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Comparative structural and functional studies of avian and mammalian hemoglobins✪

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Thermal stabilities of chicken, grey lag goose (*Anser anser*), turkey as avian hemoglobins (Hbs); and human, bovine, sheep and horse as mammalian Hbs in hemolysate form were investigated and compared with oxygen affinities taken from literature. The thermal stability was obtained from thermal profiles using temperature scanning spectrophotometry. The buffer conditions were 50 mM Tris, pH 7.2, and 1 mM EDTA. The average of the inverse temperature transitions, average hydrophobicity, total van der Waals volume, partial molal volume and hydration potential were calculated by computational methods. The hemolysed avian Hbs have a lower oxygen affinity, higher thermal stability and higher self association than the mammalian Hbs. These observations are based on amino-acid composition, influence of ionic effectors, and the presence of Hb D in several avian Hbs. The results indicate that the avian Hbs have a more tense (T) conformation than the mammalian Hbs.

The respiratory system of birds differs from that of mammals; whereas their hemoglobins are functionally similar. Oxygen affinities of mammalian and avian hemoglobins have been studied by a number of investigators who have found the presence of inositol pentaphosphate (IP_5) in the birds blood instead of 2,3-diphosphoglycerate, DPG, found in mammalian blood (Lenfant *et al.*, 1969; Christensen & Dill, 1965; Bartels *et al.*, 1966; Tuker, 1972; Knapp *et al.*, 1999). The agent that is generally used to study the hemoglobin–phosphate interactions *in vitro* is inositol hexaphosphate (IHP) (Rollema &

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Abbreviations: DPG, 2,3-diphosphoglycerate; DTAB, dodecyltrimethylammonium bromide; IHP (IP_6), inositol hexaphosphate; IP_5, inositol pentaphosphate; p_{50}, pressure at half saturation of hemoglobin; T(g), temperature at midpoint of thermal changes of turbidity, measured as variation in absorbance at 700 nm; T_m, temperature at midpoint of thermal denaturation; T_t, inverse temperature transition.

Bauer, 1979; Brygier *et al.*, 1975; Coletta *et al.*, 1993; Messana *et al.*, 1998).

Cho & Choy (1980) studied the thermal stabilities of various methemoglobin and metmyoglobin derivatives in the presence of different ligands and at different pH values by differential scanning calorimetry (DSC). As a result, they concluded that the thermal denaturation of Hb derivatives could be described by an activated two-state process and determined corresponding parameters of activation. The stability of normal and cross-linked hemoglobins was studied on the assumption of a two-state model for denaturation of Hb derivatives (Yang & Olsen, 1988). The relationship between thermal stability and hydrophobicity has been studied very extensively (Jiang *et al.*, 2001; Irback & Sanderlin, 2000; Wagschal *et al.*, 1999; Kumar *et al.*, 2000). A number of researchers believe that this relationship should be the method of choice for studying the hemoglobins of birds and mammals (Irback & Sanderlin, 2000; Petruzzelli *et al.*, 1996; Bull & Breese, 1973). For example, Bull & Breese (1973) have suggested a relationship between stability and hydrophobicity of hemoglobins from animal sources. It has been suggested that the stability of proteins at high temperature (60–70°C) is due to enhanced hydrophobic bonding interactions (Bull & Breese, 1973). Also Bigelow & Channon (1976) believed that the hydrophobic character of proteins could be the determining factor of their stability and proposed a method for the calculation of average degree of hydrophobicity of thermophilic proteins. Once the method was applied, they found the hydrophobicity of the thermophilic proteins was by 10% higher than that of normal proteins. It should be noted that the correlations between quaternary structures and stabilities and functions of proteins were also studied by the accessible and the buried surface area calculations (Chothia *et al.*, 1976). Hydrophobic interactions affect also polymerization of biomolecules (Urry *et al.*, 1992; Adachi *et al.*, 1987; Adachi *et al.*, 1993; Yohe *et al.*, 2000). The role of these interactions in increasing the association or polymerization of Hb S (Harrington *et al.*, 1997) and lamprey Hb (Andersen & Gibson, 1971) has been already investigated. Increments in phosphate concentration and temperature were found to increase the rate of polymerization (Yohe *et al.*, 2000).

