

Thermodynamic Elucidation of Binding Isotherms for Hemoglobin & Globin of Human and Bovine upon Interaction with Dodecyl Trimethyl Ammonium Bromide

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Binding of dodecyl trimethylammonium bromide (DTAB) to human and bovine hemoglobin and globin samples has been investigated in 50 mM glycine buffer pH = 10, I = 0.0318 and 300 K by equilibrium dialysis and temperature scanning spectrophotometry techniques and method for calculation of average hydrophobicity. The binding data has been analyzed, in terms of binding capacity concept (θ), Hill coefficient (n_H) and intrinsic Gibbs free energy of binding (ΔG_{br}). The results of binding data, melting point (T_m) and average hydrophobicity show that human hemoglobin has more structural stability than bovine hemoglobin sample. Moreover the results of binding data analysis represent the systems with two and one sets of binding sites for hemoglobin and globin, respectively. It seems that the destabilization of hemoglobin structure due to removal of heme group, is responsible of such behavior. The results indicating the removal of heme group from hemoglobin caused the depletion of first binding set as an electrostatic site upon interaction with DTAB and exposing the hydrophobic patches for protein.

Key words : Hemoglobin, Globin, Binding sites, Hydrophobicity, Electrostatic contribution

Introduction

Hemoglobin (Hb), the circulating red pigment of blood, is a heme protein and has a long evolutionary history as an oxygen transport protein.^{1,2} Detail studies on normal as well as mutant hemoglobins have established a structure/function relationship for human hemoglobin.³ Interestingly animal hemoglobins have amino acid differences at critical points, when compared to human Hb A. These discrepancy may suggest the differences in ability of hemoglobin samples for oxygen affinity.

The phenomenon of cooperativity in Hb arises from the coupling between ligand binding processes and the interaction between globin and heme group, subunit chains within the tetrameric Hb molecule. Comparison between thermodynamic properties of Hb and globin of different vertebrates are therefore of considerable interest in defining the energetic states and transitions which may account for cooperative events.

Comparison between thermodynamic parameters of ionic surfactants with Hb's and globins of different vertebrates can be helpful with respect to this purpose. It has been well established that most of the ionic surfactants can bind to native structure of globular proteins^{4,5} and cause denaturation for them as well as provide the information about the native state in terms of its cooperativity, intrinsic stability and the nature of the forces required to maintain its tertiary

structure.⁶⁻⁹ It has been shown that analyzing the binding data of two sets of binding systems can reveal some additional structural information.^{10,11} With respect to the mentioned facts, in the present study the binding of dodecyl trimethylammonium bromide (DTAB) as a cationic surfactant with human Hb and bovine Hb and their globins have been investigated. Calculation and comparison of thermodynamic parameters are accomplished in relation to structural stability and the role of heme group in structural integrity of Hb.

Experimental Section

Materials. Hemoglobins and globins from human and bovine, sodium azide, DTAB, glycine and orange II dye were purchased from Sigma Ltd. Visking membrane dialysis tubing (MW cut-off 10,000-14,000) was obtained from SIC (East Leigh) Hampshire, UK. 50 mM glycine buffer pH = 10.0, I = 0.0318, has been used. All other materials and reagents were of analytical grade. Double distilled water was used in the preparation of solutions.

Methods. Equilibrium dialysis was carried out at 300 K using Hb and globin solutions of concentration 0.02%(w/v), of which aliquots of 1 cm³ were placed in the dialysis bags and equilibrated with 2 cm³ of DTAB solution covering the required concentration range for 96 h, as explained previously.¹² All the measurements reported refer to DTAB concentrations below the critical micelle concentration (C.M.C), the free DTAB concentrations are in equilibrium with complexes and were assayed by the orange II dye

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method.¹³

Spectrophotometry. The Gilford as a temperature scanning spectrophotometer (model 2400-2) was used for obtaining the thermal profiles of hemoglobin samples using wavelength of 280 nm for obtaining melting point (T_m) as a criterion of protein stability. The scan was run at a rate of 1 K/min in a degassed buffer solution.

Theoretical calculation. The average hydrophobicity $H\phi$ as a criterion of the protein stability¹⁴ was calculated by means of summation of individual amino acid parameters from Kyte and Doolittle scale.¹⁵ Amino acid sequences were obtained from Swissprot database for human α (P01922) and β (P02023) chains and bovine α (P01966) and β (P02081) chains. The numbers in the parenthesis indicates the Swissprot codes for each of subunits.

