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Kinetics of Denaturation of Human and Chicken Hemoglobins in the Presence of Co-solvents

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The stability of four hemoglobins (Hb) in dimer forms (low concentration) were investigated by the kinetics of denaturation. The rate constants of denaturation were obtained by variation of 280 nm absorption versus time in 10 mM Tris-HCl, 10 mM EDTA, pH 8.0 at 45°C in the absence and presence of 0.5 M ethanol, dimethyl sulfoxide (DMSO), formamide, and glycerol. The results show the trend of rate constants in different co-solvents in the following order: chicken hemolysate < human hemolysate and chicken Hb D < chicken Hb A. The buried surface area was calculated for Hb samples in the absence of co-solvents. Accordingly, the trend points out that: chicken Hb D > chicken Hb A > human Hb A. These results suggest that both chicken hemolysate and chicken Hb D are relatively more stable than human and chicken Hb A, respectively. However, the denaturation rate constants of Hb in different co-solvents have designated the following order: ethanol > DMSO > formamide > glycerol. As a matter of fact, this phenomenon is an indication of an increase in the denaturation capacity (DC) and hydrophobicity, and a decrease in the surface tension of the solution in the preceding co-solvents.

Keywords: Denaturation capacity, Hemoglobin, Hydrophobicity, Rate constant, Solvent effect, Stability, Surface tension

Introduction

The time-course of thermal denaturation of human oxyhemoglobins A, A_2, C, and S at 45°C was studied by observing the increase in fluorescence of protein (Kinderlerer, *et al*., 1973). As a matter of fact, hemoglobins S and C were deemed to be less stable than hemoglobin A; whereas hemoglobin A_2 was considerably more stable. The time-courses of denaturation did not follow the first-order kinetics, and it could be adapted to a cooperative scheme in which the partial denaturation of the α chain proceeds the β chain. The hemoglobins under investigation have exclusively a α subunit in common; they only differ in the β subunit, or δ subunit in the case of hemoglobin A_2. Hemoglobin A_2 is the minor fraction of the adult hemoglobin which seems to be somehow more heat stable than the major fraction of hemoglobin A. However, the difference between the amino acids of hemoglobins A and A_2 appears to be due to the presence of an extra hydrogen bond between residues $114\alpha_1$ and $116\delta_2$. This finding is the major decisive factor which causes hemoglobin A_2 to be more resistant to heat denaturation (Perutz and Raidt, 1975).

The structural and functional analyses of the hemoglobin D component from chicken have been investigated (Knapp *et al.*, 1999). Accordingly, the report indicates that the difference between chicken Hb A ($\alpha_2^A\beta_2$) and Hb D ($\alpha_2^D\beta_2$) is linked to the presence of an a chain. The re-analysis of the published oxygen binding data for chicken Hbs indicates that both chicken Hb A and Hb D hold enhanced cooperativity *in vitro* in the presence of inositol hexaphosphate.

The electrostatic surface potential of a calculated model of the chicken deoxy-Hb D tetramer shows a pronounced hydrophobic patch that involves parts of the D and E helices of the β subunits (Knapp *et al.*, 1999).

The correlation between the hydration of hemoglobin and the primary structure was studied by Bonger *et al.*, 1998. They found a link between the hemoglobin primary structure, hemoglobin hydrophobicity-hydrophilicity, and erythrocyte water contents in various mammalian species. Accordingly, a number of hemoglobin molecules (such as those of camels and camelides) were more hydrophilic, containing more charged-amino acid, than the hemoglobins of humans and a number of other mammalian species. Among the investigated

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species, the hemoglobin hydrophobicity is directly related to the response of erythrocyte water fraction osmotically. Therefore, it is proposed that alternations in the hemoglobin sequences of camels and camelides could be a part of the natural selection process that is aimed at protecting these animals from the osmotic dehydration in arid environments.