Total free energy of unfolding is obtained by calculating the sum of two energy contributions, i.e conformational and hydration energy. Hydration affects the empirical conformational energy values. The free energy of hydration is composed of additive contributions of various functional groups of proteins. The hydration of each group is assumed to be proportional to the accessible surface area of the group (Ooi *et al.*, 1987). The energy of unfolding is considered as a measure of protein stability (Jiang *et al.*, 2001; Ooi *et al.*, 1987; Koehl & Levitt, 1999).

Koehl & Levitt (1999) have developed a protein design procedure that optimizes whole sequences for a given target conformation based on the knowledge of template backbone and on semiempirical potential energy function. This energy function is purely physical including steric interaction based on Lennard–Jones potential, electrostatic interaction based on a Coulomb potential, and hydrophobicity in the form of an environment free energy based on accessible surface area and interatomic contacts. The constants of proportionality, representing the free energy of hydration per unit area of accessible surface, have been evaluated for seven classes of groups (Koehl & Levitt, 1999).

In this study we have compared the stabilities and functions of several mammalian and avian hemoglobin samples by using various experimental and computational methods.

MATERIALS AND METHODS

DEAE-Sephadex A-50, and Sephadex G-25 filtration gels were obtained from Pharmacia

Fine Chemicals. Dodecyltrimethylammonium bromide (DTAB), Tris, and inositol hexaphosphate (IHP) were obtained from Sigma.

Hemoglobin samples from fresh blood of humans, bovine, sheep, horse and greylag goose were obtained from the Blood Transfusion Organization, Slaughter House, and The School of Veterinary Medicine, University of Tehran, respectively.

Preparation of Hb

The heparinized blood samples were washed three times with 0.9% NaCl. After each wash, the sample was centrifuged at 3 000 r.p.m. Afterwards, the packed cells were lysed with 5 portions of double distilled water, and the sample was centrifuged at 10 000 r.p.m. Subsequently, 5% (w/v) NaCl was added to the sample and the mixture was centrifuged at 18 000 r.p.m. All the steps were carried out at 4°C, and each centrifugation step was carried out for a period of 15 min. The method of Williams & Tsay (1973) was applied. All samples were dialyzed against a buffer: 50 mM Tris, pH 7.2, and 1 mM EDTA.

The dialyzed samples from chicken were purified by chromatography in 4.5 × 10 cm DEAE Sephadex A-50 (Cobb *et al.*, 1992). The major component of Hb A was eluted immediately and then Hb D was eluted with the same buffer solution containing 0.03 M NaCl. After separation of Hb A and Hb D, each was concentrated by ultrafiltration through the Amicon filter. The chicken hemoglobin was separated from the organic phosphate by gel filtration on Sephadex G-25 in 0.1 M Tris/HCl, pH 8.5 and 0.5 M NaCl (Rollema & Bauer, 1979).

Spectrophotometry

The Gilford spectrophotometer (model 2400-2) was used for obtaining the thermal profiles of hemoglobin solutions, using wavelengths of 280 and 700 nm for the conformational and the turbidity studies, respectively. The scan was run at a rate of 1 K/min in a degassed buffer solution.

Theoretical calculations

The single point energy was determined by the AMBER force field (scale factor 4) implemented in Hyperchem 5.02 version. Data for the human (PDB file is 1hho.pdb) and the bar-headed goose (PDB file is 1a4f.pdb) Hbs were used (Zhang *et al.*, 1996). The conformational energies (E) in folded and unfolded or extended forms ((ϕ, ψ, ω) = (–155°, 160°, 180°)) were obtained for the dimers and monomers. The hydration free energy, ΔG_{h}, was calculated by using the group hydration (g_i) values (Table 3) taken directly from (Ooi *et al.*, 1987) for the corresponding Hb conformation. The difference between the dimers (in folded conformation) and the monomers (in unfolded conformation) as well as the differences between the hydration energy, $\Delta G_{\mathrm{h}}^{\mathrm{t}}$, and the conformational energy, ΔE^{t}, were calculated as follows:

$$\Delta G_{\mathrm{h}} = \sum_i g_i (\mathrm{ASA})_i \qquad 1a$$

$$\Delta G_{\mathrm{h}}^{\mathrm{t}} = \sum_{j=\alpha,\beta} \Delta G_{\mathrm{h}}(u)_j - \Delta G_{\mathrm{h}}(f)_{\alpha,\beta} \qquad 1b$$

$$\Delta E^{\mathrm{t}} = \sum_{j=\alpha,\beta} E(u)_j - E(f)_{\alpha,\beta} \qquad 1c$$

