Hemoglobin of the Antarctic Fishes *Trematomus bernacchii* and *Trematomus newnesi*: Structural Basis for the Increased Stability of the Liganded Tetramer Relative to Human Hemoglobin†

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Received October 2, 2000; Revised Manuscript Received December 12, 2000

ABSTRACT: Hemoglobins extracted from fishes that live in temperate waters show little or no dissociation even in the liganded form, unlike human hemoglobin (HbA). To establish whether cold adaptation influences the tendency to dissociate, the dimer−tetramer association constants ($L_{2,4}$) of the carbonmonoxy derivatives of representative hemoglobins from two Antarctic fishes, *Trematomus newnesi* (Hb1Tn) and *Trematomus bernacchii* (Hb1Tb), were determined by analytical ultracentrifugation as a function of pH in the range 6.0−8.6 and compared to HbA. HbA is more dissociated than fish hemoglobins at all pH values and in particular at pH 6.0. In contrast, both fish hemoglobins are mostly tetrameric over the whole pH range studied. The extent of hydrophobic surface area buried at the $\alpha_1\beta_2$ interface upon association of dimers into tetramers and the number of hydrogen bonds formed are currently thought to play a major role in the stabilization of the hemoglobin tetramer. These contributions were derived from the X-ray structures of the three hemoglobins under study and found to be in good agreement with the experimentally determined $L_{2,4}$ values. pH affects oxygen binding of *T. bernacchii* and *T. newnesi* hemoglobins in a different fashion. The lack of a pH effect on the dissociation of the liganded proteins supports the proposal that the structural basis of such effects resides in the T (unliganded) structure rather than in the R (liganded) one.

It is well-known that in hemoglobins binding of ligands to the heme determines large structural modifications, which lead to a transition from the T (unliganded) to the R (liganded) state (*1*). The most relevant changes occur at the $\alpha_1\beta_2$ (and at the symmetrical $\alpha_2\beta_1$) interface, which comprises residues from the CD and FG corners and from the C and G helices. Dissociation of tetramers into $\alpha\beta$ dimers likewise occurs at the $\alpha_1\beta_2$ and $\alpha_2\beta_1$ interfaces, such that the state of heme ligation and the state of association are linked. Thus, vertebrate deoxyhemoglobin is essentially tetrameric, whereas oxy- and carbonmonoxyhemoglobins undergo a finite dimer−tetramer association−dissociation equilibrium.

The association−dissociation equilibrium is also affected by external factors, as ionic strength, or by allosteric effectors such as chloride ions and organic phosphates. Also variations of pH can affect the equilibrium (*2*). The binding of protons to residues at the $\alpha_1\beta_2$ and $\alpha_2\beta_1$ interfaces modifies the number and strength of hydrogen bonds that contribute to the stabilization of the tetramer. Differences in the number, polarity, or hydrophobicity and spatial orientation of the residues at the $\alpha_1\beta_2$ interface therefore can lead to a qualitatively and quantitatively different pH-dependence of dissociation also in hemoglobins with similar sequence and X-ray structure, which otherwise would be expected to have a similar behavior.

Hemoglobins extracted from fishes that live in temperate waters (dogfish, trout), unlike human hemoglobin (HbA)[1] and other vertebrate hemoglobins, show little or no dissociation, even in the liganded form (*3*). Nothing is known about the association−dissociation equilibria of hemoglobins from Antarctic fishes, whose adaptation to low temperatures (a seawater−ice mixture has a temperature of −1.87 °C, which leads to a higher percentage of physically dissolved oxygen and an increased blood viscosity) has consequences on oxygen affinity and hence could affect also dissociation into subunits.

Another characteristic of fishes from temperate latitudes is the presence in their blood of a variety of hemoglobins with different ligand binding properties to meet the physiological requirements imposed by the variability of the environment. In contrast, the blood of Antarctic fishes, probably because of the relatively stable environmental conditions, in general contains a single major component, Hb1, which accounts for at least 95% of total hemoglobin, a second, minor component, Hb2 (5%), and traces of another component, HbC (less than 1%) (*4*). Usually both major and minor components display the so-called Bohr and Root effects, which correspond to a decrease in oxygen affinity with decreasing pH in the alkaline and in the acidic range,

† This study is in the framework of the Italian National Program for Antarctic Research.

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[1] Abbreviations: HbA, human adult hemoglobin; Hb1Tb, *Trematomus bernacchii* major hemoglobin; Hb1Tn, *Trematomus newnesi* major hemoglobin.

10.1021/bi002297j CCC: $20.00 © 2001 American Chemical Society
Published on Web 02/10/2001

respectively. In some instances, however, oxygen binding is not pH-dependent.