A few studies on the effect of solvents and denaturants on the structure and oxygen affinity of hemoglobin were previously reported (Herskovits *et al.*, 1977; Colombo *et al.*, 1992; Bordbar *et al.*, 1996, 2002; Liu *et al.*, 1998; Militello, Vitrano and Cupane, 1999; Ajloo *et al.*, 2002 a, b; Dayer *et al.*, 2002). There is, however, a lack of information on the consumed co-solvents in this paper.

In this study, the stabilities of the hemoglobins (i.e., chicken Hb A, chicken Hb D, hemolysed chicken, and human Hb in dimer forms) were investigated through the denaturation kinetics in various co-solvents. The calculation of the buried-surface area of the proteins was also studied.

Materials and methods

Materials DEAE-Sephadex A-50, anion exchange, and the solvents were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and Merck Chemical Company (Whitehouse station, USA). Fresh hemoglobin samples of human and chicken blood were obtained from the Blood Transfusion Organization and the School of Veterinary Medicine at the University of Tehran.

Preparation of Hb The heparinized-blood samples were washed three times with 0.9% NaCl. After each wash, it was centrifuged at 3,000 rpm. Afterward, the packed cells were lysed with 5 portions of double-distilled water, then it was centrifuged at 10,000 rpm. Subsequently, a 5% (w/v) NaCl solution was added to the sample and it was again centrifuged at 18,000 rpm. It should be noted that all of the steps were carried out at 4°C. Each centrifugation step was maintained within a period of 15 min, accordingly to the method of Williams and Tsay (1973). All of the samples were dialyzed in a buffer condition. The biggest portion of the hemolysed hemoglobin was in the form of Hb A.

The dialyzed-chicken samples were purified by chromatography of DEAE Sephadex-A-50 in 4.5×10 cm (Cobb *et al.*, 1992). The major component of Hb A and Hb D were eluted in the identical buffer solution (0.03 M NaCl solution). After the separation of Hb A and Hb D, each sample was concentrated in an ultrafiltration process using an Amicon filter. All of the samples were dialyzed against 10 mM Tris-HCl, pH = 8.0, containing 10 mM EDTA.

Spectrophotometry All of the spectrophotometric measurements were carried out with UV-Vis spectrophotometer, double-beam Shimadzu Model 3100. The variation of absorbance versus time was examined at 280 nm and 45°C in a buffer condition.

Surface calculation Accessible surface area (ASA) was computed based on the method of Shrake and Rupley (1973). This method was implemented in the Fantom Program at http://www.scsb.utmb.edu. The buried-surface area was calculated as: $ASA_{dimer} - \Sigma ASA_{monomer}$. The three-dimensional structure of the protein was obtained from the Protein Data Bank (PDB) at the site of www.rcsb.org.

Results and discussion

Evaluation of rate constant Figure 1 shows the time-courses for denaturation of a number of oxyhemoglobins at 45°C as a prototype (the figures in the presence of co-solvents are not shown, but the data are designated in Table 1). The rate constant of denaturation of Hb was calculated (by implementing the data from the Table 1) based on the following scheme [1]:

$$\alpha\beta \xrightarrow{k_1} \alpha\beta^* \xrightarrow{k_2} \alpha^*\beta^* \qquad \text{Scheme 1}$$

Here the Hb is in a dimeric state because of the low concentration of hemoglobin (Guidotti, 1967). In this case, αβ represents a dimeric from, the star (*) designates the denatured subunits, and k_1 and k_2 are the rate constants for the first and second steps of the denaturation. Consequently, this would lead to the following expressions for the concentrations of each species at any time, t, where D represents the initial hemoglobin concentration (Kinderlerer *et al.*, 1973):

$$[\alpha\beta] = De^{-k_1 t} \quad (1)$$

$$[\alpha\beta^*] = D[k_1/(k_1 - k_2)].(e^{-k_2 t} - e^{-k_1 t}) \quad (2)$$

$$[a^*b^*] = D[k_1/(k_1 - k_2)].(k_2 e^{-k_1 t} - k_1 e^{-k_2 t}) \quad (3)$$

At first, it was assumed that these reactions are essentially irreversible when the rate constants for each reversible reaction are considerably smaller than the forward reactions at the designated temperature. According to Benson (1960), the

