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Critical Review

Occurrence and Formation of Endogenous Histidine Hexa-Coordination in Cold-Adapted Hemoglobins

Antonello Merlino[1,2], Barry D. Howes[3], Guido di Prisco[4], Cinzia Verde[4], Giulietta Smulevich[3], Lelio Mazzarella[1,2] and Alessandro Vergara[1,2]

[1]*Department of Chemistry "Paolo Corradini," University of Naples "Federico II," Complesso Universitario Monte S. Angelo, Via Cinthia, I-8012 Naples, Italy*

[2]*Istituto di Biostrutture e Bioimmagini, CNR, Via Mezzocannone 16, I-80134 Naples, Italy*

[3]*Dipartimento di Chimica "Ugo Schiff," Università di Firenze, Via della Lastruccia 3-13, I-50019 Sesto Fiorentino (FI), Italy*

[4]*Institute of Protein Biochemistry, CNR, Via Pietro Castellino 111, I-80131 Naples, Italy*

Summary

Spectroscopic and crystallographic evidence of endogenous (His) ligation at the sixth coordination site of the heme iron has been reported for monomeric, dimeric, and tetrameric hemoglobins (Hbs) in both ferrous (hemochrome) and ferric (hemichrome) oxidation states. In particular, the ferric bis-histidyl adduct represents a common accessible ordered state for the β chains of all tetrameric Hbs isolated from Antarctic and sub-Antarctic fish. Indeed, the crystal structures of known tetrameric Hbs in the bis-His state are characterized by a different binding state of the α and β chains. An overall analysis of the bis-histidyl adduct of globin structures deposited in the Protein Data Bank reveals a marked difference between hemichromes in tetrameric Hbs compared to monomeric/dimeric Hbs. Herein, we review the structural, spectroscopic and stability features of hemichromes in tetrameric Antarctic fish Hbs. The role of bis-histidyl adducts is also addressed in a more evolutionary context alongside the concept of its potential physiological role. © 2011 IUBMB

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Keywords crystallography; EPR; hemichrome; quaternary structure; resonance Raman; root effect.

INTRODUCTION

Few proteins have been studied in such a wide array of organisms as hemoglobin (Hb), and recent discoveries on its structure-function relationship have stimulated renewed interest. Hbs are very ancient proteins, probably evolving from enzymes that protected tissues against toxic oxygen levels. Hbs have been found in bacteria, fungi, plants, and animals; they serve a wide array of physiological roles, from oxygen transport in vertebrates to catalysis of redox reactions. The familiar vertebrate Hb, a tetramer of two identical α and β globin chains, developed relatively recently adaptations to widely different environmental conditions. Though Hbs carry out their function of oxygen carriers in the reduced Fe(II) state, the oxygenated forms are easily oxidized to various ferric (met) forms. Although autoxidation is inevitable in nature for all oxygen-binding heme proteins, the met-Hb content of freshly drawn blood is usually maintained within 1–2% by virtue of a strongly reducing environment (*1*). The physiological role *in vivo* of these ferric forms has been highly debated in the last decades (*2, 3*). In the Hb superfamily, and in particular in the vertebrate $\alpha_2\beta_2$ tetrameric Hbs, the ferric hexacoordinate (6c) aquo form, in equilibrium with the hydroxy form, is by far the most common one formed during the autoxidation process. However, other forms have also been detected and characterized, namely a pentacoordinate (5c) state and an endogenous hexacoordinate (6c) bis-His (hemichrome) species. The relative amounts of these states are highly variable, thus suggesting that the functional roles of these oxidation states are multiple, possibly being a tool for modulating ligand binding or redox properties.

The coordination of a protein side chain to the distal position of the heme iron is expected to influence both the dynamic and

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Address correspondence to: Alessandro Vergara, Department of Chemistry "Paolo Corradini", University of Naples Federico II, Via Cinthia, I-8016, Naples, Italy. Tel +39-081674259, fax +39081674090. E-mail: avergara@unina.it and Giulietta Smulevich, Dipartimento di Chimica "Ugo Schiff", Universita di Firenze, Via della Lastruccia 3-13, I-50019 Sesto Fiorentino (FI), Italy. Tel: +39-055-4573083. Fax: +39-055-4573077. E-mail: giulietta.smulevich@unifi.it.

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structural features of Hb. The axial ligand strength is an essential property of the molecule that must be considered capable of influencing the kinetics of ligand binding, as well as providing alternative functional roles.

In general, in all ferric Hbs, the 5c state is quite rare. It has been detected in some myoglobin distal mutants (*4, 5*), the monomeric flavoHb (*6*), and the dimeric *Scapharca inaequivalvis* Hb (*7*). More recently, the 5c species has been found in Hbs with higher structural complexity, such as tetrameric Hbs from polar fish (*8–10*) and giant Hbs (*11*).