To ascertain whether the Bohr or Root effect is correlated with differences in the pH dependence of the dimer−tetramer association−dissociation equilibrium, the carbonmonoxy derivatives of the two representative major hemoglobins of *Trematomus newnesi* (Hb1Tn) and *Trematomus bernacchii* (Hb1Tb), named *Pagothenia bernacchii* in some previous reports, were investigated. The species belong to the family *Nototheniidae*. The association−dissociation equilibria were studied by analytical ultracentrifugation in the pH range 6.0−8.6, in comparison with the behavior of human carbonmonoxyhemoglobin (HbACO).

HbA displays the Bohr effect, which is reversed at acidic pH, such that the oxygen binding curve has a minimum around pH 6.0 (*1*, *5*). Hb1Tb displays Bohr and Root effects (*6*), while Hb1Tn shows only a modest Bohr effect and no Root effect (*4*). It is worth noticing that *T. newnesi* swims actively and feeds near the surface, whereas *T. bernacchii* is a sedentary bottom dweller. Moreover, the blood of *T. newnesi* contains, in addition to Hb1 and Hb2, a large amount (20−25%) of HbC, which displays Bohr and Root effects (*4*).

The structural analysis of the liganded hemoglobins under study, based on the crystallographic coordinates available in the Brookhaven Protein Data Bank (PDB), reveals a strong similarity in the amino acids located at the $\alpha_1\beta_2$ interface. In Hb1TbCO and Hb1TnCO this interface differs only in five positions. When the two fish hemoglobins are compared to HbA, differences in 12 positions are observed. The small number of substitutions in principle allows one to interpret the respective dissociation behavior in terms of specific structural differences.

The stability of the tetramer species is known to depend on both polar interactions (e.g., hydrogen bonds) and hydrophobic interactions. In particular, a recent study conducted on native human hemoglobin and on site-specific single and double mutants at the $\alpha_1\beta_2$ interface (*7*) showed that there is a direct correlation between the association constant and the hydrophobic surface buried at this interface upon association of dimers into tetramers, after correcting for changes in the number of hydrogen bonds formed between residues at the interfaces, which contribute to the stability of the tetramer. In the hemoglobins studied in the present work, the hydrophobic and the hydrophilic contributions to the tetramer stability are both in good agreement with the experimentally determined dimer−tetramer association constants. Thus, in the liganded derivatives of fish hemoglobins, which display a stronger tendency to associate with respect to HbA, association of dimers into tetramers leads to the burial of a larger hydrophobic surface and to the formation of more hydrogen bonds than in the human protein.

MATERIALS AND METHODS

Protein Purification and Sample Preparation. HbA, Hb1Tb, and Hb1Tn were obtained as previously described (*4*, *6*, *8*) and stored at −80 °C. Aliquots were unfrozen and dialyzed for at least 12 h at 4 °C in the appropriate buffer to equilibrate them at the desired pH, after addition of sodium dithionite, to reduce traces of ferric hemoglobin eventually present, and carbon monoxide. The dialyzed hemoglobin solutions were diluted to the desired concentrations and used within 24 h or stored at −10 °C.

Analytical Ultracentrifugation. All experiments were conducted at 4 °C on the carbonmonoxy derivatives in a Beckman XL-A analytical ultracentrifuge equipped with absorbance optics and an An60-Ti rotor. The protein concentration varied in the range 0.016−1.4 mg/mL. It was calculated using the extinction coefficients 11.75 and 0.825 mL mg^{-1} cm^{-1} at 419 and 540 nm, respectively. The buffers used were 100 mM bistris HCl at pH 6.0 or 7.0 and 100 mM Tris HCl at pH 8.6. Carbon monoxide was added to the buffer to keep the hemoglobins in the carbonmonoxy form. As a control for the possible occurrence of autoxidation, absorbance spectra were measured in the ultracentrifuge cells on all samples before and after every run. The data were discarded when ferric hemoglobin exceeded 5%.

Sedimentation velocity experiments were conducted at 40 000 rpm. Data were collected at an appropriate wavelength (419 or 540 nm, depending upon the initial protein concentration) at a spacing of 0.005 cm with three averages in a continuous scan mode every 5 min and analyzed with the program DCDT, provided by Dr. Walter Stafford (Boston Biomedical Research Institute). Data analysis with this software gives a sedimentation coefficient distribution (g(s)), from which the weight average sedimentation coefficient, $\bar{s}$, can be calculated (*9*). Sedimentation equilibrium experiments were performed at 17 000 or 20 000 rpm. Data were collected at a spacing of 0.001 cm with 10 averages in a step scan mode every 3 h. Equilibrium was checked by comparing scans up to 24 h. Data sets were edited with REEDIT (J. Lary, National Analytical Ultracentrifugation Center, Storrs, CT) and fitted with NONLIN (PC version provided by E. Braswell, National Analytical Ultracentrifugation Center, Storrs, CT) (*10*). Data from different speeds were combined for global fitting. Fits to a single species give a Z-average molecular weight. For fits to a dimer−tetramer association scheme, the dimer molecular weight was fixed at the value determined from the amino acid sequence. The calculations to obtain the values of $L_{2,4}$ from the average sedimentation coefficients were carried out with a program compiled on purpose in MATLAB (The Math Works Inc.) using known equations (*11*).