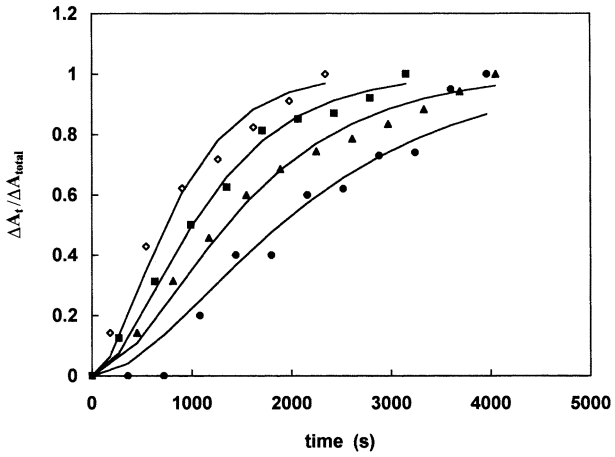

Fig. 1. Time course denaturation profiles of human hemolysate (◇), chicken Hb A (■), chicken Hb D (▲) and chicken hemolysate (●) in 10 mM Tris-HCl buffer, 10 mM EDTA, pH 8.0 in the absence of co-solvent. The concentration of Hb was 1 μM per heme. The denaturation profiles in the presence of ethanol, DMSO, formamide, and glycerol as co-solvents were examined (figures not shown).

Table 1. Rate constants and T_m for four hemoglobin samples in dimeric form in the Tris buffer and four co-solvents.

T_m (±0.1°C)*		Rate constant $k \times 10^3$ s^{-1}				
Hemoglobin	Buffer	Buffer	Ethanol	Glycerol	DMSO	Formamide
Chicken Hb A	65.2	1.67 (±0.05)	4.78 (±0.43)	2.41 (±0.06)	2.73 (±0.09)	1.98 (±0.07)
Chicken Hb D	67.0	1.25 (±0.13)	3.73 (±0.05)	2.18 (±0.06)	2.63 (±0.09)	2.21 (±0.05)
Chicken hemolysate	67.6	0.89 (±0.05)	3.53 (±0.05)	1.64 (±0.05)	2.31 (±0.04)	2.09 (±0.07)
Human hemolysate	64.0	2.28 (±.08)	4.47 (±0.42)	2.72 (±0.15)	4.45 (±0.23)	3.21 (±0.12)

*Midpoint of temperature transition of UV absorbance at 280 nm. The data were taken from Ajloo *et al.*, 2002b.

insertion of the reverse-rate constants complicates the integrated rate equations. Since the experimental data lead to an impossible reaction, its suitability may be questioned. However, the renaturation of Hb is considerably slower than the denaturation. A further complication is the time-dependent reaction, which eventually leads to precipitation.

The change in absorbance at any time t would be:

$$\Delta A_t = a[\alpha\beta] + b[\alpha\beta^*] + c[\alpha^*\beta^*] - aD \quad (4)$$

$$= D\left(\frac{ck_2 - bk_1}{(k_2 - k_1)} + a\right)e^{-k_1 t} - \frac{k_1}{k_1 - k_2}D(b - c)e^{-k_2 t} + (c - a)D \quad (5)$$

Where a, b, and c are the relative absorptions of αβ, αβ*, and α*β*, respectively, aD is the absorbance at zero time and cD is the absorbance as time tends to infinity.

If one assumes that the value b is equal or close to c, then the expression that involves a change in absorbance tends to become a hyperbolic rather than a sigmoid (see equation 5), this includes only one exponential term. Alternatively, if the value b approximates to a, then the equation (5) becomes:

$$\frac{\Delta A_t}{\Delta A_{total}} = 1 - \frac{k_1 e^{-k_2 t} - k_2 e^{-k_1 t}}{k_1 - k_2} \quad (6)$$

where $\Delta A_t / \Delta A_{total}$ is the fractional change in the absorbance for any time, t. An examination of the experimental curves suggests that the value b is closer to a than c. Also, as in the first approximation, it is assumed to be equal to a.