As compared to the 5c state, the occurrence of an endogenous hexacoordination is more widespread at least among invertebrates, plants, and bacteria. Therefore, hexacoordinated Hbs are universally distributed throughout the living world and, thus, may have essential function(s) in cell metabolism.

The residue that can occupy the sixth axial coordination site under native conditions depends on the nature of the distal residues. Although in the majority of cases the sixth ligand is a His residue (*12*), recently, in 2/2 bacterial Hbs (*3, 13–15*) or in naturally occurring mutants of human Hb (HbA) (*16*), a Tyr has been found to be the sixth ligand.

In the present review, only the bis-His (hemichrome) derivatives will be described, focusing on their structural, spectroscopic and stability features.

Occurrence of bis-Histidyl Adducts

Although the formation of hemichrome in a native Hb structure is rare, the presence of bis-histidyl hexacoordination has been discovered in different monomeric Hbs in solution, namely the plant legHbs (*17*), barley Hb (*18*), cyanobacterium *Synechocystis* PCC6803 Hb (*19*), and human Hb subunits (*20*). To date, only a few Hb structures with bis-histidyl endogenous coordination have been deposited in the PDB (*10, 21–29*).

The first discovery of the hemichrome state in tetrameric Hbs was associated with a partial unfolding of the native structure (*30*). However, it was soon recognized that the hemichrome was an accessible structural substate of the native-like Hb population. In tetrameric HbA in solution, the hexacoordination is favored when the chains are separated, suggesting a possible role of the quaternary structure in the destabilization of the hemichrome species (*30*).

A tetrameric Hb in a partial hemichrome state was first observed by some of the present authors while investigating air-oxidized crystals of the main Hb component isolated from the Antarctic fish *Trematomus newnesi* (Hb1*Tn*) (*23*). Successively, the formation and stability of hemichrome species in solution, as well as their structural features in the solid state were extensively investigated for several Antarctic fish Hbs. In particular, a detailed analysis of the hemichrome species was obtained by complementing X-ray crystallography with optical and resonance Raman (RR) spectroscopy in solution (*29, 31–33*) and in the solid state (*2, 34*), and with electron paramagnetic resonance (EPR) (*8–10*). The crystal structure of the hemichrome of Hb*Tb*, the Hb from the Antarctic fish *Trematomus bernacchii,* has been elucidated (*8*). Crystallographic detection of the hemichrome has also been reported in horse Hb (*24*) and in the α chain of HbA complexed with the α-Hb-stabilizing-protein (AHSP) (*35*). Representative examples of Hb structures, showing bis-histidyl endogenous coordination, are summarized in Table 1. The histidyl coordination has always been interpreted as due to $N\varepsilon_{2His\text{-}proxymal}$-Fe-$N\varepsilon_{2His\text{-}distal}$ binding (Fig. 1). However, His bonding via a ring carbon has been recently suggested as a possible alternative coordination mode, particularly in a local slightly acidic environment (*36*), but a carbene-Fe binding mode of His to heme in hemoproteins is not supported by experimental observations.

Structural Features

Statistics on the bis-Histidyl Stereochemistry. The Heme Environment. As in the usual pentacoordinate (5c) or exogenous hexacoordinate (6c) state the distal histidyl $N\varepsilon_2$ is located at about 4 Å from the iron atom, its coordination to the heme iron requires significant rearrangement of the distal site. Moreover, since both the proximal and distal His are embedded in a α-helical environment (helices E and F, respectively), it is likely that the perturbation produced by the formation of the internal covalent linkage is not confined to the distal pocket. How far from the heme pocket this conformational readjustment will be sensed depends on the protein matrix rigidity and on the aggregation state of Hb. In turn, since protein rigidity may cause a pronounced distortion from the ideal coordination geometry at the heme site, heme distortion from planarity may result from the formation of the bis-His complex.

In the following, we first examine the local geometrical features of the bis-histidyl coordination in Hbs, bearing in mind that a meaningful comparison should be based on a more populated set of structures. High-resolution diffraction data are particularly important, because the presence of the electron-rich iron ion increases the positional errors of the bonded lighter atoms. Relevant parameters of the bis-histidyl complex (defined in Fig. 1) of various Hbs are collected in Table 1 and compared with those obtained from three different structures of the closely related bis-histidyl cytochrome b_5. In detail, Table 1 reports the tilt angles of the distal (θ_d) and proximal (θ_p) imidazole with the heme plane, the dihedral angle formed between the distal and the proximal imidazole groups (ω), the bond length between the iron atom and the $N\varepsilon_2$ of the proximal and distal His (Fe-$N\varepsilon_{2im}$), the $N\varepsilon_{2prox}$-Fe-$N\varepsilon_{2dist}$ bond angle and the angle φ formed by the sp^2 orbital direction of the $N\varepsilon_2$ atom (of both His) and the $N\varepsilon_2$-Fe bond (*8*). The heme distortion from planarity was monitored via the root mean square deviations (RMSD) from the least squares plane through the 24 atoms of the porphyrin skeleton (RMSD24A) and through the four pyrrole nitrogen atoms of the heme (RMSD4N). Finally, the distances of the iron atom from the least squares plane through the 24 atoms of the porphyrin skeleton were evaluated.

