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Thermodynamic Denaturation of Human Hemoglobin A in Aqueous Sodium *n*-Dodecyl Sulphate as a Function of pH

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In this paper, thermodynamic denaturation of human hemoglobin A derivatives (oxyhemoglobin and methemoglobin) in aqueous sodium n-dodecyl sulphate as a function of pH has been studied. The thermodynamic analysis reveals that maximum stability, $\Delta G_{D(H_2O)}$, and minimum stability, $\Delta G_{D(hc)}$, of hemoglobin A derivatives in aqueous sodium n-dodecyl sulphate is both pH and temperature dependent. Variation of activation parameters (ΔG_D, ΔG_D and ΔS_D) in the transition region depends on the presence of salt bridge, hydrogen bonding, van der Waal forces, changes in solvation and polarity at the reactive centers, as well as conformational mobility of hemoglobin A and sodium n-dodecyl sulphate within the active sites. The thermodynamic denaturation of hemoglobin A derivatives in aqueous anionic surfactant is independent of the spin state of the protein.

INTRODUCTION

Hemoglobin (MW 64,500) is a respiratory molecule. It contains four polypeptide chains and four heme prosthetic groups, in which the iron atoms are in the ferrous (Fe(II)) state. The protein portion called globin, consists of 2 $\propto$ chains (141 amino acid residues each) and 2β-chains (146 amino acid residues each). X-ray analysis reveals that hemoglobin molecule is roughly spherical, with a diameter of about 5.5 nm. Each of the four chains has the characteristic of a tertiary structure, in which the chain is folded [1].

It has been, recently, established that the following ionizable groups are electrostatically linked to the sulphydryl reactivity of the Cys (93) β-sulphydryl group of hemoglobin with 5, 5^1-dithiobis (2-nitrobenzoic acid) in the presence of inositol-P6 namely: (i) Val NA1 (1) β, (ii) His NA2 (2) β and (iii) His H21 (143) β [2].

From the previous work, pKa of 6.4 is assigned to Val. NA1 (1) β [2]. Inositol-P6 binds with positive amino acid residues on hemoglobin which can be likened to sodium n-dodecyl sulphate, a negative surfactant.

In the reaction of $2, 2^1$-dithiobispyridine with Cys F9 (93) β sulphydryl group of hemoglobin in the absence or presence of sodium n-dodecyl sulphate, SDS, simple pH-dependence profiles are obtained resembling the titration curves of either a monoprotic acid or a diprotic acid [3].

It has been shown that His HC3 (146) β also influences the sulphydryl reaction of hemoglobin, with pKa value of ca. 6.0 [3]. These studies have provided valuable opportunities for the study of the dependence of catalytic site chemistry on specific binding interactions.

Among the least well-understood aspects of hemoglobin chemistry are the roles of binding interactions and active center desolvation and their interdependence. To understand molecular recognition in hemoglobin-ligand binding system, it is necessary to consider not only binding perse, but also the interdependence of binding interactions and the covalency changes that play a central role in catalytic-site chemistry.

In this paper, an attempt is made to interpret the SDS denaturation of hemoglobin A derivatives.

MATERIALS AND METHODS

Blood from normal donors was obtained from the Blood Bank at the Central Hospital Cape Coast, Ghana. Hemoglobin was prepared according to normal

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laboratory procedures [4] . SDS was purchased from British Drug Houses and used as supplied. All the salts used in the preparation of the buffer were analytical grade and were made up in distilled water.

DETERMINATION OF SDS DENATURATION CURVE

Solutions of hemoglobin A and SDS were prepared in phosphate buffers with pH of 6.0, 7.0, 8.0 and total ionic strength of 0.05 M. SDS denaturation curves were determined by measuring the maximum optical density of the solutions containing 10 μM hemoglobin at 405 nm using a Jenway model UV/Vis 6100 spectrophotometer and 1-cm cuvette thermostated to maintain the temperature at 20°, 28° and 34° ± 0.1°C

All measurements were made after incubation of hemoglobin A and SDS for two hours when the spectral absorbance did not change.