Where, $f, u, \alpha, \beta, \alpha\beta$ represent the folded and unfolded conformation, monomers and dimer subunits, respectively. The accessible surface area (ASA) was computed based on the methods of Shrake & Rupley (1973). This method was implemented in the Fantom (http://www.scsb.utmb.edu) and Naderi-manesh and coworkers (Naderi-manesh *et al.*, 2001) programs. The total energy of unfolding was obtained as follows:

$$\Delta G_u = \Delta G_{\mathrm{h}}^{\mathrm{t}} + \Delta E^{\mathrm{t}} \qquad (2)$$

The buried surface area, ΔASA, for the oligomers is defined as the sum of the accessible area of the monomers minus that of the dimer, and correlates with the stability. The gap volume is a void space in the interface between the subunits. It was calculated for the same PDB files of human oxy Hb and the bar-headed goose Hb using the web site (www.biochem.ucl.ac.uk).

All of the physical parameters for proteins such as average hydrophobicity, Hϕ, average inverse transition temperature, $<T_t>$, total van der Waals volume, V_t, total hydration potential, HP^t, and average partial molal volume, $<v>$, were calculated by using amino acid parameters, i.e. hydrophobicity index, HI (Argos *et al.*, 1982) inverse of transition temperature, T_t, (Urry *et al.*, 1992), ($T_t \equiv T_{(g)}$ is the temperature at which the turbidity measured at 700 nm reaches the half-maximal value), van der Waals volume, V_v (Chalikian *et al.*, 1996), hydration potential, HP (Argos *et al.*, 1982), and partial specific volume, v (Prakash, & Timasheff, 1985), respectively. The cited average parameters were calculated as the sum of the parameters for amino acids (Table 2) divided by the number of amino acids.

RESULTS

Figure 1 shows the denaturation profiles at 280 nm for the oxyhemoglobin samples. It points to the higher melting temperature, T_m, considered as a stability criterion, for the avian as related to the mammalian Hb samples. Figure 2 shows the direct relationship between T_m of hemoglobins in buffer condition and p_{50} of mammalian and avian blood, taken directly from literature and collected in Table 1. The data indicate that lower oxygen affinity is consistent with the higher stability of hemoglobins. The hydrophobicity, Hϕ, of 33 hemoglobins (Hb A), downloaded from (Swiss-Prot) data base, was calculated as the sum of the amino acids hydrophobicity index, HI, which is tabulated in Table 2. Figure 3 shows the average hydrophobicity, Hϕ, and the average inverse transition temperature, $<T_t>$, for the avian and the mammalian hemoglobins. This Figure also points to a higher hydrophobicity and a lower $<T_t>$ for the association criterion of the avian as compared with the mammalian Hbs. The variation of absorbance at 700 nm as a turbidity parameter *versus* temperature and (DTAB) (Fig. 4) points to a higher turbidity of the avian as

