a stable molten globule state can be detected.[5] The molten globule concept is widely accepted and molten globules are assumed to be of general importance to the folding and processing of proteins.[6] It is also reported that the molten globule (for example, α-La) is a third thermodynamic state of the protein.[7]

The binding of ionic surfactants to water-soluble proteins has been extensively studied.[8-12] The elucidation of binding sites is the heart of protein structural information. In protein denaturation, surfactant is directly bound to protein. The unfolding mechanism of proteins is dominated by electrostatic and hydrophobic interaction.[12] The directly bound surfactant helps to identify the effected binding sets.

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 protein.[13] Usually the binding of the ionic head group of surfactants to sites with opposite charge at the surface of the protein initially occurs, followed by more extensive hydrophobic binding to hydrophobic residues in the protein.[14]

A common method, which has been used, for evaluating the binding of surfactants to proteins is the Scatchard plot.[15] 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.[16-18]

In the present article, the results provide evidence for the existence three sets of binding sites concerning the interaction between α-La and tetradecyl trimethyl ammonium bromide, TTAB, as a cationic surfactant. Here we fit a threestate equation for the cited interaction to find the sets of binding sites to elucidate unfolding of α-La in interaction with TTAB.

Materials and Methods

Materials. α-La was purchased from Merck Chemical Co. Before use it was exhaustively dialyzed against the buffer. *n*-Tetradecyl trimethyl ammonium bromide (TTAB) was obtained from Sigma. Visking membrane dialysis tubing (molecular weight cut-off 10000-14000) was from SIC (Eastleigh, Hampshire, UK).

The buffer solution was tris-NaOH (50 mM), pH = 9. It also contained 0.02% w/v sodium azide to inhibit bacterial growth. All the reagents were of analytical grade and solutions were made up with double-distilled water.

Methods

Isothermal titration calorimetry (ITC). Enthalpy measurements were made with a LKB microcalorimeter (2277 Thermal Activity Monitor, Boromma, Sweden). The enthalpy of interaction between TTAB and α-La was measured by adding 25 μL portions of TTAB 40 mM solution to the 0.2% (w/v) solution of the protein in the titration cell. The enthalpy of demicellization of TTAB due to injection was corrected by measuring the enthalpy changes after injections of TTAB solution into the buffer solution using the same procedures and experimental conditions. The heat of dilution of α-La has been neglected.

Equilibrium dialysis. Equilibrium dialysis was carried out to determine the concentration of free TTAB in equilib-

rium with the complexes at 37 °C and hence the amount of TTAB bound to the protein. The experiments were carried out with dialysis bags made from Visking tubing (9/16 inch) as semipermiable membrane. Volumes of 2 cm^3 aliquots of 0.05% (w/v) α-La solution, or as required, were dialyzed against 2 cm^3 aliquots of TTAB solution in the concentration range 2×10^{-4}-3.3×10^{-3} mol dm^{-3}. Equilibration times were in excess of 96 h. The free TTAB concentrations in equilibrium with the complexes were assayed by the orange II method of Few and Ottewill.[19]

Spectrophotometry. These experiments were performed using a recording spectrophotometer (UV-3100 Shimadzu model, Japan). Initially the absorbance reading at 274.5 nm was made with a sample containing 2 mL aliquots of 0.04% (w/v) α-La solution and the reference cell containing 2 mL of the same buffer solution, subsequent reading was made after each addition of surfactant to both cells.

Results and Discussion

Figure 1 shows binding isotherm for interaction between TTAB with α-La at pH = 9 and 37 °C.

Figure 2 shows the unusual Scatchard plot. This may be due to the existence of more than one set of binding sites.[16-18] As regards the Figure 2, the existence of three sets of binding sites is suggested. In this case the Hill equation can be written:

$$\nu = \frac{g_1(K_1[\mathrm{TTAB}])^{n_{1H}}}{1 + g_1(K_1[\mathrm{TTAB}])^{n_{1H}}} + \frac{g_2(K_2[\mathrm{TTAB}])^{n_{2H}}}{1 + g_2(K_2[\mathrm{TTAB}])^{n_{2H}}} + \frac{g_3(K_3[\mathrm{TTAB}])^{n_{3H}}}{1 + g_3(K_3[\mathrm{TTAB}])^{n_{3H}}} \quad (1)$$

Where n_{1H}, n_{2H}, n_{3H}, K_1, K_2, K_3 and g_1, g_2, g_3 are the Hill coefficients, the binding constants and binding capacities of first, second and third sets of binding sites, respectively. Here the

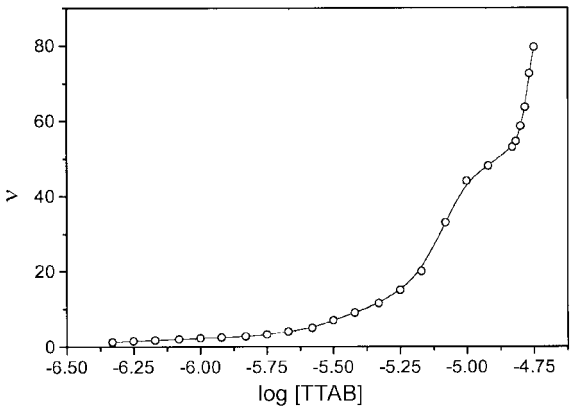

Figure 1. Binding isotherms for interaction of TTAB with α-La.