Table 1
Relevant stereochemical parameters (see text) of various bis-histidyl heme proteins.

Hemoprotein (PDB code)	Res. (Å)	θ_p (°)	θ_d (°)	ω (°)	$N\varepsilon_{2im}$-Fe-$N\varepsilon_{2im}$ (°)	φ (°) Prox.	φ (°) Dist	$C\alpha_{prox}$-$C\alpha_{dist}$ (Å)	Fe-$N\varepsilon_{2im}$ (Å) Prox.	Fe-$N\varepsilon_{2im}$ (Å) Dist	RMSD4N (Å)	RMSD24N (Å)	Distance Fe-heme plane[a] (Å)
Tetrameric Hbs													
T. bernacchii (Hb*Tb* β chain) (2 PEG)	1.4	88.5	68.8	57.7	173	12	16	12.6	2.02	1.98	0.022	0.101	0.02
T. newnesi (Hb1*Tn* β chain) (1LA6)	2	76.8	73	62.7	165	15	11	12.4	2	2	0.036	0.133	0.08
Equus caballus (Hb α chain) (1NS6)	2.1	83.1	67.6	22.7	173	4	16	12.5	2.11	2.18	0.011	0.045	0.03
H. sapiens	2.4	83.2	85.1	89.1	174	5	3	12.8	2.13	2.1	0.015	0.027	0
[(*AHSP) HbA* α chain] (1Z8U)		89.8	85.7	68.6	175						0.010	0.021	0
Dimeric globins													
Oryza sativa (Nonsymbiotic plant Hb) (1D8U)	2.3	87.1	88.4	64.4	178	3	4	12.1	2.08	2.08	0.015	0.124	0.05
		87.9	87.2	63.8	177	4	4	12.1	2.11	2.04	0.012	0.123	0.09
H. sapiens	2	83.4	82.4	67.4	171	13	12	12.3	1.96	2.2	0.007	0.04	0.03
cytoglobin (1URV)		84.3	81.3	67.4	174	3	5	12.6	2.21	2.26	0.019	0.082	0.04
Monomeric globins													
H. sapiens	1.9	88.4	81.8	65.5	175	8	2	12.3	2.09	2.15	0.003	0.065	0.04
neuroglobin (1OJ6)		89.3	78.1	67.3	175	8	3	12.2	2.02	2.17	0.010	0.067	0.03
		88.5	79.1	66.1	175	10	9	12.1	2.1	2.06	0.002	0.09	0.05
		88.8	79.4	60.9	176	6	5	12.3	2.02	2.08	0.021	0.092	0.02
Synechocystis s. (1RTX)	1.8	79	72.7	77	175	17	15	11.5	2.11	1.99	0.017	0.06	0.01
Caudina arenicola (1HLB)	2.5	89.3	77	65	177	14	9	11.6	2.06	2.07	0.048	0.092	0.07
Mus musculus neuroglobin (1Q1F)	1.5	89.5	79.6	61.1	177	4	11	12.1	2.12	1.91	0.012	0.145	0.14
D. melanogaster (2BK9)	1.2	86.9	88.5	89	179	1	2	12.2	1.97	2.02	0.080	0.104	0.10
Cytochrome b5													
Bovis taurus cyt b_5 (1CYO)	1.5	85.9	86.3	21.2	178	4	5	11.7	2	2.07	0.002	0.106	0.02
Rattus norvegicus cyt b_5 (1B5M)	2.7	89.1	81.3	11.8	166	9	10	11.5	1.82	1.92	0.045	0.172	0.10
H. sapiens cyt b_5 (sulfite oxidase domain) (1MJ4)	1.2	87	86.3	0.7	178	3	2	11.6	2.01	2.01	0.015	0.111	0.02

The first two entries refer to the β chains of tetrameric Hbs of Antarctic fish; the third entry refers to the α chains in the tetramers of horse met-Hb at acidic pH and the fourth to the ferric α chains of human HbA complexed with the α-Hb stabilising protein (AHSP). The other entries refer to other Hbs, such as cytoglobin and neuroglobin. Several cytochrome b_5 data are also reported for comparative analysis (adapted from Vergara et al., 2007).
[a]Distance of the iron atom from the least square plane through the 24 atoms of the porphyrin skeleton.