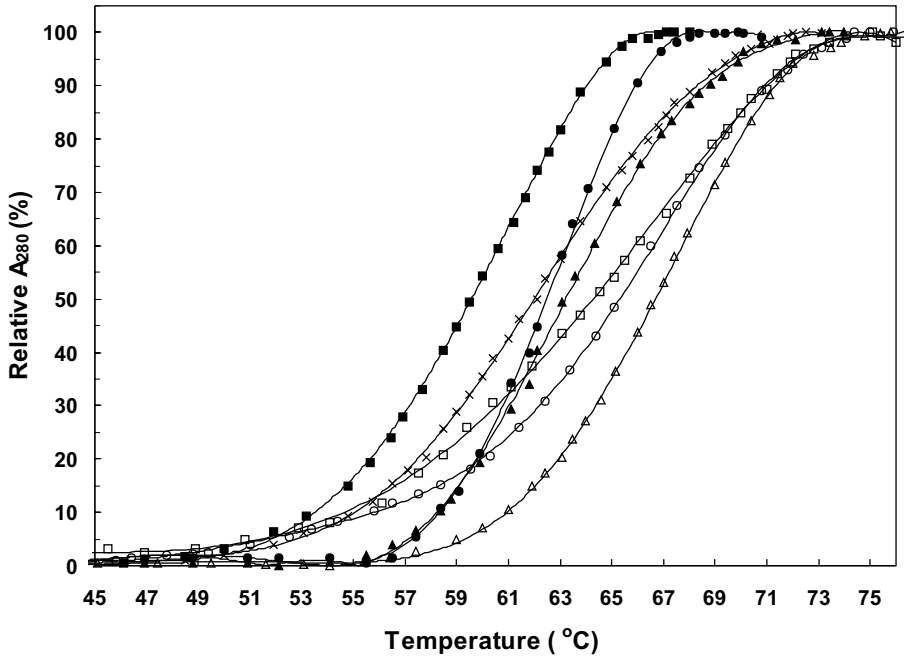

Figure 1. Thermal stability profiles of oxyhemoglobin in 50 mM Tris buffer, pH 7.2, and 1 mM EDTA at 280 nm, for chicken (□), turkey (○), goose (Δ), human (▲), bovine (●), horse (■), sheep (✖) hemoglobins.

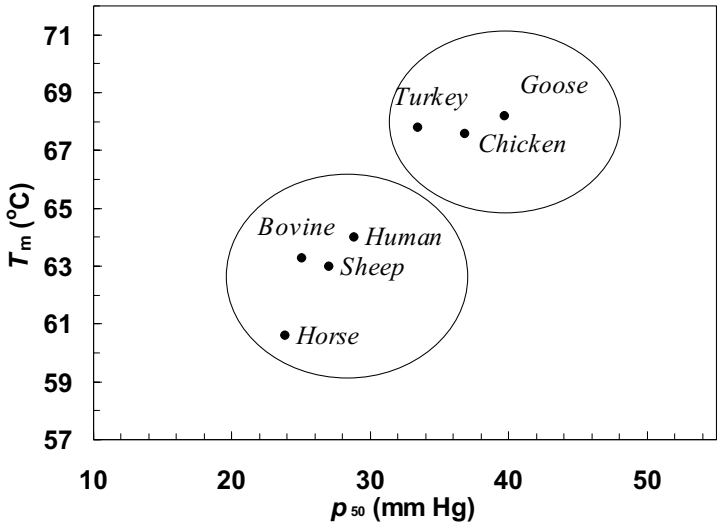

Figure 2. The relationship between midpoint of thermal transition, T_m (our experiment) at 280 nm and blood oxygen affinity (p_{50}) taken directly from literature (Lenfant *et al.*, 1969; Christensen & Dill, 1965; Bartels *et al.*, 1966; Tuker, 1972; Knapp *et al.*, 1999; Adachi *et al.*, 1987; Yohe *et al.*, 2000; Harrington *et al.*, 1997; Andersen & Gibson, 1971).

compared to the mammalian Hbs. The experimental and calculated parameters for avian and mammalian hemoglobins are listed in Table 1.

The conformational energy, ΔE^t, for the bar-headed goose and human Hbs were obtained by theoretical calculations. The free energy of unfolding, ΔG_u, is the sum of the hydration and conformational energies (Ooi *et al.*, 1987). The difference between the dimer and the monomer hydration energies were obtained from the group hydration data taken from Ooi *et al.* (1987) (see Table 3). The conformational, ΔE^t, unfolding, ΔG_u, electrostatic and the van der Waals energies of the samples are tabulated in Table 4.

The results show that the stability parameter, ΔG_u, has a higher value for birds (e.g. goose) than for mammals (e.g. humans). In general, a higher conformational stability for dimer goose Hbs, from our experiment, could be deemed as a characteristic feature for the avian compared with the mammalian Hbs.

The buried surface area, ΔASA, gap volume, V_G, melting temperature, T_m, for some representative oxyhemoglobins are listed in Table 5. These data will be discussed further in the text.

Table 1. Experimental and calculated parameters for avian and mammalian hemoglobins

	Goose	Chicken	Turkey	Human	Sheep	Bovine	Horse
p_{50}[a]	39.7	36.8	33.4	28.8	27	25	23.8
T_m[b]	68.2	67.6	67.8	64	63	63.3	60.6
T(g)[c]	62.2	58.8	59.4	66.4	66.4	65.4	64.4
$\langle T_t \rangle$[d]	47.8	47.5	47.0	47.9	49.7	50.2	49.8
Hϕ[e]	538	544	547	516	502	49077	505
$\langle v \rangle$[f]	0.745	0.746	0.746	0.743	0.738	0.738	0.741
V_t[g]	59736	59838	59836	58372	58664	58280	58644
HP[t,h]	341.1	341.5	341.4	339.5	334.0	340.0	338.0