For noninteracting proteins, $\bar{s}$ is inversely correlated to protein concentration, due to the influence of the solute on the density and viscosity of the solution, according to the equation

$$\bar{s} = s_0(1 - g\bar{w}) \quad (1)$$

where s_0 is the sedimentation coefficient at zero protein concentration, $\bar{w}$ is the protein concentration, and g is a factor that depends on the hydrodynamic properties of the protein. For uncharged spherical globular proteins g has a value of 0.007 L g^{-1}.

For reversibly associating proteins, the total flux of sedimenting material, $\overline{sw}$, is given by the sum of the fluxes of the different components, i.e.:

$$\overline{sw} = \sum_i s_i w_i \quad (2)$$

As the concentration of the i^{th} product of association can be

related to the monomer concentration through the association constant $L_{1,i}$ (where L is expressed in mL mg^{-1}) with the expression

$$w_i = L_{1,i} w_1^{\,i} \tag{3}$$

Equation 2 becomes

$$\overline{sw} = \sum_i s_i L_{1,i} w_1^{\,i} \tag{4}$$

that can be modified by introducing the concentration dependence described by eq 1 into:

$$\overline{sw} = (1 - g\bar{w}) \sum_i (s_i)_0 L_{1,i} w_1^{\,i} \tag{5}$$

assuming that g has the same value for all the products of oligomerization.

For a monomer−dimer system, in particular

$$\bar{s} = \frac{1 - g\bar{w}}{w} (w_1 (s_1)_0 + w_2 (s_2)_0) \tag{6}$$

where

$$w_1 = \frac{-1 + \sqrt{1 + 4L_{1,2}\bar{w}}}{2L_{1,2}} \tag{7a}$$

and

$$w_2 = L_{1,2} w_1^{\,2} \tag{7b}$$

These equations, introduced into an appropriately written program, allow the calculation of $L_{1,2}$ from the dependence of $\bar{s}$ on the total protein concentration $\bar{w}$. The sedimentation coefficients of monomer and dimer (hemoglobin dimer and tetramer, respectively), $(s_0)_1$ and $(s_0)_2$, were kept constant and determined the extreme values for the fit.

Structural Analysis. The protein X-ray structures used, identified by their Brookhaven Protein Data Bank code, are 1HHO, human oxy HbA; 1T1N, *T. newnesi* Hb1CO; and 1PBX, *T. bernacchii* (formerly *Pagothenia*) Hb1CO.

The solvent accessible surface area (SAS), which corresponds to the area traced out by the center of a solvent sphere rolled over the surface of the molecule according to Lee and Richards (*12*), was calculated on a Silicon Graphics O2 workstation using the program Homology (ProStat/Access Surf) within the InsightII package (BIOSYM/MSI, San Diego, CA). Values of tetramer and dimers were obtained by summation of the single amino acid SAS.

The molecular surface (MS) area, defined as the contact surface between the solvent probe and the molecule and computed using the Connolly surface algorithm, was calculated for dimers and tetramer using the Viewer Module (Molecule/Surface) of InsightII.

The buried hydrophobic surface area at the interface was estimated by subtracting the value computed for the tetramer from the sum of the values computed for dimers.

Comparison and drawing of the $\alpha_1\beta_2$ interfaces was performed by superimposition of the structures of fish hemoglobins to that of human HbA at the conserved region formed by the B, G, and H helices (BGH core). Computation of electrostatic interactions at the interfaces was performed using InsightII. Identification of all the residues involved at the interfaces (residues of one chain that are within 5 Å from those of the other chain) was made with the program Swiss-PdbViewer (*13*).

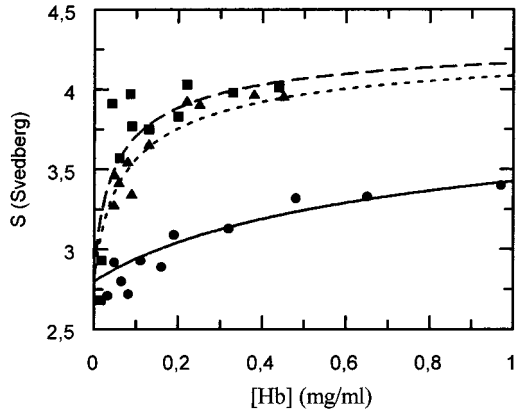

FIGURE 1: Sedimentation coefficients of HbACO (● and —), Hb1TbCO (■ and — —) and Hb1TnCO (▲ and - - -). Samples were in 100 mM bistris buffer at pH 6.0. The data yield $L_{2,4}$ values of 1.0, 24.8, and 14.9 mL mg^{-1}, respectively.