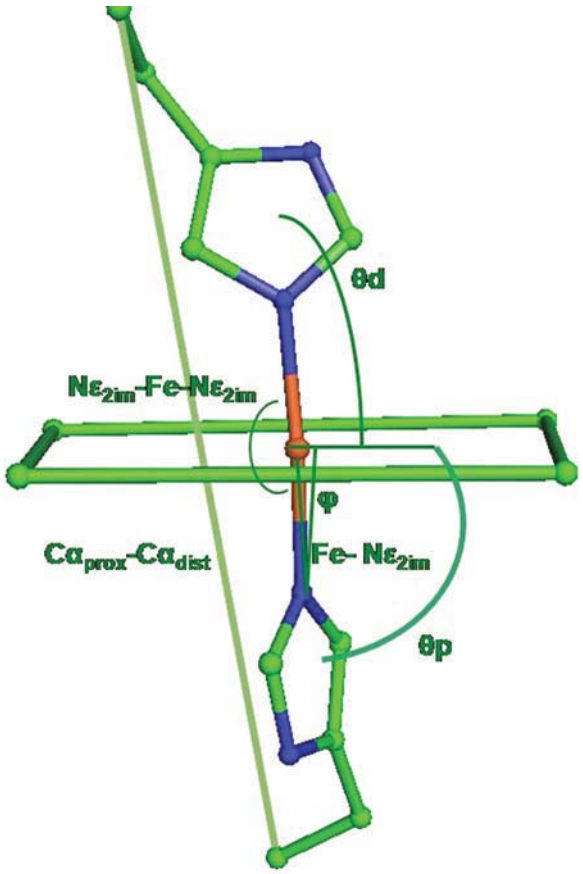

Figure 1. Description of the bis-histidyl coordination parameters. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Although only a few structures have been considered, some general indications can be drawn.

The cytochrome b_5 hemichromes and model compounds with unhindered imidazoles display the expected unconstrained coordination geometry with values of θ_d and θ_p close to 90° (values in the range 85.6°–89.1°), and maximal π overlap between the axial imidazole planes and the Fe dπ orbitals, which is achieved for ω close to 0° (values span the range 0.7°–21.2°) (*2, 8*). The unconstrained geometry of cytochrome b_5-like hemichromes is also inferred from the values of the Nε_{2prox}-Fe-Nε_{2dist} angle, which measures the deviation from linearity of the apical bonds at the iron atom. These values are very close to 180°, if we do not consider the case of *Rattus norvegicus* cyt b_5 (Nε_{2prox}-Fe-Nε_{2dist} = 166°), whose structure has been determined at a resolution significantly lower than the other members of the set (*37*). Deviations from the ideal His coordination are generally pronounced in Hbs, and more so in tetrameric than monomeric Hbs. In fact, in these cases, the Nε_{2prox}-Fe-Nε_{2dist} angle assumes values in the range 165–175°. These findings suggest a steric contribution to the orientation of the axial ligands induced by the protein environment. However, there is no clear indication whether the distal His, as monitored by the angle φ, deviates from the ideal coordination more than the proximal His, although this appears to be the case in tetrameric Hbs. A His coordination geometry close to the ideal has been observed only in Hb isolated from *Drosophila melanogaster* (*26*) and in the HbA α-chain complexed with AHSP (*35*). Furthermore, in these two proteins, the formation of the bis-histidyl adduct induces large heme distortions. The mean value of the RMSD from the best plane of RMSD24 and RMSD4N is 0.08 ± 0.04 Å in *D. melanogaster* Hb and 0.02 ± 0.01 Å for the HbA α-chain complexed with AHSP (*12*). Albeit significant, these values are smaller than those observed in other hemoproteins, mainly in bis-His multiheme cytochromes (RMSD24 = 0.17 ± 0.06 Å) and bis-His metallohydrolase (RMSD24 = 0.17 ± 0.08 Å). Thus, the distortion of the heme plane in bis-His globins seems to be less pronounced with respect to the other bis-His hemoproteins and even less than that of hemoproteins containing a sixth ligand different from His.

The elucidation of the crystal structure of Hbs in a bis-histidyl state together with the stereochemical parameters provided an important guide for the interpretation of the spectroscopic features observed in solution, especially for tetrameric Hbs. Electron paramagnetic resonance (EPR) data correlate well with the X-ray data collected for several Hbs and model systems (*8, 38*). Indeed, EPR provides unique data on the oxidation, coordination, and spin state of the heme group. Particularly, depending on the protonation state of His side chains and the geometry of bis-His coordination, the dispersion of the *g*-factor values (*g* anisotropy) for the signal associated with the hemichrome moiety may vary significantly (*39*). With the exception of human cytoglobin, *g* anisotropy is found to increase: (i) with deviation of θ_d from 90°, i.e., with increase in the tilt angle of the distal imidazole plane with respect to the heme plane (*8*); (ii) with deviation of dihedral angle ω, between the proximal- and distal-imidazole planes, from 0° or 90°.