[a] p_{50}, pressure at half saturation of hemoglobin directly taken from literature (Lenfant *et al.*, 1969; Christensen & Dill, 1965; Bartels *et al.*, 1966; Tuker, 1972; Knapp *et al.*, 1999; Gustin *et al.*, 1988; Clerboux *et al.*, 1993; Bard *et al.*, 1976); [b] T_m obtained from Fig. 1; [c] T_t(g), midpoint of absorbance at 700 nm *vs* temperature plot (turbidity) for oxyhemoglobin; [d] $\langle T_t \rangle$, calculated average inverse of transition temperature; [e] Hϕ, calculated average hydrophobicity; [f] $\langle v \rangle$, calculated average partial molal volume; [g] V_t, total van der Waals volume; [h] HP[t], calculated hydration potential.

Table 2. Parameters for amino acids used in this work

Amino acid	HI	T_t	V_v	HP	ν
Ala	0.61	45	67	2.00	0.74
Arg	0.60	60	148	0.00	0.70
Asn	0.06	50	96	0.20	0.63[a]
Asp	0.46	150	91	0.01	0.60
Cys	1.07	30	86	1.51	0.61
Gln	0.00	60	114	0.25	0.66[a]
Glu	0.47	250	109	0.13	0.66
Gly	0.07	55	48	2.07	0.64
His	0.61	30	118	0.12	0.67
Ile	2.22	10	124	0.23	0.90
Leu	1.53	5	124	2.06	0.90
Lys	1.15	35	135	0.23	0.82
Met	1.18	20	124	1.47	0.75
Phe	2.02	−30	135	1.58	0.77
Pro	1.95	−8	90	0.93	0.76
Ser	0.05	50	73	0.92	0.63
Thr	0.05	50	93	0.94	0.70
Trp	2.65	−90	163	0.79	0.74
Tyr	1.88	−55	141	0.75	0.71
Val	1.32	24	105	2.00	0.86

HI, Hydrophobicity index (Argos *et al.*, 1982); T_t, inverse of temperature transition (Urry *et al.*, 1992); V_v, van der Waals volume (Chalikian *et al.*, 1996); HP, hydration potential; υ (ml/g), partial molal volume (Prakash & Timasheff, 1985); [a]Values estimated from a direct correlation between van der Waals volume and partial molal volume.

Table 3. The computed *g* coefficient for free energy of hydration[a]

Class of chemical group	g (kcal/mol · Å^2)
Aliphatic carbon	0.008
Aromatic carbon	−0.008
Hydroxyl	−0.172
Amide and amine	−0.132
Carboxyl and carbonyl carbon	0.427
Carboxyl and carbonyl oxygen	−0.038
Sulfur and thiol	−0.021

[a]Data are taken from (Ooi *et al.*, 1987).

DISCUSSION

Our results indicate a reversed relationship between the oxygen affinity and the melting temperature, T_m, i.e., an increase in the stability is directly related to a decrease in the oxygen affinity (Fig. 2). In our experiments, we have used seven hemoglobin samples (avian and mammalian) in hemolysate form. Our data show that higher temperature Hbs stability in birds is related to lower oxygen affinity. The higher stability of avian as compared to mammalian Hb is believed to be due to the differences in their amino-acid composition (Kumar *et al.*, 2000), the presence of ionic effectors (Rollema & Bauer, 1979; Brygier *et al.*, 1975) and/or the influence of Hb D (Knapp *et al.*, 1999).

Avian Hbs contains more hydrophobic amino-acid residues than mammalian Hbs (Fig. 3). This observation is consistent with the presence of a higher content of hydrophobic residues in avian hemoglobin, its higher thermal stability and attainment of a tense (T) state. The conservation of hydrophobic domains in proteins such as the avian Hbs might have, in fact, required the stabilization of tertiary structure, in order to maintain the function of the protein through a long period of evolution (Perutz, 1983).