RESULTS

Analytical Ultracentrifugation. Sedimentation velocity experiments were conducted at pH 6.0, 7.0, and 8.6 and 4 °C over the widest range of protein concentration allowed by the instrumentation, namely, 0.016−1.4 mg/mL. These experiments yield a weight average sedimentation coefficient, $\bar{s}$, which depends on protein concentration according to the tendency of the protein to undergo an association−dissociation equilibrium (*11*). The association constant $L_{2,4}$ is calculated from the values of $\bar{s}$ as a function of the total protein concentration, $\bar{w}$. It follows that the sedimentation velocity method works best at concentrations where both dimers and tetramers are present in significant amounts. This situation applies to the experiments performed at pH 6.0 and 7.0. The experiments at pH 6.0 are presented in Figure 1. At pH 8.6, the hemoglobins analyzed are mainly tetrameric even at the lowest accessible concentrations. Therefore, the sedimentation velocity experiments were supplemented by sedimentation equilibrium experiments. This technique allows the analysis of the protein distribution through the whole ultracentrifuge cell (Figure 2), i.e., over a wide and continuous concentration range, and yields directly $L_{2,4}$ values. Its drawback, however, is the long time required to reach equilibrium (about 24 h). Moreover, as experiments are usually conducted at various speeds to get more information, they can last for two or three days. Hence, they are limited only to stable systems. In our case, the $L_{2,4}$ values obtained with both techniques are in good agreement (Table 1).

The three hemoglobins have the same dependence of $L_{2,4}$ on pH, in keeping with the structural similarity at the interfaces (Table 1). Thus, an increase in pH from 6.0 to 8.6 determines an increase in the stability of the tetramer toward dissociation into $\alpha\beta$ dimers, such that at pH 8.6 the proteins are almost entirely tetrameric, as evidenced by the values of $L_{2,4}$ that are all around 100 mL mg^{-1}. This behavior is in agreement with published data on the liganded (R) form of HbA (*14*).

From a quantitative point of view, lowering the pH from 8.6 to 7.0 causes a 5-fold decrease in the association constant