RESULTS AND DISCUSSION

SDS Denaturation of Hemoglobin A

The SDS denaturation curves for human hemoglobin A derivatives (oxyhemoglobin A; O_2HbA; and methemoglobin A; MetHbA) are shown in Figure 1. In all cases, denaturation was followed by measuring the optical density at 405 nm.

The free energy of unfolding ΔG was calculated as a function of SDS concentration by assuming a two-state mechanism [5,6] and using the following equations [7]:

$$F_D = (Y_N - Y_{obs})/(Y_N - Y_D), \quad (1)$$

$$\Delta G_D = -RT\ InK = -RT\ In[F_D/(1 - F_D)]$$

$$= -RT\ In\ [(Y_N - Y_{obs})/(Y_{obs} - Y_D)], \quad (2)$$

where K is the equilibrium constant for the transition region and Y_{obs} is the observed value of the parameter used to follow unfolding in the transition region. Y_N and Y_D are the values of Y characteristic to the native and denatured conformations of the protein, respectively. The values of Y characteristic varies inversely with the SDS concentrations.

Figure 1 shows the SDS denaturation curve for O_2HbA and MetHbA in 0.05 M phosphate buffer with pH of 6.0 and at 20°, 28° and 34° ± 0.1°C. Equation 1 was used to estimate the fraction denatured in Figure 1.

The free energy, ΔG_D, is calculated from Equation 2 based on the data in Figure 1 which varies linearly with SDS concentration in the limited region as shown in Figure 2. The simplest method of estimating the conformational stability in the absence of SDS, $\Delta G_{D(H_2O)}$, is to assume that this linear dependence