For the various values of k_1 and k_2 of each of the hemoglobins, it was found that the value of k_1 tended to k_2, although they were different for different hemoglobins. Assuming the same arguments as above, one may use different solutions of differential equations (equations 1, 2, and 3) to derive an expression for the fractional change corresponding to equation (6):

$$\frac{\Delta A_t}{\Delta A_{total}} = 1 - (1 + kt)e^{-kt} \quad (7)$$

and $k = k_1 = k_2$.

If the assumption that the chains are denatured consecutively rather than in parallel holds true, then the denaturation of the second chain is enhanced by the first. This would appear to be related to a rate constant which is apparently the same as the original reaction. The rate constants for hemoglobins in the absence and presence of co-solvents as well as T_m (midpoint of temperature transition at 280 nm) are tabulated in Table 1. The variation of $\frac{\Delta A_t}{\Delta A_{total}}$ versus time for 1 μM chicken Hb A, Hb D, hemolysate, and human hemolysate in the buffer condition and 0.5 M of various co-solvents (ethanol, methanol, glycerol, formamide, and dimethyl sulfoxide (DMSO)) were obtained. The results of $\frac{\Delta A_t}{\Delta A_{total}}$ fitted to equation (7) by means of the SigmaPlot 2000 program. The results show the trends for the rate constants (k) and T_m as follows: chicken hemolysate < human hemolysate and chicken Hb D < chicken Hb A, and chicken hemolysate > human hemolysate and chicken Hb D > chicken Hb A respectively.

The higher value of the T_m parameter indicates higher protein stability. The corresponding protein stability trend is accompanied by decrease in the rate constants of these Hb samples. The reason for these trends (T_m and k) for the Hb samples is determined by the calculation of the surface area as follows: The accessible and buried surface areas for the dimers were calculated by FANTOM software which was implemented in http://www.scsb.utmb.edu. This program calculated the accessible surface area from the X-ray crystallography data in the Protein Data Bank (PDB). In these studies, we used the bar-headed goose X-ray structure instead of chicken Hb A to compare with human dimeric hemoglobin because a three-dimensional structure data for chicken Hb A in PDB was not available. Sequence identity of chicken and bar-headed goose Hb A are about 80% (data not shown). Table 2 includes the accessible and buried surface of the hemoglobins. It indicates that the buried surface area of the chicken $\alpha^D\beta$ dimer as: dimer of Hb D (1HBR.PDB) > $\alpha^A\beta$ (dimer of Hb A, 1A4F.PDB) > human dimer hemoglobin (1HHO.PDB). It appears to be more stable than the others. However, it could be linked to the salt bridge or hydrogen bonding during the stabilization of this hemoglobin, as it was found in human Hb A_2 and Hb A (Perutz and Raidt, 1975,

Table 2. Calculated accessible and buried surface area

Oxy-Hemoglobin	PDB code	αASA (Å^2)	βASA (Å^2)	αβASA (Å^2)	Buried surface Area (Å^2)
Chicken Hb A*	1A4F	7635	7873	13660	1848
Chicken Hb D	1HBR	7293	7835	13269	1859
Human Hb A	1HHO	7626	7935	13764	1798

*Data calculated from three-dimensional structure data in PDB bank of bar-headed goose that had about a 81% sequence identity with chicken Hb A.

Knapp *et al.*, 1999; Kumar *et al.*, 2000).