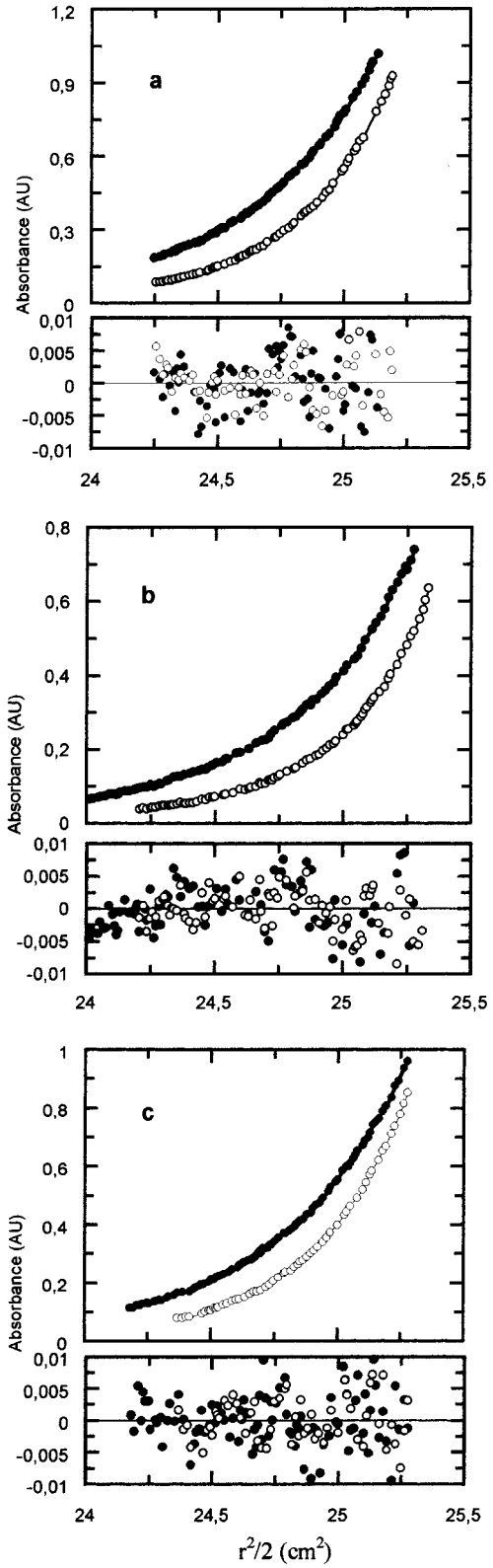

FIGURE 2: Sedimentation equilibrium of HbACO (a), Hb1TbCO (b), and Hb1TnCO (c) at 17 000 (●) and 20 000 (○) rpm. The initial protein concentration was 0.16 mg/mL in 100 mM Tris buffer at pH 8.6. The data yield a $L_{2,4}$ value (mL mg^{-1}) of (a) 140 ± 10; (b) 330 ± 40; and (c) 119 ± 9. The error distributions are shown.

of HbACO, whereas the decrease is less marked (2-fold) in the fish hemoglobins. At pH 6.0, dissociation is much higher than at pH 7.0 in HbACO (about 30-fold). The same pH change has a lesser effect on Hb1TbCO and Hb1TnCO (about 3-fold). Thus, the overall decrease of $L_{2,4}$ is 2 orders of magnitude for HbACO but is only 10- and 5-fold for Hb1TbCO and Hb1TnCO, respectively. As a result, the $L_{2,4}$ values of the three hemoglobins are rather similar at pH 7.0 and 8.6, but differ at pH 6.0, where HbACO is significantly more dissociated than the two fish hemoglobins. The similarity in behavior of Hb1TbCO and Hb1TnCO reflects the very high sequence and structure homology between these two proteins, whereas the differences with HbACO can be ascribed to the larger number of substitutions at the relevant $\alpha_1\beta_2$ interface (Table 2).

Table 1: Dimer−Tetramer Association Equilibrium Constants, $L_{2,4}$ (mL mg^{-1}), in HbACO, Hb1TbCO, and Hb1TnCO Derived from Sedimentation Velocity and Equilibrium Studies[a]

	pH 6.0	pH 7.0	pH 8.6
HbACO	1.0	28.3	164.6 (140 ± 10)
Hb1TbCO	24.8	91.7 (150 ± 10)	259.1 (330 ± 40)
Hb1TnCO	14.9	46.1	76.4 (119 ± 9)

[a] Values are calculated from 10 to 16 independent sedimentation velocity experiments. Numbers in parentheses refer to sedimentation equilibrium experiments. Buffers used are 100 mM bistris (pH 6.0 and 7.0) and 100 mM Tris (pH 8.6). Protein concentration varies in the range 0.016−1.4 mg/mL.

Table 2: Contacts at the $\alpha_1\beta_2$ Interface in the Carbonmonoxy Derivatives of Human, *T. bernacchii* and *T. newnesi* Hemoglobins[a]

HbACO		Hb1TbCO		Hb1TnCO	
α chain	β chain	α chain	β chain	α chain	β chain
Pro 37	His 97	Pro 37	His 97	Pro 37	His 97
Thr 38	His 97	**Gln 38**	His 97	**Gln 38**	His 97
	Val 98				
	Asp 99		Asp 99		Asp 99
			Pro 100		Pro 100
	Tyr 145				
Thr 41	Arg 40	Thr 41	Arg 40	**Ile 41**	Arg 40
	Leu 96				Leu 96
	His 97		His 97		His 97
Tyr 42	Arg 40	Tyr 42	Arg 40	Tyr 42	Arg 40
					Tyr 41
Leu 91	Arg 40	Leu 92	Arg 40	Leu 92	Arg 40
Arg 92	Pro 36	Arg 93	Pro 36	Arg 93	Pro 36
	Trp 37		Trp 37		Trp 37
	Gln 39		Gln 39		Gln 39
	Arg 40		Arg 40		Arg 40
Val 93	Trp 37	Val 94	Trp 37	Val 94	Trp 37
Asp 94	Trp 37	Asp 95	Trp 37	Asp 95	Trp 37
			His 41		**Tyr 41**
	Asp 99		Asp 99		Asp 99
	Asn 102		Asn 102		Asn 102
Pro 95	Trp 37	Pro 96	Trp 37	Pro 96	Trp 37
Val 96	Asp 99	**Ala 97**	Asp 99	**Ser 97**	Asp 99
	Glu 101		**Asp 101**		**Asp 101**
Asn 97	Asp 99	Asn 98	Asp 99	Asn 98	Asp 99
		Lys 100	**Asp 101**	Lys 100	**Asp 101**
Leu 100	Asp 99	Ile 101	Asp 99	Ile 101	Asp 99
Tyr 140	Pro 36	Tyr 141	Pro 36	Tyr 141	Pro 36
	Trp 37		Trp 37		Trp 37
		Arg 142	**Ile 33**	Arg 142	
			Val 34		Val 34
			Tyr 35		Tyr 35
			Pro 36		Pro 36
			Trp 37		Trp 37

[a] Residues within 5 Å distance at the $\alpha_1\beta_2$ interface. In the α chain of Hb1Tb and Hb1Tn, due to the insertion of an amino acid at position 47, numbers of residues after this position are increased by one unit. Residues in Hb1TbCO and Hb1TnCO that differ from HbA are in bold-face.