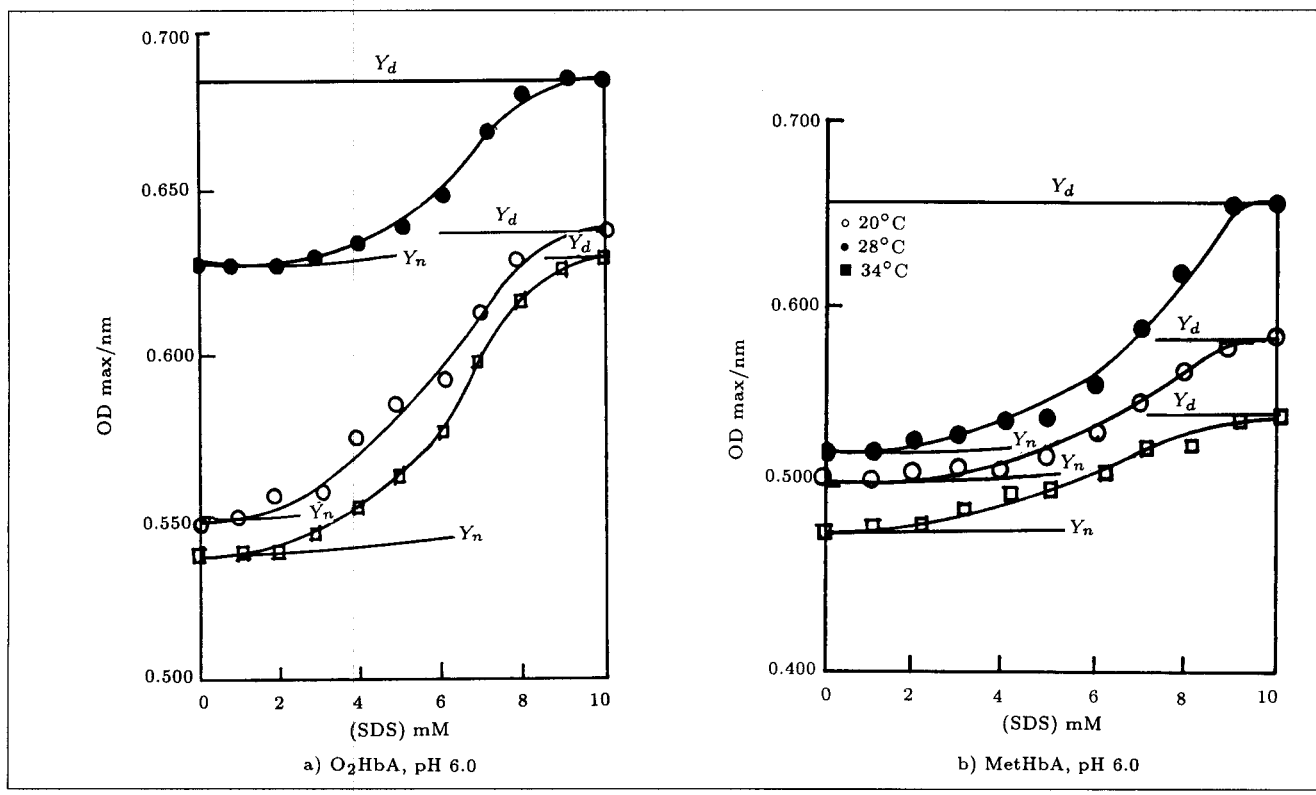


Figure 1. Thermodynamic denaturation of hemoglobin A with SDS: dependence of maximum optical density on SDS concentration.

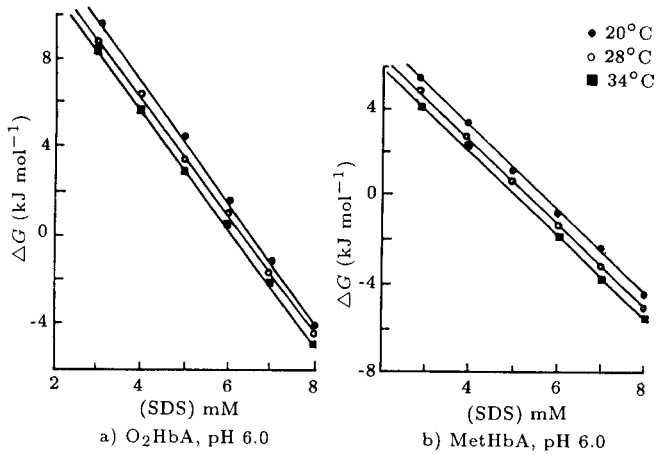


Figure 2. Dependence of ΔG_D on SDS concentration.

continues to zero concentration and to use a least squares analysis to fit the data into Equation 3 [8]:

$$\Delta G_D = \Delta G_{D(H_2O)} - m[\text{SDS}] = 0, \qquad (3)$$

where m is a measure of the dependence of ΔG_D on SDS concentration, obtained from the slope of Figure 2 depicting the closeness to linearity of Figure 2.

The values of m (measure of the dependence of ΔG_D on SDS concentration) are shown in Table 1. It is observed that at pH of 6.0 and 7.0 for O_2HbA, cooperativity decreases with increasing temperatures. It implies that the denaturation process of O_2HbA by SDS denaturant is temperature-dependent. The observed differences in the degree of cooperativity for O_2HbA can be explained in terms of ionizable groups involved in the catalytic binding. At pH of 7.0, the isoelectric point of hemoglobin makes the positively charged amino acid residues to interact with the SDS. High values of ΔG reported in Table 1 are attributed to the hydrophobic interactions that exist between the subunits of hemoglobin. AT pH of 6.0, His NA2(2) β, His H21 (143) β and His HC3 (146) β bind with SDS. SDS denatures hemoglobin and also binds with histidine moieties [3]. Decrease in the cooperativity with an increase in temperature might be due to the fact that the binding capacity of histidine moieties decreases with increasing the temperature as a result of denaturation. This explanation is also true for the case observed at pH of 7.0 where Val NA1 (1) β binds with SDS. In addition, the accessibility to the surface areas of the binding sites decreases with increasing the temperature because increasing temperature probably leads to the shrinking of the molecule, thereby reducing the pore size and causing a lot of diffusional limitation to occur. At pH of 8.0, cooperativity increases with increasing the temperature. This might be due to the conformational changes of the tertiary structure of the protein.

In the case of MetHbA, at pH of 6.0 and 8.0, cooperativity decreases with increasing the temperature. This is due to the fact that at pH of 6.0, histidine moieties bind with SDS.