Results and Discussion

Figures 1 and 2 are the binding isotherms (the average number of DTAB ions bound per molecule of protein (ν) as a function of logarithm of the free DTAB concentration $[\text{DTAB}]_f$ at specified conditions) of hemoglobin-DTAB and globin-DTAB interaction respectively.

For analyzing the binding data, the concept of Wyman binding potential, $\Pi(P, T, \mu_1, \mu_2, \dots)$ which is at pressure P and temperature T relates to ν_i and chemical potential, μ_i as¹⁶

$$\nu_i = \left(\frac{\partial \Pi}{\partial \mu_i} \right)_{T, P, \mu_{j \neq i}} \quad (1)$$

the homotropic second derivative of the binding potential with respect to chemical potential of ligand for ideal solution is as follow:

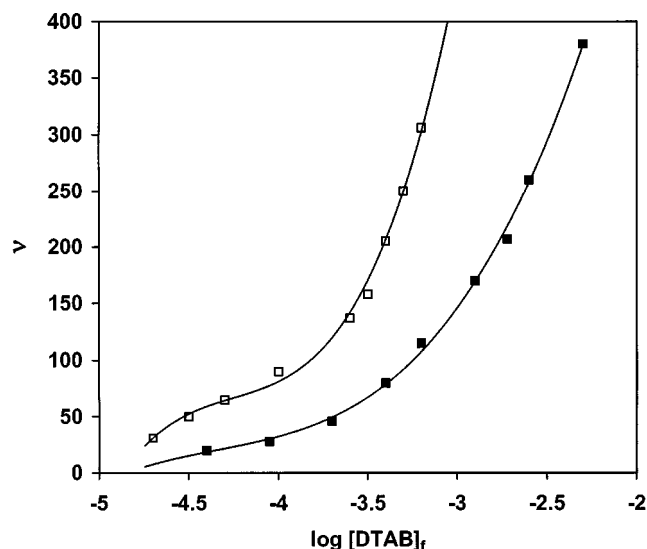


Figure 1. Binding isotherms (average number of bound DTAB to one macromolecule, ν against logarithm of free concentration of DTAB) of Bovine (□) and human (■) hemoglobins on interaction with DTAB in 50 mM glycine buffer, pH=10 and 27 °C. Each experiment in equilibrium dialysis method repeated three times and the data were consistently reliable.

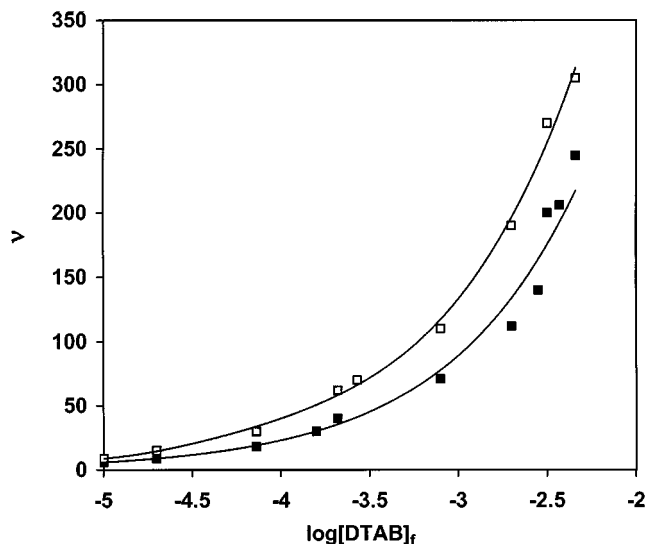


Figure 2. Binding isotherms of bovine (□) and human (■) globin on interaction with DTAB in 50 mM glycine buffer pH=10 and 27 °C. Each experiment in equilibrium dialysis method repeated three times and the data were consistently reliable.