Structural Analysis. The hydrophobic surface buried at the $\alpha_1\beta_2$ interface upon association of dimers into tetramers has been calculated in two different ways that yield the SAS (solvent accessible surface) and the MS (molecular surface)

Table 3: Solvent Accessible Surface (SAS) and Molecular Surface (MS) Area Buried at the Interfaces in Liganded HbA, Hb1Tb, and Hb1Tn[a]

	SAS		MS	
	$\alpha_1\beta_2 + \alpha_2\beta_1$	$\alpha_1\beta_1 + \alpha_2\beta_2$	$\alpha_1\beta_2 + \alpha_2\beta_1$	$\alpha_1\beta_1 + \alpha_2\beta_2$
$HbAO_2$	2512	4143	1007	2301
Hb1TbCO	3227	4413	1481	2703
Hb1TnCO	3143	4246	1537	2516

[a] Extension of the $\alpha_1\beta_2$, $\alpha_2\beta_1$, $\alpha_1\beta_1$, and $\alpha_2\beta_2$ interfaces (Å^2). Values are calculated according to Lee and Richards (SAS) or Jackson and Sternberg (MS).

area. The SAS area is widely used in protein modeling to describe the entropy driven hydrophobic effect, whereas the MS area is related to the free energy of processes in solution (*15*). Values from SAS measurements are always higher than those from MS, due to the contribution of the probe radius. Moreover, the two sets of values are not simply correlated by a constant of proportionality (*15*).

The data obtained on Hb1TnCO, Hb1TbCO, and liganded HbA (given in Table 3) are in agreement with the experimental dimer−tetramer association constants. Thus, in HbACO, which is characterized by a lower $L_{2,4}$ value, association of dimers into tetramers leads to the burial of a smaller hydrophobic area than in Hb1TbCO and Hb1TnCO. Moreover, for the two fish hemoglobins the area of the surface buried upon association of dimers is very similar, as expected from the higher structural similarities between these hemoglobins relative to HbACO. Calculations on the $\alpha_1\beta_1$ and $\alpha_2\beta_2$ interfaces were also made. The hydrophobic surface area buried at such interfaces is essentially the same in the three hemoglobins (Table 3), indicating that these interfaces have no influence on the differential stabilization of the three tetrameric structures.

The state of association of hemoglobins is known to depend on hydrophilic interactions, and hydrogen bonds in particular. The X-ray structures of the liganded hemoglobins under study show that in HbA five hydrogen bonds are formed between residues of the α- and β-subunits, two of which are mediated by ordered water molecules at the interface, whereas in the fish hemoglobins there are eight hydrogen bonds, of which two and three are mediated by water, respectively, in Hb1TbCO and Hb1TnCO (Figure 3, Table 4). The hydrogen bonding contribution to the tetramer stability, therefore, is in agreement with the association constants measured through analytical ultracentrifugation, just as the hydrophobic contribution. In the liganded fish hemoglobins, the number of hydrogen bonds formed upon association of dimers into tetramers is the same and this number is larger than in HbA.

DISCUSSION

Association of hemoglobin dimers into tetramers is driven primarily by the interactions between residues present at the newly formed interfaces, $\alpha_1\beta_2$ and $\alpha_2\beta_1$. Also, other contacts are formed, namely, those at the $\alpha_1\alpha_2$ and $\beta_1\beta_2$ interfaces (the latter only in the R state), but they are less relevant as they involve a much smaller number of residues. The contribution of the $\alpha_1\beta_1$ (and $\alpha_2\beta_2$) interfaces likewise can be disregarded, as these two "stationary" contacts do not change upon ligand binding. As a matter of fact, recent studies on HbA mutants carrying specific mutations show that changes, e.g., at position β112, may have small effects on the $L_{2,4}$ value (*16*, *17*).

Residues at the relevant $\alpha_1\beta_2$ interface influence the association−dissociation equilibrium through distinct types of interactions. The strongest ones involve charged or polar residues, and in particular hydrogen bonding interactions, as they contribute to the protein stability for about 1.5 kcal/mol (*7*). The free energy gain due to hydrophobic interactions, in turn, is of about 15−25 cal/mol·Å^2 of hydrophobic surface buried at the interface (*7*, *18*). This apparently low value becomes relevant if one considers that the extension of the interface amounts to thousands of Å^2 (Table 3).