UV–vis and RR spectroscopy have also been used to characterize hemichrome states. As the Q bands of the bis-His species at 530 and 565 nm are distinct from those of hydroxy low-spin complexes or other 6cLS species, observation of such bands together with the characteristic RR bands of LS heme states enables the unambiguous detection of hemichrome states (*40*). The use of these techniques on solution samples to reveal hemichrome states offers an opportune means of complementing and validating the information obtained from the X-ray data, enabling identification of the heme spin states that form progressively during the autoxidation of the tetrameric Antarctic fish Hbs. It has been shown that autoxidation through formation of an intermediate hemichrome state is a common mechanism for a number of Antarctic fish Hbs (*29, 32–34*). Furthermore, it was demonstrated that these hexacoordinate states could be reversibly reduced to deoxy and carbonmonoxy forms, suggesting that they may have a functional role. RR spectroscopy (*32, 33*) and EPR (*10*) have also been applied to monitor the formation and stability of hemichrome states of tetrameric Hbs in solution as a function of pH and other environmental parameters.

Modification of the Polypeptide Chain. Interesting modifications of polypeptide chain arrangements are produced by endogenous hexacoordination of the iron. The formation of the covalent bonds between the distal His and the iron necessarily requires a scissor-like closure of the fork formed by the helices E and F: the two helices hold the heme moiety by means of the distal and proximal His. The scissor-like movement of the EF segment is provided by a change of distances between the Cα carbon atoms of the distal and proximal His residues, which belong to helices E and F, respectively. Depending on the protein, this distance, which is sharply distributed around a value of 14.5 Å in the α and β chains of Hb from different organisms and in a variety of ligated states (*2, 32*), shrinks to 11.5–12.5 Å in bis-histidyl hemes. The movement of the two helices is accompanied by a sliding of the heme toward the solvent. However, in the majority of the structures, the largest modifications of the polypeptide chains are observed in the nearby CD region, in line with the greater mobility of this segment. This result is of great interest as this region is directly involved in the allosteric interface of tetrameric Hbs.

Quaternary Modifications. For tetrameric Hbs of Antarctic fish, interesting observations have been obtained regarding the quaternary-structure (*8, 23, 24, 29*). These Hbs form only partial hemichrome states either in the α or in the β chain, strongly supporting the intrinsic differences between the two chains in terms of backbone flexibility. Interestingly, while in horse Hb, the hemichrome is formed at the α heme (*24*), in Antarctic fish Hbs (Hb*Tb* and Hb1*Tn*) only the β heme is involved (*8, 23, 29*). As the quaternary assembly is very similar, these findings suggest a change in the relative flexibility of the two chains. In fact, horse Hb and Antarctic fish Hbs differ markedly. In the former, the hemichrome state was obtained as a result of a pH-induced transition starting from a met-Hb single crystal. The solid-state transformation is coupled with extensive modifications of the packing contacts and tertiary structure. In common with other cases, the largest modifications are observed in the CDα region that converts from an extended loop at pH 7.1 to a helix at pH 5.4 (Fig. 2A), and packs against helix C of the β chain of a symmetry related molecule. In particular, Phe46α moves more than 6 Å away from its position in the hydrophobic distal cavity to the surface, forming a π-stacking interaction with the side chain of symmetry related Arg40β, and the α-heme propionate groups of two adjacent tetramers form an inter-tetramer hydrogen bond. Thus, the conformational change at the CD corner is intimately linked to the packing interactions, and favors the transition to the hemichrome state. Despite this remarkable change, which affects a region directly involved in the $\alpha_1\beta_2$ ($\alpha_2\beta_1$) interface, the quaternary-structure modifications are negligible. The molecule fully maintains the standard R-state organization of the original met-Hb form (*24*).

Unlike horse Hb, hemichrome is formed in the β subunits of Antarctic fish Hbs (Fig. 2B), where the approach of the two His is favored by Tyr85 in position F2, which replaces Phe in HbA.