In addition, the accessibility of SDS to the catalytic binding sites of hemoglobin A decreases with increasing the temperature. In order to account for these similarities at pH of 6.0 and 8.0 for MetHbA, it is necessary to consider the hemoglobin spin states. Methemoglobin assumes a two-transition state mechanism namely acid-alkaline methemoglobin transition. It is known that water molecules bound to the sixth coordination position of the heme iron atom; aquomethemoglobin ionizes with a pKa of 8.1 [9]. In acidic state, the presence of a water molecule at this position confers an extra positive charge on each iron atom of aquomethemoglobin which shows that the aquomethemoglobin is in low-spin state [9]. In case of the alkaline state, the hydroxyl group replaces the water molecule at the sixth coordination position of the heme iron atom of methemoglobin. It implies that the iron atom of hydroxy methemoglobin is in the high-spin state. The similarities in the degrees of cooperativity of MetHbA with SDS at pH of 6.0 and 8.0 cannot be accounted for in terms of spin states. The similarities might be due to the fact that MetHbA at pH of 6.0 and 8.0 assumes the same tertiary structure.

At pH of 7.0, cooperativity increases with an increase in temperature. It should be noted that inositol-P6 binds with cationic amino acid residues such as His NA2 (2) β, His H21(143) β, His HC3 (146) β and Val NA 1 (1) β [2]. It has been established that inositol-P6 shifts the equilibrium between hemoglobin

Table 1. Parameters characterizing the SDS-hemoglobin A unfolding at temperatures between 20°C and 34°C.

	T°C	pH 6.0				pH 7.0				pH 8.0			
		m/ (k.Jmol^{-1} M^{-1})	$\Delta G_{D(H_2O)}$ (kJ mol^{-1})	$\Delta G_{D(hc)}$ (kJ mol^{-1})	$\Delta\Delta G$ (kJ mol^{-1})	m/ (kJ mol^{-1} M^{-1})	$\Delta G_{D(H_2O)}$ (kJ mol^{-1})	$\Delta G_{D(hc)}$ (kJ mol^{-1})	$\Delta\Delta G$ (kJ mol^{-1})	m/ (kJ mol^{-1} M^{-1})	$\Delta G_{D(H_2O)}$ (kJ mol^{-1})	$\Delta G_{D(hc)}$ (kJ mol^{-1})	$\Delta\Delta G$ (kJ mol^{-1})
O_2HbA	20	2880	18.89	-77.04	-95.93	3140	17.00	-88.79	-105.79	2010	10.42	-55.54	-65.96
	28	2660	17.01	-69.41	-86.42	2900	15.13	-79.98	-95.11	2060	10.81	-55.56	-66.37
	34	2430	15.16	-64.49	-79.65	2670	13.27	-69.28	-82.55	2214	10.86	-60.90	-77.76
MetHbA	20	2810	19.56	-65.21	-84.77	870	5.38	-21.75	-27.13	2040	11.88	-56.17	-68.05
	28	2300	15.54	-59.21	-74.75	1200	6.61	-30.98	-37.59	1670	9.01	-45.81	-54.82
	34	1900	12.98	-51.48	-64.46	1330	6.95	-34.77	-41.72	1590	8.66	-40.51	-49.17

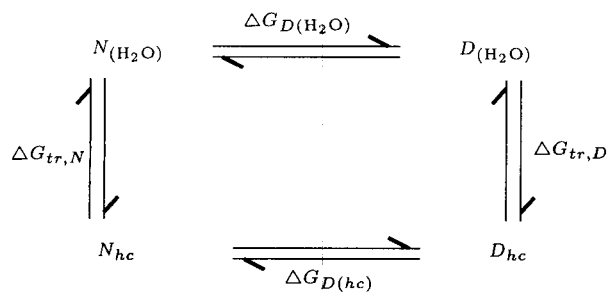
dimers and tetramers in favor of tetramers at low concentrations (10 μM inositol-P6) [10]. It is been assumed here that inositol-P6 characteristic could be likened to SDS. If the assumption is valid, it implies that SDS may shift the equilibrium hemoglobin dimers and tetramers in favor of dimers at high concentration of SDS (1 mM to 10 mM). At pH of 7.0, cooperativity increases with increasing the temperature which might be due to the dimer-form of MetHbA, in line with the previous findings presented in [2]. Dimer molecules of hemoglobin are more stable than that of tetramer molecules [3].