Chicken Hb D differs from human oxyhemoglobin in the AB and GH corners of the α subunits and EF corner of the β subunits. The electrostatic surface potential for a calculated model of chicken deoxy-Hb D tetramer shows a pronounced hydrophobic patch that involves parts of the D and E helices of the β subunits (Knapp *et al.*, 1999)

The $\alpha_1\beta_1$ is similar to other vertebrate Hbs. However, out of the 36 residues that form the $\alpha_1\beta_1$ interface in chicken Hb D, 21 (58%) were identical to their counterparts in human Hb; whereas 29 residues (81%) were identical with bar-headed goose Hb A. Although most of these subunits do not alter the stability of the $\alpha_1\beta_1$ interface, there are three substitutions, such as $Gly^{\alpha114}$(GH2), $Gln^{\alpha103}$(G10), and $Ser^{\beta119}$(GH2) in chicken Hb D that need to be explored. The replacement of proline by a glycine at α114 in chicken Hb D results in the reorientation of the α114 main chain torsion angle, it thereby eliminates an intersubunit hydrogen bond. In place of this hydrogen-bond, the $\alpha_1\beta_1$ interface is stabilized by two additional hydrogen bonds. One is formed between the hydroxyl group of $Ser^{\beta119}$ (GH2) and the carbonyl oxygen of $Val^{\alpha111}$ (G18), and the other one is formed between the side chains of $Gln^{\alpha103}$ (G10) and $Asp^{\beta108}$ (G10). The addition of an extra hydrogen-bond stabilizes both the R- and T-states of chicken Hb D. $Gln^{\beta103}$ is conserved in all of the avian α^D sequences and about half of the avian α^A sequences; whereas $Asp^{\beta108}$ is conserved in all of the 20 β sequences that were surveyed. $Ser^{\beta119}$ is only present in four of the avian β sequences (Knapp *et al.*, 1999).

Table 1 shows the rate constant of the solvent effect on the protein with the following order: ethanol > DMSO > formamide > glycerol > buffer. The denaturation capacity (DC), the strength of biomolecules denaturation by the co-solvent (Khmelnitsky *et al.*, 1991), surface tension (σ), and hydrophobicity criterion (log *P*) of the co-solvent, (*P* is the partition coefficient of co-solvent between octanol and water) are tabulated in Table 3. Figure 2 shows the correlation between DC, σ, and log *P* with the rate constants, which are tabulated in Table 4. This indicates higher rate constant which corresponds to higher DC and log *P*, while σ remains inverted. Figure 2 and Table 4 show a good linear correlation between the rate of denaturation and log *P* as a criterion of hydrophobicity, i.e. a higher hydrophobic solvent coincides with a higher rate of denaturation. Abraham *et al.* (1994)

Table 3. Experimental parameters for co-solvents

	DMSO	EtOH	formamide	Glycerol
DC[1)]	30.3	54.4	0	20.2
Surface tension[2)]	43.54	24.05	59.13	62.5
log *P* [3)]	-2.03	-0.32	-1.51	-2.66

(1) Khmelnitsky *et al.*, 1991, (2) Dean, 1992, (3) Abraham *et al.*, 1994

calculated the log *P* from several descriptors, such as excess molar refraction (R_2), solute dipolarity/polarizability (π_2^H), effective solute hydrogen-bond acidity and basicity ($\sum\alpha_2^H$ and $\sum\beta_2^H$), and the characteristic volume of McGowam (V_x) (Abraham *et al.*, 1994). They used the statistical method and derived the following equation for 200 solutes as follows:

$$log\ P = -0.02 - 0.74\pi_2^H - 0.15\sum\alpha_2^H - 3.51\sum\beta_2^H + V_x \quad (8)$$

This equation shows that log *P* (hydrophobicity) decreases with the increase of the polarizability, refractivity, hydrogen-bond acidity, and basicity. It increases with the decreasing size of co-solvent molecules.

Hence, the hydrophobicity and denaturation capacity of the solvents are an important factor for enhancing the rate constant of Hb, whereas the surface tension decreases the rate constant. The reason for this diminishing phase is that a decrease in the surface tension of water and a transfer of amino acid from the protein interior to water facilitate protein unfolding (Rosell *et al.*, 1995; Baldwin, 1996). These results indicate that a lower surface tension accelerates the rate of denaturation in the same way as a surfactant decreases the surface tension and activates protein denaturation.