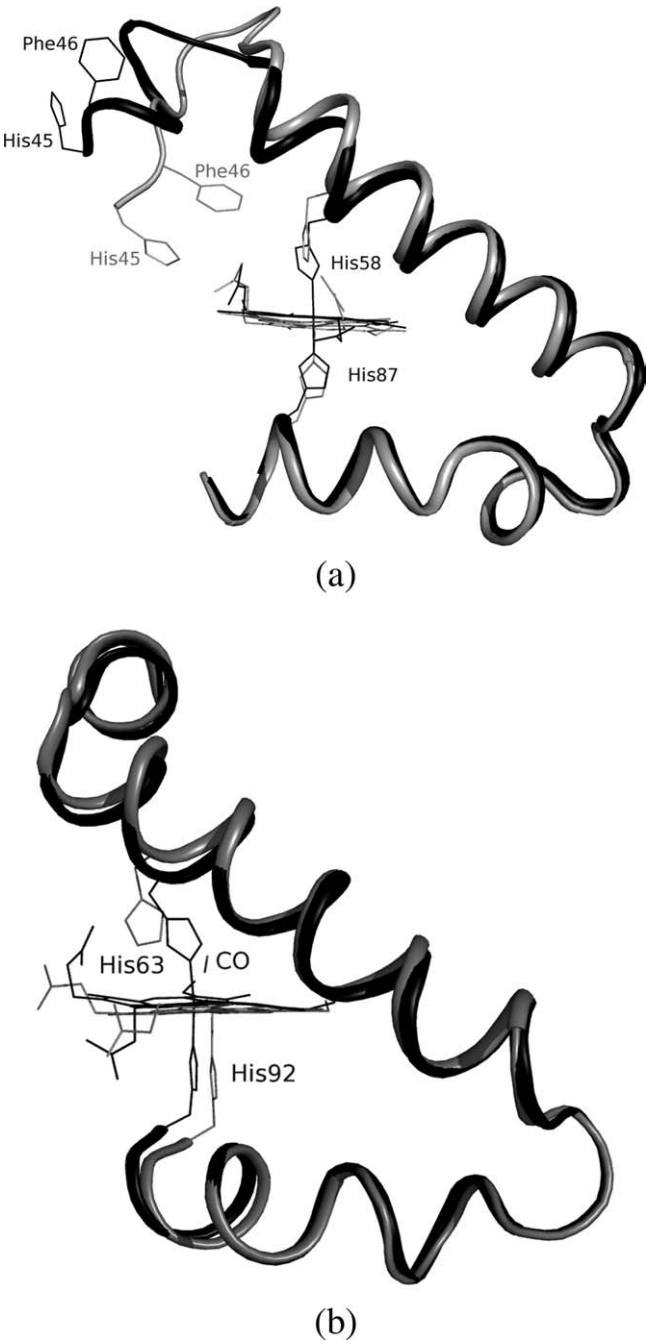

Figure 2. Superimposition of 6c hexogenous state (grey) and 6c endogenous state (black) for horse Hb (A) and Hb*Tb* (B).

The hydroxyl group of Tyr causes a kink in the facing helix E by forming two H-bonds (*23*) with the oxygen carbonyl atom of Gly70 and the N atom of Gly74, thus facilitating the movement of the distal His positioned almost at the other end of the same helix E. In addition, the CD corner of the β chain becomes highly disordered, whereas the same region in the α chain of horse Hb is well ordered. However, in the latter, the position of

the CD corner (*24*) suggests that its ordered structure is merely the result of packing interactions, and that this region could be at least partially disordered in solution.

In Hb*Tb* and Hb1*Tn*, the closure movement of the EF fragment associated with hemichrome formation propagates to other structural elements of the tertiary structure of the β subunit, directly involved in the quaternary assembly of the molecule. In particular, the FG corner assumes a conformation that is more similar to that found in the T state than the R state. The displacement of the FG corner produces variations in the position of His97β, whose location is crucial in stabilizing the quaternary structure of liganded and unliganded forms of tetrameric Hbs. Indeed, Antarctic Hbs adopt a quaternary assembly (denoted H state) that is intermediate between the classic R and T states. A way to distinguish the H state from canonical R and T states is the quantification of the relative orientation between dimers $\alpha_1\beta_1$ and $\alpha_2\beta_2$. Indeed, on superimposition of the $\alpha_1\beta_1$ dimer of ferric Hb*Tb* with the deoxy (T) structure (*8*), a further rotation of 6.7° is required to superimpose the $\alpha_2\beta_2$ dimers. Similar values (about 4.7°), but with a rotation in the opposite direction, have been obtained superimposing ferric Hb*Tb* with its carbonmonoxy (R) structure (*41*), indicating that the H state has a quaternary structure that is intermediate between those of the high affinity R and low affinity T states. The combined structural variations induced by hemichrome formation in the β chain and in the quaternary assembly cause significant changes of the FG region of α subunits that extend up to the FG region in contact with the α heme. Therefore, the structural data on Antarctic fish Hbs indicate that the scissoring-like motion of the EF pocket may initiate a cascade of events that favor the switch from the R to the T state. The hypothesis that reversible bis-histidyl complexes, such as hemichrome, could populate the landscape of native Hbs in solution is particularly attractive as it suggests a functional role of this form in tetrameric Hbs. In all cases, the study of hexacoordinate Hbs must demonstrate that cells and tissues are able to express significant Hb-reductase activity, necessary to restore the reduced state required for oxygen binding.

bis-Histidyl coordination is strongly affected by mutations at the heme distal pocket. Indeed, EPR analysis of both cathodic Hb of *T. newnesi* (HbC*Tn*) (*8*) and Hb of the Arctic fish *Liparis tunicatus* (*9*) revealed a 5c ferric state, presumably preferred to the bis-histidyl adduct at the β heme. Probably, the replacement of Val67β with the bulkier Ile generates unfavorable interactions in the heme pocket, which negatively affects hemichrome formation (*8*). The 5c state of a ferric β subunit, having the more common Val residue in E11, has also been observed for the AFHb Hb*Tb*. In this case, the quaternary structure at acidic pH is pushed toward the T state (*10*) by the strong Root effect characteristic of this Hb (see below). The hemichrome-pentacoordinate transition results from the break of the iron-distal histidine axial bond, due to the higher tension of the distal coordination compared to the proximal site.