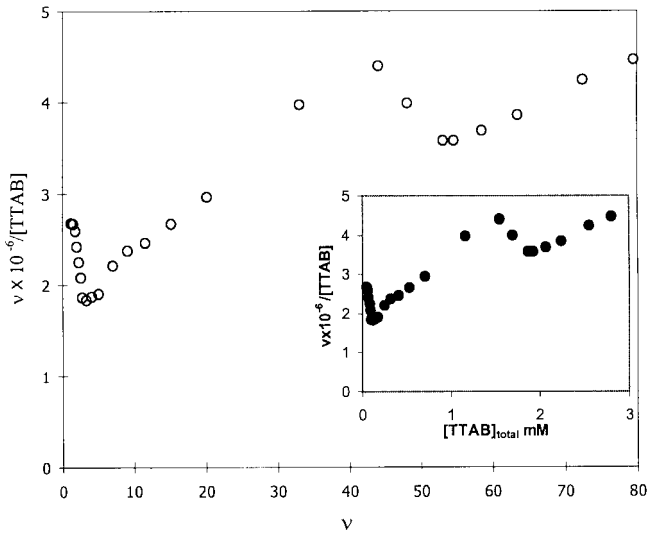

Figure 2. Scatchard plots for interaction of TTAB with α-La.

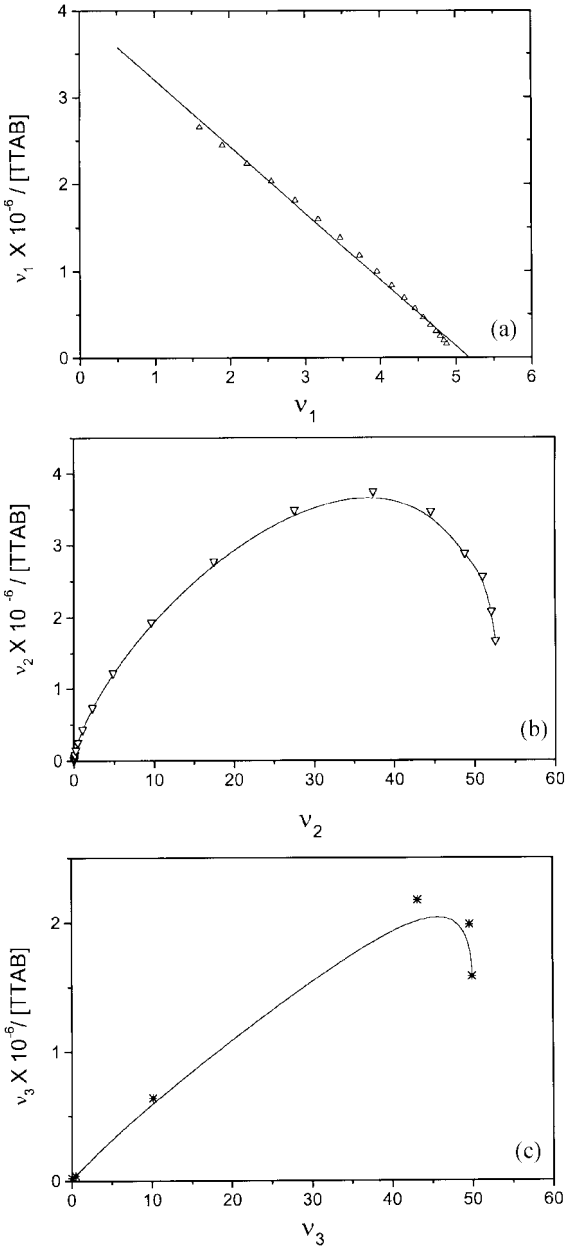

Figure 3. The analyzed Scatchard plots by Hill equation for three sets of binding sites for interaction of TTAB with α-La: (a) first set; (b) second set; (c) third set.

Table 1. Parameters derived from equation (1) for the binding of n-tetradecyl trimethyl ammonium bromide to α-La at 37 °C and pH = 9.