The structural analysis of the $\alpha_1\beta_2$ interface on the three hemoglobins under study shows that in the two fish hemoglobins hydrophobic and polar contributions to the tetramer stability are essentially the same and are larger than in HbA. Thus, in both Hb1TbCO and in Hb1TnCO association of dimers into tetramers leads to the burial of a more extensive hydrophobic surface with respect to liganded HbA, as well as to the formation of more hydrogen bonds. A quantitative estimate of the free energy gain cannot be made, as it should be diminished by the energy loss due, for example, to steric interactions, which are difficult to measure.

The stability of the $\alpha_1\beta_2$ interfaces in HbACO, Hb1TbCO, and Hb1TnCO as apparent from the analytical ultracentrifugation experiments is in good agreement with the structural analysis. The hemoglobins from the two Antarctic fishes studied are more associated than human HbA and their tetramers are quite stable even in the ligand bound carbonmonoxyderivative. This behavior is similar to that of hemoglobins from fishes that live at temperate latitudes (*3*, *19*). Hence, cold adaptation of Antarctic fishes does not appear to influence the association−dissociation equilibrium of their hemoglobin significantly.

A further distinctive feature of both fish hemoglobins relative to HbACO is that the stability of the Hb1TbCO and Hb1TnCO tetramers is not pH-dependent. The factors that may contribute to the destabilization of the tetrameric structure at low pH are difficult to bring into focus on the basis of the available structural data that refer to crystals grown under alkaline conditions (*6*, *20*, *21*). Some hypotheses can nevertheless be made. The pH dependence of association can be ascribed, *ceteris paribus*, to the breaking or to the formation of hydrogen bonds upon protonation/deprotonation of one of the residues involved or of a residue in their proximity. Hydrophobic interactions would have only minor contributions and can be neglected. Among the three hemoglobins under study, the dimer−tetramer equilibrium is strongly pH-dependent only in HbACO. Therefore, a more detailed analysis of the residues involved in the formation of hydrogen bonds at the $\alpha_1\beta_2$ interface of liganded HbA has been conducted, in comparison with the two fish carbonmonoxyhemoglobins (Figure 3, Table 4). Such analysis reveals differences in the hydrogen bonds formed, in particular those involving His β97 and Asp β99. His β97, in fact, is hydrogen bonded through the backbone carbonyl group to Thr α38 in liganded HbA but not in Hb1TbCO and Hb1TnCO. This is due to the substitution of Thr α38 with Gln both in Hb1Tb and in Hb1Tn, which leads to the formation of two hydrogen bonds between Gln α38 and Asp β99. These hydrogen bonds are not present in HbA. Ordered