Mechanism of Hemichrome Formation in Tetrameric Hbs of Antarctic Fish. A detailed study by X-ray crystallography and electronic absorption and RR spectroscopy has been carried out on the formation of hemichromes in Hb*Tb* (*33*) and Hb1*Tn* (*32*), following autoxidation of carbonmonoxy complexes by air exposure. The comparison of the crystallographic structures obtained within the first hours of air exposure reveals that both Hb*Tb* and Hb1*Tn* undergo a quaternary structure modification toward the H structure; the β heme slides out by 0.5 Å, then contraction of the β heme pocket and the Cα–Cα distance shortening from 14.5 to 13.5 Å take place. Figure 3 reports the RR spectra of Hb*Tb* and Hb1*Tn* observed during the autoxidation process of these proteins at the times indicated. The spectra of the deoxy and met forms are also shown for comparison. In both Hbs, careful inspection of the RR spectra reveals the immediate appearance of an aquo hexacoordinate high-spin ferric form. The spectra after 3 h show a band assigned to the ν_3 6cHS mode, that appears as a shoulder of the deoxy band at ν_3 1470 cm^{-1}, due to laser-induced photolysis of the CO ligand. The frequency shifts of the ν_3 and ν_2 bands upon autoxidation to 1505 and 1579 cm^{-1} (1578 cm^{-1} for Hb1*Tn*) are consistent with the formation of a hexa-coordinate bis-His (hemichrome) form. In both proteins, the hemichrome occurs via the formation of an intermediate, which is different in the two proteins. In Hb1*Tn*, a 5c form appears in the RR spectra, which is not observed in Hb*Tb*. Indeed, the X-ray crystallographic data reveal that after 3 h of air exposure the crystal of Hb1*Tn* contains a 5c ferric coordination at the β heme that is not observed in Hb*Tb*. Presumably, in the latter, the hemichrome is formed through an aquomet form, as suggested for HbA (*20*). The analysis also reveals that Hb*Tb* and Hb1*Tn* show a difference in the α/β heterogeneity in oxidation kinetics. Indeed, during air exposure, the β chain of Hb1*Tn* reacts much faster than the α chain, and oxidation is virtually complete within 3 h. Moreover, in the autoxidation process of both Hbs monitored by RR, the spectra show a progressive concomitant increase of both hexa-coordinate high-spin and low-spin forms. In addition, the spectra of Hb1*Tn* also show the progressive increase of a 5c high-spin state (Fig. 3). In line with these results, chemical oxidation yields two ferric derivatives in Hb*Tb* (the aquo 6cHS and the hemichrome states) and three ferric derivatives in Hb1*Tn*, where a 5c state is also observed.

Physiological Role of bis-Histidyl Adducts

The finding that hemichromes can be reversibly formed in Antarctic fish, as well in many other heme proteins, including neuroglobins and cytoglobins (*25, 42*), in marked contrast with the behavior of human HbA, suggests a putative physiological role of the *in vivo* formation of this form. Many hypotheses on the role of bis-histidyl coordination in organism physiology have been suggested (*2*). However, a full understanding of the physiological functions of these proteins remains elusive. Although in vertebrates endogenous coordination is generally