No. of set	g	n_H	K (M^{-1})
1	5	1.12	8.2×10^5
2	53	3.44	1.29×10^5
3	50	14	5.7×10^4

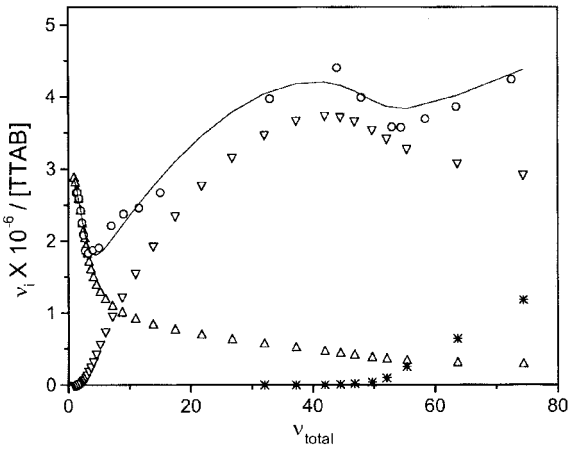

Figure 4. Scatchard plots for interaction of TTAB with α-La, (○); (Δ) first set; (∇) second set; (∗) third set.

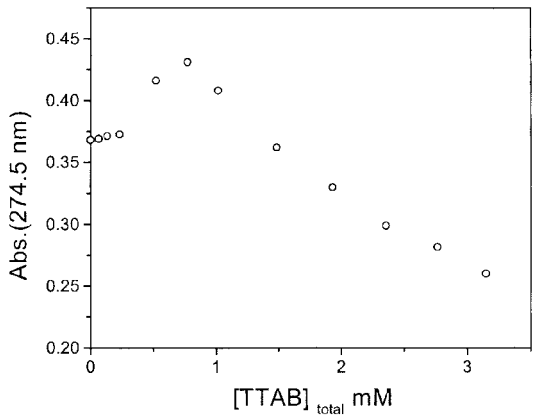

Figure 5. Variation of absorbance at 274.5 nm versus the total concentration of TTAB in interaction with α-La, at 0.04% (w/v).

data are analyzed in terms of the Eq. (1) using a computer program for nonlinear least-square fitting,[20] and the results are listed in Table 1.

It is known that the Hill coefficient could be a criterion for the type of interaction.[9] The first set of sites are occupied with specific interaction ($n_H \cong 1$), however, the other two sets of sites are occupied with positive cooperative interactions ($n_H > 1$).

The Scatchard plots, for each set of sites, are shown in Figure 3. The unusual plots are produced from superposition of those plots depicted in Figure 4.

Figure 5 shows the absorption change for the cited interaction at 274.5 nm. It is seen that at low concentration of TTAB the absorption change is negligible, while the absorption change between 0.2 mM and 2 mM concentration of TTAB (ν is between 5 to 53) is quite considerable. This implies that at concentration range 0.2-2 mM of TTAB, corresponding to g_2, the conformation change of the protein is occurred, *i.e*. the protein unfolding. In fact, the TTAB binding by its tail to the second sets of sites, likely hydrophobic, increases the absorption. In contrast, the unfolding decreases the absorption. As a result, a maximum is observed on the above concentration range. Figure 6 depicts the enthalpy curve for the interaction of the TTAB with α-La, obtained by ITC. It is observed that the measured enthalpy is endothermic on the TTAB concentration range. The maximum on this curve may be due to endothermic unfolding process,[21] along with the binding of TTAB to the second set of sites, which is likely endothermic.

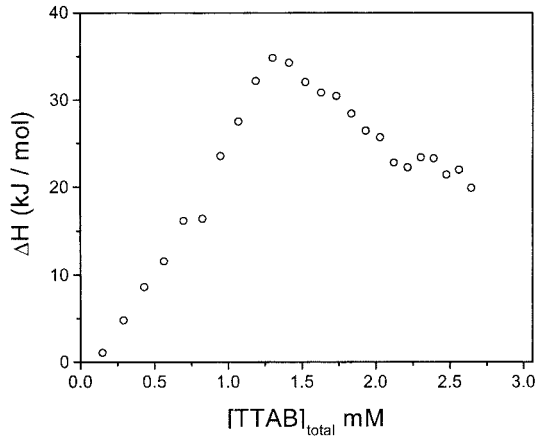

Figure 6. The enthalpy value versus total concentration of TTAB in interaction with α-La, at 0.2% (w/v).

It may be proposed that at low concentration, TTAB binds specifically to the anionic sites exposed on the surface of the protein. At higher concentrations (0.2-2 mM of TTAB concentration), the binding to the second sets of sites, likely hydrophobic, causes the protein unfolding. There may be a third set of sites, which is available once the protein is quite unfolded.

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