It has been reported that an extra positive charge in the cavity site of avian Hb (Moss & Hamilton, 1974) led to a stronger binding of ionic effectors in birds than it has been observed in mammals (Brygier *et al.*, 1975). It led also to a higher R→T transition for the avian Hb. In fact, the ionic effectors, which for mammals and birds are DPG and IP_5, respectively, exhibit a stronger binding in deoxy forms leading to decreased Hbs oxygen affinity. The number of negative charges in IP_5 seemed to be higher than in DPG. As a result, the binding constant for IP_5 with Hb is higher than that for DPG (Rollema & Bauer, 1979; Brygier *et al.*, 1975). It has been noted that the ionic effectors increase the Hill coefficient (Knapp *et al.*, 1999), tetramer association

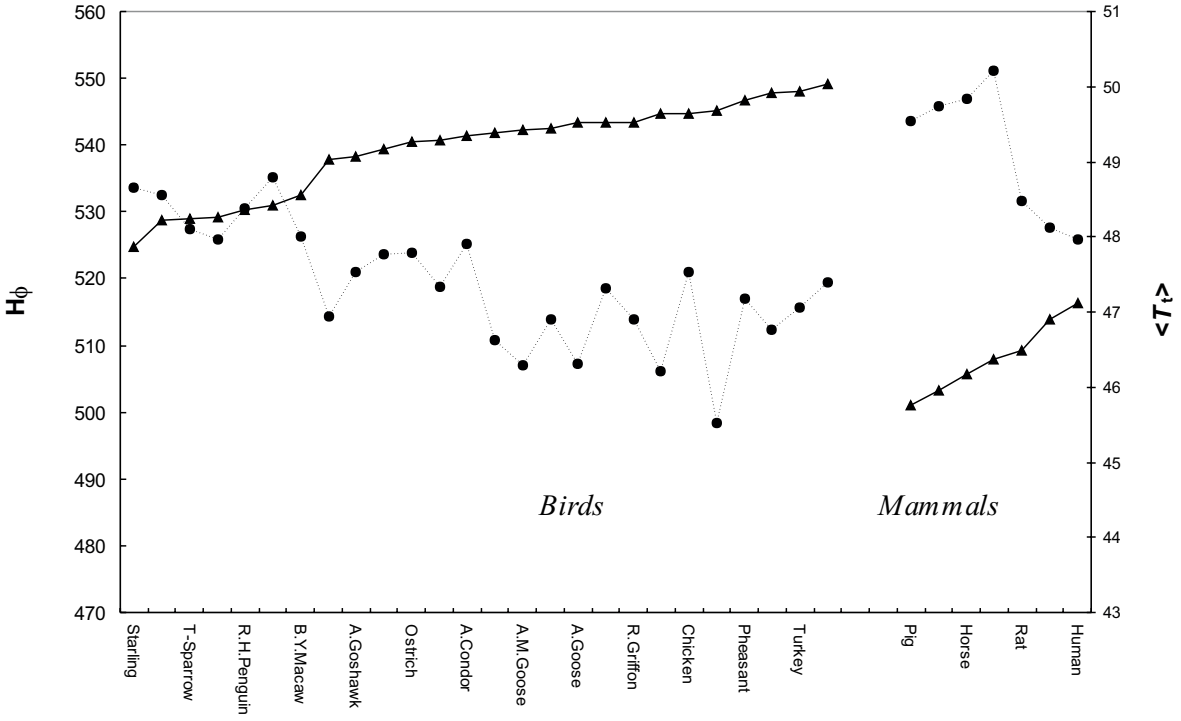

Figure 3. Hϕ (▲) and <T_t> (●) values for 33 hemoglobins (from birds and mammals) calculated from amino-acid parameters, as described under Methods.

(Yohe *et al.*, 2000) and stability of Hb (cf. data for IHP in Table 5).

Knapp and coworkers (Knapp *et al.*, 1999) have reported that the presence of Hb D component in Hb from several birds induces a lower oxygen affinity and that formation of a large tetramer-tetramer interface in chicken Hb D mediates a shift in the helix of $\alpha\beta$ subunits in such a way that the distal histidine is pushed further into the heme pocket, as it was also observed in lamprey deoxy Hb. Since the resulting position of the distal histidine hinders the oxygen binding, the oxygen affinity is reduced. Avian Hbs have a lower oxygen affinity than the mammalian Hbs. One reason for the reduction of oxygen affinity in the birds is the presence of Hb D. Our results also indicate that Hb D has a higher stability than Hb A (see

Table 4. Calculated energy of unfolding (kcal/mol) for human and bar-headed goose hemoglobins

Chains	$E(f)$	$\Delta G_h(f)$	$E(u)$	$\Delta G_h(u)$	ΔE^t	ΔG_h^t	ΔG_u	E_v	E_e
$\alpha\beta$(G)	–2208	–274	–309	–741	1899	–467	1399	–1435	–1455
α(G)	–990	–128	–123	–347	868	–219			
β(G)	–1102	–156	–186	–394	916	–238			
$\alpha\beta$(H)	–2042	–211	–235	–669	1807	–458	1349	–1390	–1406
α(H)	–732	–106	–98	–318	635	–212			
β(H)	–816	–118	–137	–350	679	–232			