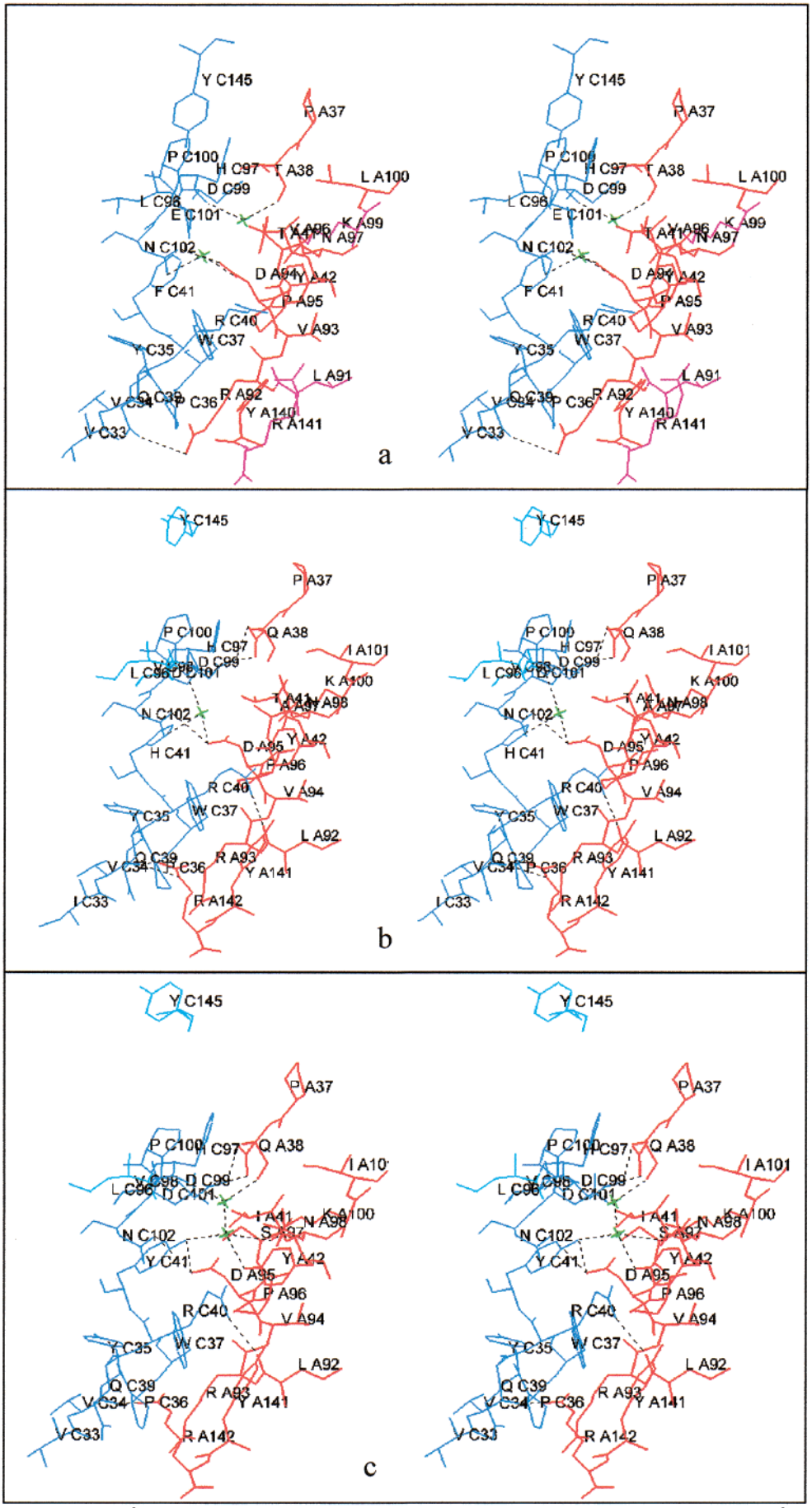

FIGURE 3: Stereo diagram of the $\alpha_1\beta_2$ interfaces of liganded HbA (a), Hb1Tb (b), and Hb1Tn (c). Residues of the α- and β-chains are in blue and red, respectively. Residues that are out of the interface in at least one species are in a lighter shade. Structural water molecules are in green and hydrogen bonds in black.

Table 4: Hydrogen Bonds at the $\alpha_1\beta_2$ Interface in the Carbonmonoxy Derivatives of Human, *T. bernacchii* and *T. newnesi* Hemoglobins

	donor	acceptor	distance (Å)
HbACO	Thr α38	His β97	3.22
	Arg α92	Gln β39	3.18
	Asp α94	Asn β102	2.80
	H_2O	Asp α94	3.25
	Asn β102	H_2O	3.20
	H_2O	Thr α38	2.75
	H_2O	His β97	2.58
Hb1TbCO	Gln α38	Asp β99	3.28
	Arg α142	Val β34	3.13
	Arg α142	Val β34	3.13
	Arg β40	Leu α92	2.96
	Asp β99	Gln α38	3.11
	Asn β102	Asp α95	3.21
	Asp α95	H_2O	3.26
	His β41	H_2O	3.09
	Asp β99	H_2O	2.92
Hb1TnCO	Gln α38	Asp β99	3.08
	Arg α142	Val β34	2.84
	Arg β40	Leu α92	3.24
	Asp β99	Gln α38	2.95
	Asn β102	Asp α95	3.24
	H_2O	Tyr α42	2.59
	Tyr β41	H_2O	2.58
	H_2O	Asn α98	3.24
	H_2O	Gln α38	2.64
	H_2O	His β97	2.52

water molecules also contribute differently to the formation of the hydrogen bond network. The observed pH dependence of $L_{2,4}$ in HbACO below pH 7.0 can thus be ascribed to modifications in the environment of His β97 and Asp β99 due to the increased proton concentration.

Last, the possible correlation between association−dissociation equilibria and the Bohr/Root effects requires a comment. Hb1Tb displays both Bohr and Root effects, whereas Hb1Tn has only a modest Bohr effect and no Root effect. Yet, both give rise in the carbonmonoxy form to tetramers that are stable in the pH range 6.0−8.6. In turn, HbA displays a Bohr effect and dissociates into dimers below pH 7.0. The lack of correlation between the pH dependence of the stability of the ligand-bound tetramer and the functional properties confirm the idea (*21*) that the structural basis of Bohr and Root effects has to be looked for in the T (unliganded) structure rather than in the R (liganded) one.

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BI002297J