$$\theta = \frac{\partial \nu_i}{\partial \mu_i} = \frac{\partial \nu_i}{RT \partial \ln[\text{DTAB}]_f} = \frac{\partial^2 \Pi}{\partial \mu_i^2} \quad (2)$$

it provides a measure of the steepness of the binding curve and may be designed as the binding capacity.¹⁷ It depicts the change in the number of mole of ligands per mole of macromolecule that accompanies a change in the chemical potential of that ligand and thus, from slightly different point of view is a measure of macromolecular capacity for grabbing ligand at any specified binding state, so it can be a measure of cooperativity as expected by the Hill coefficient, n_H , to binding capacity in order to extract a relationship between them. n_H is defined as the slope of the Hill plot.⁸

$$n_H = \frac{d \ln(\nu/(1-\nu))}{d \ln[\text{DTAB}]_f} = \left(\frac{1}{\nu(1-\nu)} \right) \left(\frac{d\nu}{d \ln[\text{DTAB}]_f} \right) \quad (3)$$

where ν is the fractional saturation of protein by ligand which is defined as follows:

$$\nu = \frac{v}{g} \quad (4)$$

where g is the number of binding sites. From the definition of binding capacity, equation (1), the following equations can also be written:

$$n_H = \left(\frac{1}{g\nu(1-\nu)} \right) RT \theta \quad (5)$$

$$\theta = \frac{n_H \nu(1-\nu)}{RT} \quad (6)$$

Equation (6) is rearranged to the following form:

$$\frac{RT \theta}{\nu} = n_H - n_H \left(\frac{\nu}{g} \right) \quad (7)$$

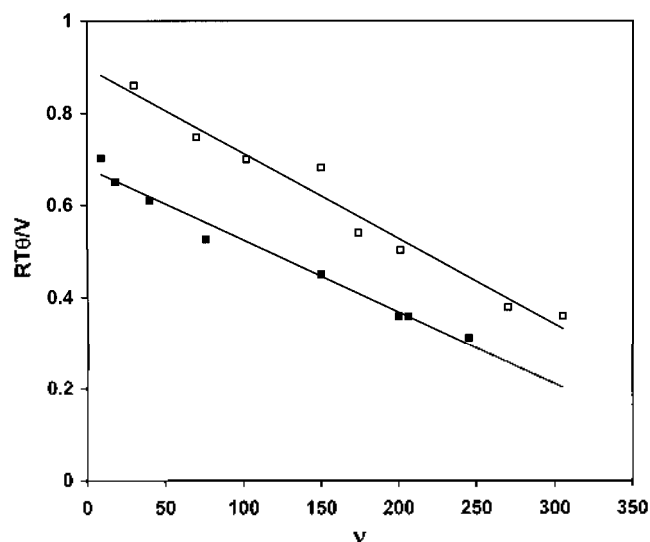


Figure 3. The plot of $RT\theta/v$ versus v for interaction of DTAB with bovine (□) and human (■) globin in mention experimental conditions. Where R , T , θ and v are gas universal constant, absolute temperature, binding capacity and average number of DTAB to one molecule of globin, respectively.

for a system with one set of binding sites and identical n_{H1} , it can be suggested that the plot of $(RT\theta/v)$ versus v should be linear, where the slope, y and x -intercepts are $-n_{H1}/g$, n_{H1} and g , respectively.

Figure 3 shows the variation of $(RT\theta/v)$ versus v for interaction of DTAB with bovine and human globin. It contains two linear plots which represent the existence of only one set of binding sites on globin samples upon interaction with DTAB. With respect to equation (7), the values of n_{H1} and g can be estimated from this plot. Using the Hill equation for one set of binding sites, equation (8) as following¹⁸:

$$v = \frac{g(K[DTAB]_f)^{n_H}}{1 + (K[DTAB]_f)^{n_H}} \quad (8)$$

the value of Hill binding constant K is also estimated. Figure 4 shows the variation of $(RT\theta/v)$ versus \bar{v} for interaction of DTAB with bovine and human hemoglobin samples with DTAB. In contrast to figure 3, these plots are not linear representing more than one set of binding sites for interaction of DTAB with the Hb samples. However, the initial and final points of these plots are fitted as a linear equations with high correlation coefficients. Each linear line can be correlated to one set of binding sites. With respect to the

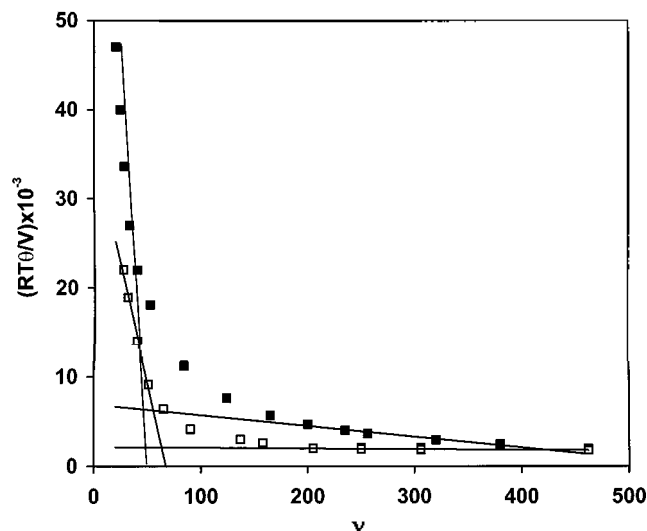


Figure 4. The plot of $RT\theta/v$ versus v for interaction of DTAB with bovine (□) and human (■) hemoglobins in mention experimental conditions. Where R , T , θ and v are gas universal constant, absolute temperature, binding capacity and average number of DTAB to one molecule of globin, respectively.