Conclusion

The results show that chicken Hb is more stable than human Hb because of higher T_m and buried surface of chicken Hb when compared with human Hb. The chicken Hb D is more stable than chicken Hb A because of lower rate constant of denaturation and higher T_m, hydrogen bond, and buried surface area. The solvent effect is interpreted according to the denaturation capacity (DC), surface tension (σ), and

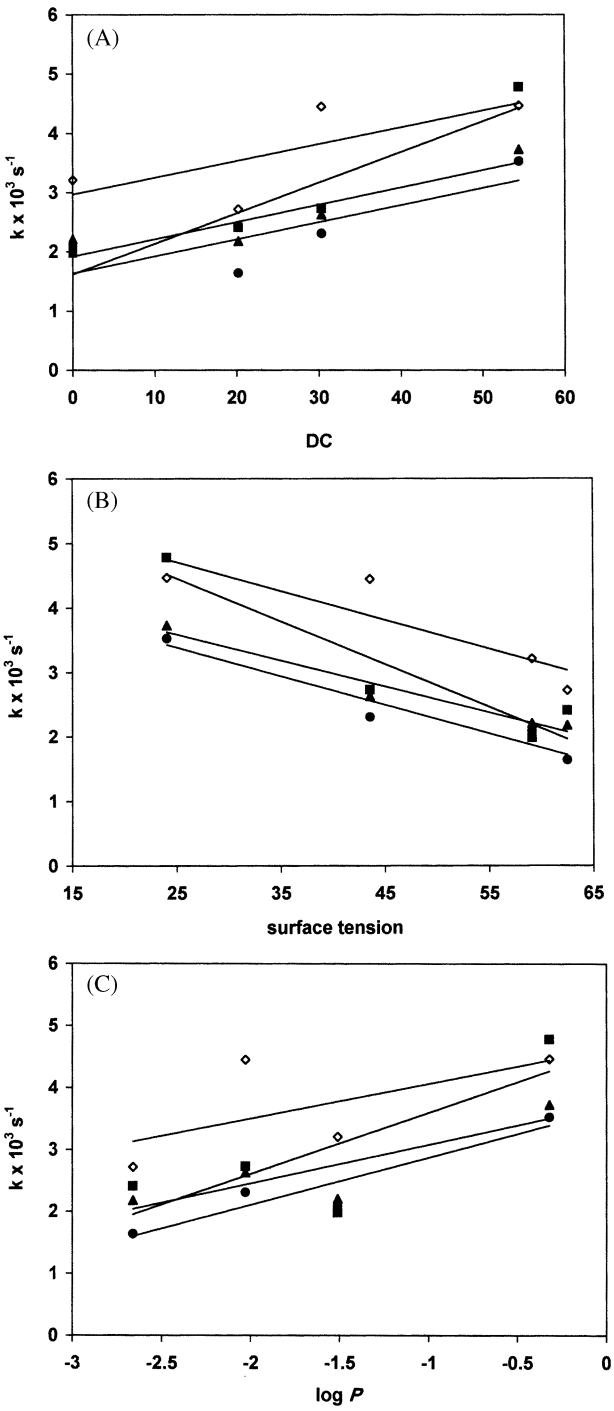

Fig. 2. Correlation between rate constant of denaturation with denaturation capacity (DC) (A); surface tension (B); log *P* (C) for human hemolysate (◇), chicken Hb A (■), chicken Hb D (▲), and chicken hemolysate (●).

hydrophobicity (log *P*). Higher hydrophobicity and DC as well as lower surface tension values of co-solvents are related to higher rate constants of denaturation. Each co-solvent with higher DC and log *P* and lower σ makes the protein more unstable. The foregoing parameters increase the similarity of the buffer medium with the protein interior. They subsequently facilitate the transformation of amino acids from the inside of a protein to the buffer solution, which results in the protein being unfolded.

Table 4. Linear correlation coefficient (R^2) between rate constants and experimental parameters for co-solvents

	DC	Surface tension	Log *P*
Hb A	0.94	0.94	0.98
Hb D	0.91	0.98	0.98
Chicken hemolysate	0.81	0.97	0.93
Human hemolysate	0.72	0.88	0.80

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