$\alpha\beta, \alpha, \beta$, refer to dimer and monomers, respectively. Human (H), bar-headed goose (G) oxyhemoglobin E_v, E_e, f and u, are van der Waals energy, electrostatic energy, folded and unfolded states, respectively. The energy of unfolding of the bar-headed goose hemoglobin was calculated because of the existence of the three dimensional data in Protein Data Bank and the high homology (%) relative to graylag goose hemoglobin.

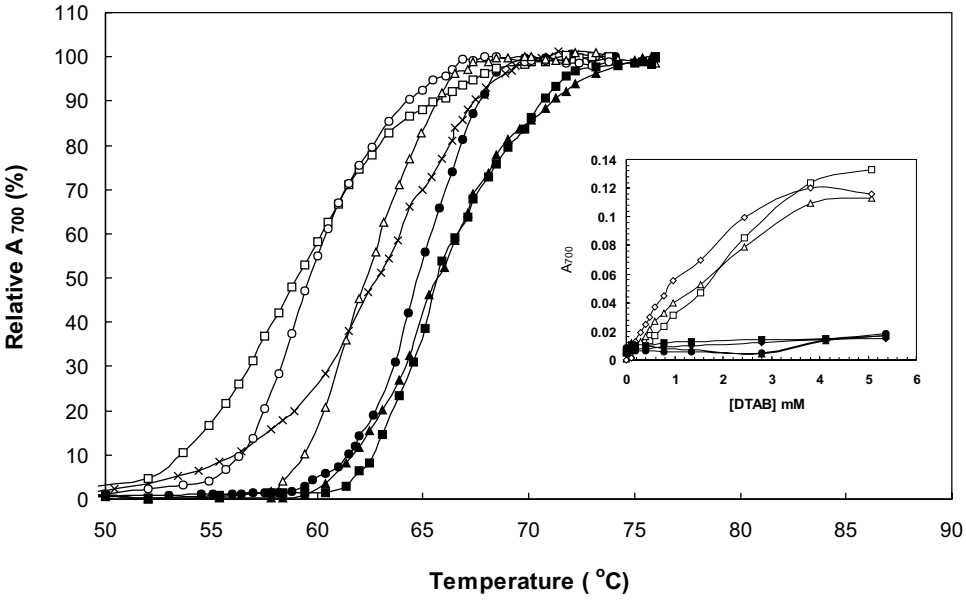

Figure 4. Variation of absorbance at 700 nm *versus* temperature for oxyhemoglobin as a measure of turbidity, and *versus* DTAB concentrations (inset).

The protein concentration for thermal profiles and DTAB curves was 6 mg/ml and 2 mg/ml, respectively. Chicken (❑), turkey (○), goose (Δ), human (▲), bovine (●), horse (■), sheep (✖) hemoglobins.

Table 5). We may conclude that a lower oxygen affinity of hemoglobins corresponds to their higher stability (both in birds and mammals).

Chothia and coworkers (Chothia *et al.*, 1976), reported that the T state conformation was more stable (5 kcal/mol) than the R state long before our findings regarding the presence of forms of the T state in the avian Hbs (Chothia *et al.*, 1976). This leads us to believe that the structural conformation of avian Hbs has a higher content of T state (deoxy form) in relation to R state (oxy form).

Based on the foregoing facts, we can conclude that the higher hydrophobicity of avian Hbs is a factor that contributes to higher stability of the T structure, nearly 20 kcal/mol (Chothia *et al.*, 1976). Accordingly, the avian Hb may have a higher T/R state ratio than the mammalian Hb.

Table 5. Comparison of surface and stability of some hemoglobins and the effect of inositol hexaphosphate (IHP)

	Chicken		Human		Goose
	Hb A	Hb D	without IHP	10 mM IHP	
T_m	65.2	67	65	68	–
T(g)	60.0	58.3	–	–	–
ΔASA[a]	–	–	885	–	946
V_G[b]	–	–	4619	–	4134

[a]ΔASA (in Å^2) was calculated for $\alpha\beta$ dimer of human and bar-headed goose oxyhemoglobins by the method of Shrake & Rupley (1973) implemented in Fantom program using (http://www.scsb.utmb.edu) web site; [b]gap volume, V_G (in Å^3) calculated for the dimer of human and bar-headed goose by web site (http://www.biochem.ucl.ac.uk). Temperatures are in °C.