In molecular biological studies, changes in physiological functions are explained in molecular terms in ways that tacitly assume a direct relationship between structure and function. No experimental evidence has yet been found that is contradictory to this view. On the contrary, cummulative evidence from the study of hemoproteins [11], especially hemoglobin, shows that in its different physiological states the molecule does indeed assume different tertiary and, in appropriate cases, quarternary structures.

At pH of 6.0, the values of ΔG for the interaction between O_2HbA and SDS are higher than that for the MetHbA. The iron atom in O_2HbA is in the low spin state whereas the iron atom in MetHbA is in the high spin state. It implies that the differences might be due to the different spin contributions during denaturation. In addition, the tertiary structure of O_2HbA and MetHbA differs. The differences observed in the values of ΔG for the hemoglobin derivatives might be due to the structural conformation of the hemoprotein.

The results from analysis of the absorbance denaturation curves (Figure 1) is virtually consistent with the previous works [8,12], which may provide a good test for a two-state mechanism [II].

1M concentration of SDS is in good agreement for the transition of the native state of protein (from water) to the denatured state of protein (to hydrocarbon). The unfolding of a protein by a chemical denatured at constant temperature and pH can be classified into thermodynamically defined stages according to the scheme [12]:



The denaturation process can be viewed as a process in which the native protein starts in water (N_{H20}) and is treated conceptually in one of the following two ways. Either, it is first transferred to the denaturing solution without unfolding (N_{hc}), after which the denatured is transferred to the solvent (D_{hc}), or it is first denatured in water and then transferred to the solvent. Thus, for example, ΔG of the N_{H20}, to D_{hc} process, is given by:

$$\Delta G_{tr,N} + \Delta G_{D(hc)} = \Delta G_{D(H_2O)} + \Delta G_{tr,D},$$

or:

$$\Delta\Delta G = \Delta G_{D(hc)} - \Delta G_{D(H_2O)} = \Delta G_{tr,D} - \Delta G_{tr,N},$$

where $\Delta G_{tr,N}$ and $\Delta G_{tr,D}$ represent the free energies of transfer of the protein, respectively, from water to denaturant solution at a given concentration of SDS.

Positive values for $\Delta G_{D(H_2O)}$ and negative values for $\Delta G_{D(hc)}$ and $\Delta\Delta G$ are shown in Table 1. For O_2HbA at pH of 6.0 and 7.0, the maximum $\Delta G_{D(H_2O)}$ and the minimum $\Delta G_{D(H_2O)}$ occur at 20°C while at pH of 8.0, the maximum $\Delta G_{D(H_2O)}$ and minimum $\Delta G_{D(hc)}$ occur at 34°C. The denaturating temperature for MetHbA at pH of 7.0 is 34°C. Maximum stability means that the protein is surrounded by water while minimum stability means that the protein is completely surrounded by denaturant. This implies that the hydrocarbon is exposed to water. $\Delta\Delta G$ values for O_2HbA and MetHbA are negative at the pH range studied, impling that the denaturation of these hemoglobin derivatives in aqueous SDS solution is feasible.

Changes in free energy, heat and entropy in chemical reactions, at constant temperature and pressure, are related to each other quantitatively by the Gibbs free energy equation:

$$\Delta G_D = \Delta H_D - T\Delta S_D, \tag{4}$$

in which ΔG_D is the change in free energy of the reacting system during denaturation, ΔH_D is the change in its heat content or enthalpy during denaturation, T is the absolute temperature at which the denaturation process is taking place and ΔS_D is the change in entropy during denaturation. The values of ΔS_D were estimated from the plots of ΔG_D against Y using a least-squares analysis. Figure 3 illustrates the linearity of the plot of ΔG_D versus T.

The change in heat capacity at constant pressure, ΔG_{PD}, during denaturation can be estimated using Kirchoff equation:

$$\Delta C_{PD} = (\Delta H_D/dT)_p. \tag{5}$$

Figure 3 represents the plot of ΔG_D versus T_D using a least-squares analysis, from which ΔH_D and ΔS_D values are estimated (see Table 2).