slope and intercepts of these lines, the values of n_{H1} and g for each set, have been estimated. The Hill equation for two sets of binding sites is as following⁵:

$$v = \frac{g_1(K_1[DTAB]_f)^{n_{H1}}}{1 + (K_1[DTAB]_f)^{n_{H1}}} + \frac{g_2(K_2[DTAB]_f)^{n_{H2}}}{1 + (K_2[DTAB]_f)^{n_{H2}}} \quad (9)$$

where g_1 , K_1 , and n_{H1} are the number of binding sites, binding constant and Hill coefficient for the first binding set, respectively and g_2 , K_2 , and n_{H2} are the corresponding parameters for the second binding set. The estimated binding parameters of Hill equation are listed in Table 1.

The intrinsic Gibbs free energy of binding per mole of surfactant for the first, $\Delta G_{b,v}^{(1)}$, and the second, $\Delta G_{b,v}^{(2)}$, binding sets can be obtained by the following equations¹⁰:

$$\Delta G_{b,v}^{(1)} = -RTn_{H1}\ln K_1 + RT(1-n_{H1})\ln[DTAB]_f \quad \text{if } 0 < v < g_1 \quad (10)$$

$$\Delta G_{b,v}^{(2)} = -RTn_{H2}\ln K_2 + RT(1-n_{H2})\ln[DTAB]_f \quad \text{if } g_1 < v < g_1 + g_2 \quad (11)$$

Figures 5 and 6 show the variation of $\Delta G_{b,v}$ versus $\log[DTAB]_f$ for interaction of DTAB with hemoglobins and globins, respectively. With respects to intrinsic nature of $\Delta G_{b,v}^{(2)}$ its variation during the progress of binding reveals the kind and extent of interaction between sites. It is well

Table 1. Binding parameters of Hill equation for interaction of DTAB with hemoglobin and globin samples in 50 mM glycine buffer pH=10 and 27 °C

Protein	g_1	$K_1 (M^{-1})$	n_{H1}	g_2	$K_2 (M^{-1})$	n_{H2}
Bovine Hb	66 ± 3	53454 ± 2745	3.011 ± 0.014	457 ± 27	36.1 ± 4.4	0.96 ± 0.02
Human Hb	50 ± 3	7943.3 ± 413	1.192 ± 0.067	432 ± 24	31.7 ± 7.3	0.70 ± 0.05
Bovine globin	498 ± 56	382.3 ± 31.3	0.72 ± 0.02	—	—	—
Human globin	430 ± 35	467.6 ± 43.1	0.90 ± 0.02	—	—	—

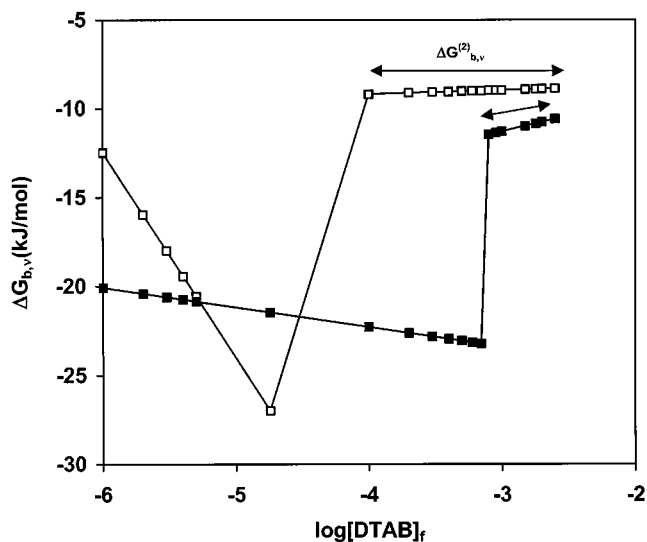


Figure 5. The variation of intrinsic Gibbs free energy of binding per mole of DTAB $\Delta G_{b,v}$ as a function of $\log [\text{DTAB}]_f$ for binding of DTAB to bovine (\square) and human (\blacksquare) hemoglobins. The initial plateau of curve belongs to first binding set $\Delta G_{b,v}^{(1)}$ and the later to the second one, $\Delta G_{b,v}^{(2)}$ (the free energy of second binding set, hydrophobic site).