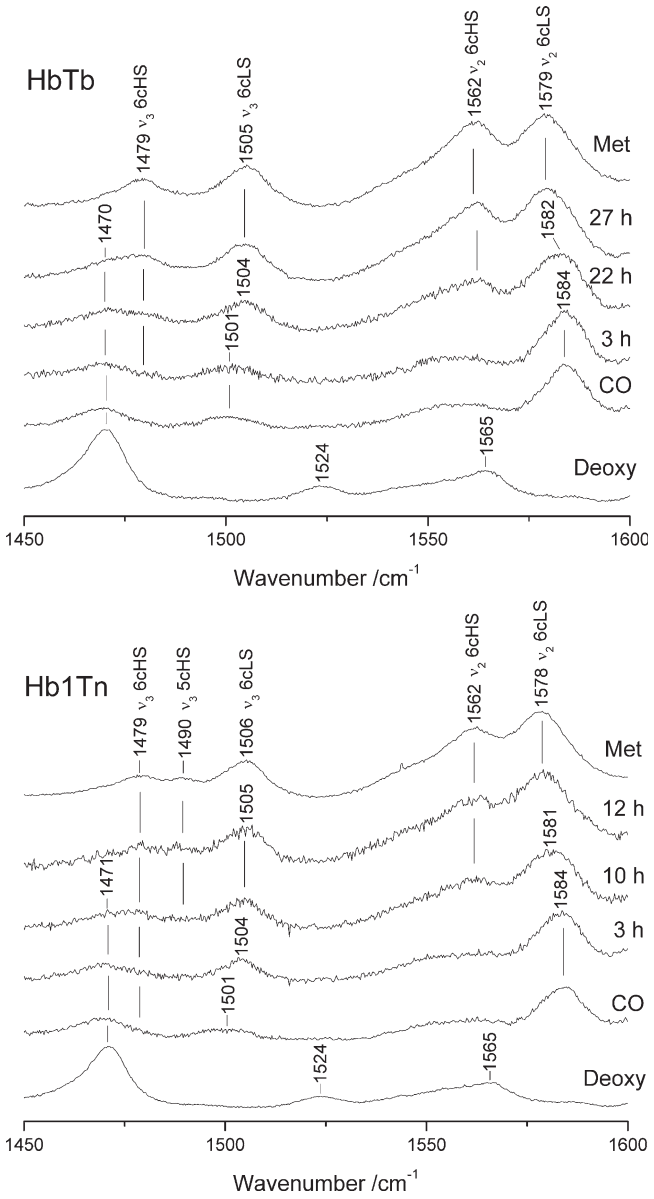

Figure 3. The autoxidation of 10 μM Hb1*Tn*CO and 50 μM Hb*TbCO* in 50 mM Tris-HCl pH 7.5 followed by RR spectroscopy (413.1 nm excitation wavelength, 1 cm^{-1} spectral resolution). For experimental conditions, see refs. *32* and *33*.

associated with impaired functions, hemichrome or hemochrome reversible formation is neither exceptional among invertebrate, plant, and bacteria globins, nor globins expressed in low amounts in some tissues of higher vertebrates. The ferric forms of Hb are physiologically inert to further oxygenation, but several subsequent side reactions in Hb autoxidation may interfere with or merge into other biochemical pathways, including formation of hemichrome. The relevance of hemichrome spans from biomedical to physiological aspects (*30*), some of which have been reviewed recently (*2*). For example, autoxidation is a severe problem that limits the storage time of cellular Hb-based blood substitutes (*43*). However, hemichrome detection has been suggested as a valuable tool for tumor diagnosis (*44*); the reaction of acetylphenylhydrazine with erythrocytes leads to hemichrome formation in healthy people but not in breast-cancer patients. The bis-histidyl complex can be involved in ligand binding (*42, 45*), in facilitating the *in vivo* reduction of met-Hb (*46*), in Heinz body formation via copolymerization with the cytoplasmatic domain of band 3 (*30*) and in nitrogen monoxide scavenging (*47*). Recently, hemichrome in human HbC was observed to interact *in vivo* with erythrocytes, modifying their membrane rigidity (*48*), and affecting its ability to release oxygen. It has also been suggested that hemichrome can be involved in the protection of the Hb molecule from peroxidation attack (*35*). Unlike the hemichrome derivative of the isolated human α subunits complexed with AHSP (*35*), Antarctic fish Hbs exhibit peroxidase activity, which is higher not only than that of HbA but also than that of temperate fish Hbs (*10*). Indeed, while it is possible that hemichrome formation in Antarctic fish is simply a fortuitous occurrence in structurally flexible cold-adapted globins without having a specific function, we can speculate that its opportunistic retention provides protection from the increased oxidative pressure due to high oxygen solubility in cold waters.

The accessibility of an oxidation hybrid (α ferrous/β ferric) in Hb*Tb* and Hb1*Tn* suggests possible superoxide-dismutase activity in these proteins that, however, has not been found (*33*). Further work on differences in electrochemical behavior of α and β chains in these fish Hbs would be beneficial. It seems clear that if the only function of these Antarctic proteins is to carry oxygen, the best coordination state to satisfy this role is the 5c state.

Interestingly, hemichrome stability has also been correlated to the Root effect (*10*). The Root effect is a peculiar property of some fish Hbs that is associated with extremely low affinity for oxygen at low pH values. The Root effect has been functionally related to buoyancy (filling of the swimbladder with gas) and to the supply of oxygen to the typically uncapillarized fish retina. Fish Hbs endowed with a Root effect (e.g. Hb*Tb*, HbC*Tn*, Hb from *Cottoperca gobio,* a sub-Antarctic fish) showed, by EPR spectroscopy, a strong decrease of the hemichrome content at acidic pH in favor of a 5c ferric adduct (*10*). Accordingly, the X-ray structure of ferric Hb*Tb* at acidic pH revealed an α(aquomet)/β(5c) coordination in a T quaternary state. In contrast, fish Hbs not endowed with Root effect (Hb1*Tn* and the single Hb of *Gymnodraco acuticeps*) do not show any pH dependence of the hemichrome stability. The interplay of these two important features might have significant consequences in cold-adapted fish physiology. The crystallographic/EPR evidence of pH-induced hemichrome $\rightarrow$ 5C conversion in Root-effect Hbs suggests that hemichrome is not compatible with the T quaternary structure. Therefore, the Root effect, by overstabilization of the T state at acidic pH, may also work in the ferric state modulating hemichrome stability (*10*).

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