Table 2. Activation parameters characterizing the SDS-hemoglobin A unfolding at temperatures between 20°C and 34°C.

	[SDS] mM	pH 6.0			pH 7.0			pH 8.0		
		ΔH_D (kJ mol^{-1})	ΔS_D (JK^{-1} mol^{-1})	ΔC_{PD} (kJK^{-1} mol^{-1})	ΔH_D (kJ mol^{-1})	ΔS_D (JK^{-1} mol^{-1})	ΔC_{PD} (kJK^{-1} mol^{-1})	ΔH_D (kJ mol^{-1})	ΔS_D (JK^{-1} mol^{-1})	ΔC_{PD} (kJK^{-1} mol^{-1})
O_2HbA	3	35.45	88.00	5.91	71.09	212.00	11.85	17.91	45.00	2.99
	4	74.81	228.00	12.47	42.89	168.00	7.15	22.79	69.00	3.88
	5	43.75	134.00	7.21	30.51	101.00	5.09	22.16	74.00	3.69
	6	24.52	77.00	4.09	14.07	54.00	2.35	35.69	124.00	5.95
	7	5.42	23.00	0.90	10.67	52.00	1.78	44.56	159.00	7.43
	8	-3.94	1.00	-0.66	8.01	45.00	1.34	37.42	146.00	6.24
MetHbA	3	112.77	345.00	18.80	5.62	12.00	0.94	103.68	330.00	17.28
	4	73.44	220.00	12.44	6.14	14.00	1.02	45.32	142.00	7.55
	5	30.11	80.00	5.02	6.07	14.00	1.01	33.65	123.00	5.61
	6	19.07	60.00	3.18	28.70	95.00	4.78	21.07	75.00	3.51
	7	19.81	70.00	3.30	36.02	126.00	6.00	19.87	71.00	3.31
	8	11.51	50.00	1.92	46.06	165.00	7.68	13.55	57.00	2.26

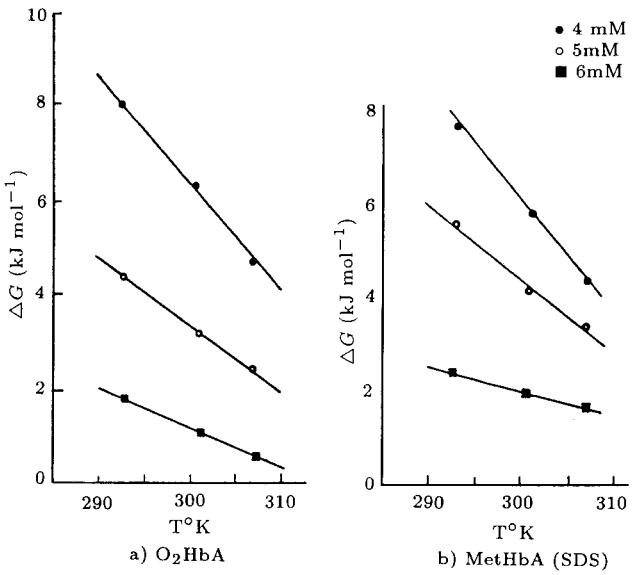


Figure 3. Dependence of ΔG_D on T at pH of 6.0.

ENTHALPIES AND HEAT CAPACITIES OF ACTIVATION

ΔH is defined as the amount of heat the reacting system releases or absorbs from its surrounding at constant temperature and pressure. Table 2 shows that the ΔH_D values are positive, demonstrating that heat is being absorbed from the surroundings. For O_2HbA at pH of 6.0 and 7.0 and MetHbA at pH of 6.0 and 8.0, ΔH_D decreases with increasing SDS concentration. This suggests that at these pH values and low SDS concentrations, the hydrophobic side chains are more exposed to the polar solvent medium. It also indicates that there is an increase in the rupturing of salt bridges, and peptide-peptide hydrogen bond breaking and decrease in van der Waals forces. It should be noted that SDS binds with hemoglobin A and, also, denatures the protein. For O_2HbA at pH of 8.0 and MetHbA at pH of 7.0, ΔH_D increases with increasing SDS concentration. This means that the hydrophobic side chains are less exposed to the solvent medium; it also shows decrease in the rupturing of salt bridges and peptide-peptide hydrogen bond breaking and increase in van de Waals forces [13-15].