The gap volume between $\alpha\beta$ subunits was found to be higher in human Hb than in the bar-headed goose Hb. Table 1 also shows a higher V_t for the avian Hb. Partial specific volume was also determined from the amino-acids constituents according to Zamyatnin

(1972). Since the partial specific volume is associated with average hydrophobicity, Hϕ, and the compressibility factor is dependent on the partial molal volume v, accordingly the empirical formula can be obtained (Gekko & Hasegawa, 1986) as follows:

$$v = 1.58 \times 10^{-4}\ H\phi + 0.578 \qquad (3)$$

Thus we can conclude not only that a higher Hϕ value corresponds to a higher partial specific volume, but we can also conclude that the partial molal volume of deoxy (T-state) is higher than that of the R state (Bureau & Banerjee, 1976). Thus, the higher stability of protein is probably related to a higher partial specific volume. It has been found that the partial specific volume has both a direct and a reverse relationship to the areas of the nonpolar and polar surfaces, respectively (Chalikian *et al.*, 1996). Also our study indicates that the total hydration potential (HP^t) has a direct relationship with the polar surfaces, and it has been determined to be higher for the avian Hb. This fact leads to the conclusion that the avian Hbs have a lower tendency to be hydrated (Kharakoze & Sarvazyan, 1993). The buried surface area of the oligomers is associated with their stabilities. Accordingly, the calculated ΔASA values of alpha-beta dimers for the bar-headed goose Hb are higher than for the human Hb (Table 5). The buried surface areas are apparently higher in T state than they are in R state (Chothia *et al.*, 1976). Janin & Chothia (Janin *et al.*, 1988; Janin & Chothia, 1990) reported twenty three oligomeric proteins with high stabilities which were assumed to be associated with the higher buried surface areas. It has been suggested that the stability of proteins at high temperatures (60–70°C) is due probably to increasing hydrophobic bondings in the protein (Goldsack, 1970). It is important to note that, in addition to hydrophobic interactions, the electrostatic and van der Waals forces also play substantial roles in the stabilization of protein structures (Chothia *et al.*, 1976). Table 5 shows the calculated values for each energy contributing source for two Hb samples (bar-headed goose, human).

Another reason for a higher T/R state form for the avian Hb in relation to mammalian Hb is a higher turbidity factor and self association of proteins which is believed to be due to a higher hydrophobicity. Figure 4 shows the variation of the absorbance at 700 nm with temperature and the effect of DTAB as a surfactant factor. We have assumed that these changes are associated with the degree of turbidity. In this case, the protein association seems to be higher for the avian Hb. It is believed that the surfactant either increases the surface hydrophobicity or, may be, neutralizes the surface charges of the hemoglobin of birds, so that this surface hydrophobicity is intrinsically higher in the avian Hb than it is in mammalian Hb. The literature data indicate that the increment of the surface hydrophobicity raises the association and polymerization of proteins such as Hb S (Harrington *et al.*, 1997), and Hb of lamprey (Andersen & Gibson, 1971). These Hbs become more associated in the deoxy form (T state) because the surface hydrophobicity in this state is probably higher than that in the oxy forms. Accordingly, Hb D induces a turbidity and polymerization in the avian Hb. The results of differential scanning calorimetry (not shown) at three scan rates (2, 1, 0.5 K/min) based on the method of Cho & Choy (1980) gave identical results for three Hb samples. This finding leads us to believe that the kinetics of association of the avian Hb (including Hb D) is biphasic. This phenomenon has been also observed for Hb S (Reddy *et al.*, 1996; Mu *et al.*, 1998). We have also used T_t to estimate the magnitude of self-association of Hbs. T_t depends on the hydrophobicity of the constituent amino-acid residues (Urry *et al.*, 1992) and seems to be lower for the hydrophobic amino acids. Once the value of T_t is above that for the physiological temperature, the polypeptide or protein will be unfolded (disassembled); and once T_t is below

that for the physiological temperature, the polypeptide or protein will be folded (assembled). The calculated average T_t, $<T_t>$, given in Fig. 3 indicates that avian Hbs proteins characterized by higher hydrophobicity have lower $<T_t>$ values. Accordingly, the higher hydrophobicities of avian Hb have a lower $<T_t>$.

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