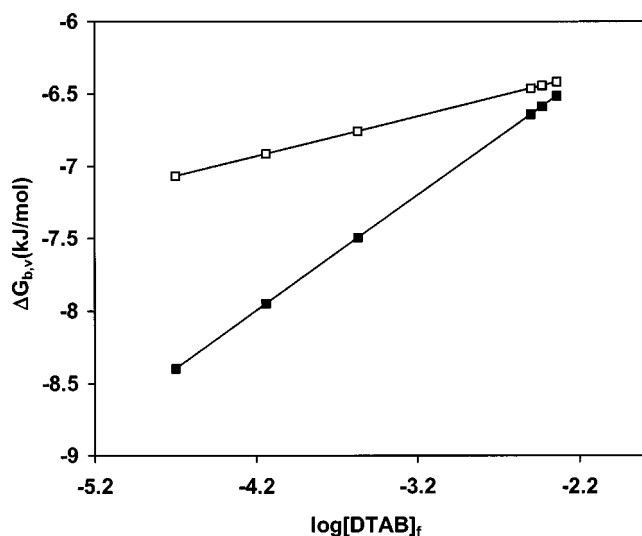


Figure 6. The variation of intrinsic Gibbs free energy of binding per mole of DTAB $\Delta G_{b,v}$ as a function of $\log [\text{DTAB}]_f$ for binding of DTAB to bovine (\square) and human (\blacksquare) globins.

known that both electrostatic and hydrophobic forces are involved in the interaction of ionic surfactants with globular proteins.^{5,11} It is also suggested that the mechanism of interaction is due to binding charge head groups of the surfactant to the sites with opposite charge at the protein surface, accompanying with simultaneous interaction of hydrophobic tail of the surfactant to hydrophobic patches. Such mechanism is confirmed by modification of ionic sites, due to acetylation of lysyl residues, which reduce the number of binding sites of first binding set, and weakening of the binding resulted from reducing the alkyl chain length.¹⁹ In figure 5 the initial binding sites in bovine hemo-

globin are more interactive (highly cooperative) and stronger than the human Hb. The positive cooperativity ($n_H > 1$) in the first binding set of bovine hemoglobin may be related to the high extent of hydrophobic interaction at the protein surface relative to human hemoglobin sample. On the basis of this interpretation, it may be concluded that the area of hydrophobic patches at the surface of bovine hemoglobin is greater than human sample. This is a good reason for higher stability for human Hb relative to bovine Hb.

For binding of ionic surfactants to globular proteins, the above statements of these initial interactions are followed by the unfolding and exposure of the hydrophobic interior and hence generation of numerous hydrophobic binding sites which can be related to the second binding set.^{20,21} Comparison of $\Delta G_{b,v}^{(2)}$ in figure 5 shows also a big jump after the occupation of the first binding set. This usually corresponds to the unfolding region.^{8,10,22} This jump starts for bovine hemoglobin at lower DTAB concentration relative to human hemoglobin sample.

Figure 6 shows the trend of variation of $\Delta G_{b,v}$ for globin samples look like with $\Delta G_{b,v}^{(2)}$ hemoglobin which is plotted in figure 5. This shows that the nature of binding forces in globin samples is more hydrophobic than electrostatic contribution. In literature also cited the removal of heme from hemoglobin induced the hydrophobic forces to come in access with water partially and suppress the electrostatic contribution.²³⁻²⁸ Therefore the plots in the figure 6 resemble to second binding set of hemoglobin-DTAB complexes in figure 5 that belongs to hydrophobic interaction.

Figure 7 shows the thermal profiles for human and bovine hemoglobin samples. The figure indicates that melting point (T_m , midpoint of thermal transition at absorbance of 280 nm) as a criterion of protein stability is higher for human hemoglobin relative to bovine hemoglobin sample, that is tabulated in Table 2.