Change in heat capacity at constant pressure, C_p, depends on:

1. The amount of the substance present,
2. The chemical nature and physical state of the substance.

During the denaturation process, ΔC_{PD} is a measurable quantity and is estimated from Equation 5. The variation of ΔC_{PD} with increasing SDS concentration is shown in Table 2. For O_2HbA at pH of 6.0 and 7.0

and MetHbA at pH of 6.0 and 8.0, ΔG_{PD} decreases with increasing SDS concentration, illustrating a decrease in binding constant of O_2HbA.

At pH of 6.0, the interaction of anionic detergent, SDS, with hemoglobin is shown by unfolding of hemoglobin (positive ΔC_P) which is equivalent to decrease in the stability of the hemoprotein. This indicates that the surface of hemoglobin has a high positive charge which can bind SDS and, subsequently, expose the non-polar side chains to the solvent. The large and positive value of ΔC_P is commonly attributed to the hydrophobic interaction, although other factors may contribute to ΔC_P as well [16,17].

When hemoglobin unfolds, the buried non-polar side chains are brought into contact with water. In order to accommodate these side chains, they are surrounded by cages of water molecules so that the extent of hydrogen bonding is increased.

Conversely, for O_2HbA at pH of 8.0 and MetHbA at pH of 7.0, ΔC_{PD} increases with increasing SDS concentration, showing that SDS binds more with O_2HbA and MetHbA in this pH range.

ENTROPIES OF ACTIVATION

The entropy of activation, ΔS, may be positive or negative and reflects the difference in the number and character of the translational, rotational and vibrational degrees of freedom between transition state and reactants. Factors determining the size and magnitude of ΔS include changes in polarity at the reactive center, changes in solvation and for hemoglobin reactions, in particular, changes in conformation of the hemoglobin and associated changes in the structure of ordered networks of bound water molecules [13-15].

For O_2HbA at pH of 6.0 and 7.0 and MetHbA at pH of 6.0 and 8.0 (see Table 2), ΔS_D values are positive and the magnitude of ΔS_D decreases with increasing SDS concentration, since hemoglobin A derivatives assume a more open conformation at low SDS concentration. Cationic amino acid residues possess more freedom of movement during complexation and charges are neutralized resulting into release of solvent molecules, accounting for the observed relationship between ΔS_D and SDS concentration. The decrease in the magnitude of ΔS_D might be due to differences in permitted conformational mobility of hemoglobin and SDS within the reactive sites.

For O_2HbA at pH of 8.0 and MetHbA at pH of 7.0, ΔS_D magnitude increases with increasing SDS concentration. This might be the result of permitted conformational mobility of hemoglobin and SDS within the active sites. Since SDS concentration increases with increasing ΔS_D, the rate of desolvation increases with increasing SDS concentration. It implies that accessibility of the surface area of hemoglobin A to SDS increases with increasing SDS concentration. These findings are in perfect agreement with previous findings on the dissociation of hemoglobin tetramers to dimers using a negatively charged sulphydryl reagent 5-5[1]-dithiobis (2 nitrobenzoic acid) [10,18].

The differences observed in the values of ΔH_D, ΔC_{PD} and ΔS_D, for O_2HbA and MetHbA, as a function of pH at a given SDS concentration (see Table 2) might be due to differences in permitted conformational mobility of hemoglobin and SDS within the active sites.

CONCLUSION

The denaturing temperatures for O_2HbA and MetHbA are 27°C and 34°C, respectively, at pH of 7.0. The differences in the activation parameters observed might be due to differences in permitted conformation mobility of hemoglobin and SDS within active sites. The polypeptide chains of hemoglobin are held together by salt bridges, hydrogen bonds and van der Waal bonds. SDS is a negative denaturant and binds with hemoglobin resulting in breaking the salt bridges, hydrogen and van der Waal bonds, thereby, exposing the hydrophobic moeities to the solvent.

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