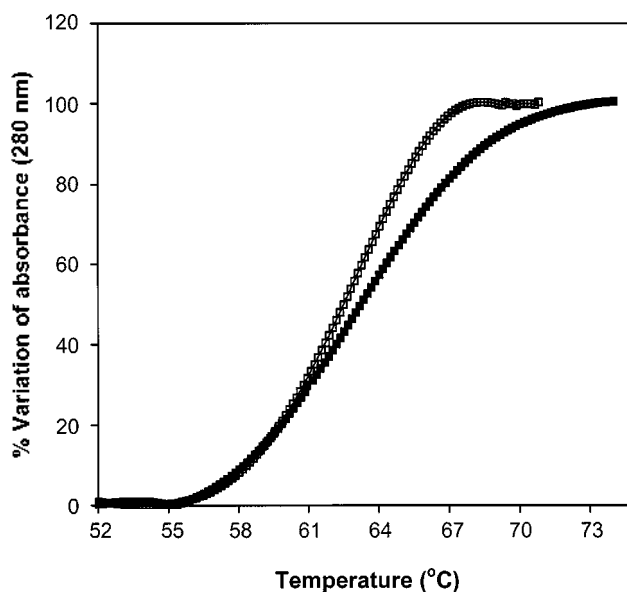


Figure 7. Percent of variation of absorbance (280 nm) versus temperature for human (\blacksquare) and bovine (\square) hemoglobins. The data for this experiment was consistently repeatable.

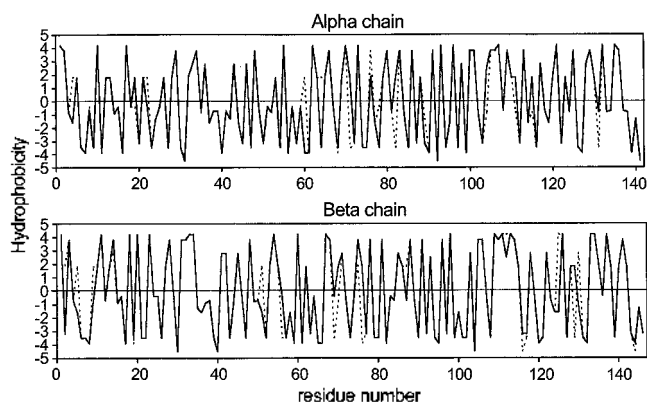


Figure 8. Hydrophobicity profiles for α , β chains of human (solid line) and bovine (dashed line) hemoglobins or globins.

Figure 8 shows the hydrophobicity profiles for α , β chains of human and bovine hemoglobin. The higher positive values of profiles show the more hydrophobicity. The average hydrophobicity ($H\phi$) as another protein stability criterion¹⁴ is calculated and the data were tabulated in Table 2.

The results indicate that the human hemoglobin has more thermodynamic stability relative to bovine hemoglobin. However, the forces responsible for the stability of the three-dimensional structure are stronger for human hemoglobin. The calculated average hydrophobicity ($H\phi$) and measured melting point (T_m) for bovine and human hemoglobin sample as the protein stability criteria have been supported the binding analysis interaction. The $H\phi$ shows also the higher value for human globin and hemoglobin than for bovine sample. This means the human globin stability is higher than bovine globin.

The removal of the heme from hemoglobin destabilized the protein structure.²⁽³⁾ This subject may cause the difference in the binding strength behavior of hemoglobin and globin samples upon interaction with DTAB. The hemes lie in nonpolar pockets of the globin chains and having about sixty interactions between atoms of the globins and hemes, all but one of those in the alpha chain and two of those in the beta chain are nonpolar.²⁸ Therefore it can be concluded that removal of heme groups induced the exposing of hydrophobic patches into water for globin. Although the addition of heme to globin is incorporated with hydrophobic contribution, these conclusions are made on the basis of analysis of binding data obtained from protein-DTAB interaction.

Conclusion

The binding set analysis having high potentiality for discrepancy for protein unfolding by surfactants. The inter-

Table 2. Stability parameters for human and bovine globins and hemoglobins

	Human	Bovine
$H\phi$	10	6.6
T_m (°C)	64 (\pm 0.1)	63 (\pm 0.1)

actions of dodecyl trimethylammonium bromide as a cationic surfactant with hemoglobins show two sets of binding sites (first set is mostly electrostatic and the second one is hydrophobic moiety), while the cited interaction for globin is including one set of binding site just as hydrophobic interaction (like the second set of binding site for hemoglobin). This means that removal of heme from hemoglobin result in the sharp reduction of the electrostatic contribution for apohemoglobin (globin).

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