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Structural and thermodynamic aspects of cooperativity in the homodimeric hemoglobin from *Scapharca inaequivalvis*☆

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Abstract

The homodimeric cooperative hemoglobin from the mollusk *Scapharca inaequivalvis* displays an unusual subunit assembly with respect to vertebrate hemoglobins. The intersubunit contact region is formed by the two heme-carrying E and F helices, which bring the two hemes in contact with each other. At variance with tetrameric vertebrate hemoglobins, the ligand binding is not accompanied by a significant quaternary transition. The major ligand-linked changes are tertiary and are limited to the heme pocket and subunit interface. These unique structural features of HbI are not easily reconciled with the classical thermodynamic models used to describe cooperative ligand binding in vertebrate hemoglobins. The lack of distinct quaternary states and the absence of allosteric effectors suggested that cooperativity in HbI is entirely homotropic in origin. Thereafter, high resolution X-ray crystallographic data displayed the preferential binding of water molecules at the intersubunit interface in the unliganded protein with respect to the liganded one. These ordered water molecules were thus proposed to act as heterotropic effectors in HbI. The contribution of specific water binding to the observed cooperativity in HbI is discussed in the framework of the enthalpy–entropy compensation effect emerging from previous accurate equilibrium oxygen binding measurements. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: *Scapharca inaequivalvis*; Hemoglobin; Thermodynamic

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☆ Dedicated to Heini Eisenberg.

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1. Introduction

The homodimeric hemoglobin from the mollusk *Scapharca inaequivalvis* (HbI) provides a striking demonstration that cooperativity in oxygen binding can be achieved by a quaternary assemblage of the globin fold, distinct from that of tetrameric vertebrate hemoglobins [1,2]. In HbI, the two heme groups are almost in direct contact and are not exposed to solvents, as in the vertebrate hemoglobin tetramer, since the heme-carrying E and F helices form the subunit interface.

The unique mode of assembly of HbI requires the structural basis of cooperativity to be different from that operative in tetrameric vertebrate hemoglobins. Cooperative oxygen binding to HbI, therefore, cannot be described in terms of the well-known stereochemical model of Perutz [3]. The mechanistic aspects of this model are based on the crystallographic structures of deoxy- and ligand-bound human hemoglobin, and can be summarized as follows: ligand binding is accompanied by the displacement of the iron atom towards the heme plane, a motion that pulls the proximal histidine (position F8) and results in a shift of the whole F helix. The tertiary changes in the ligated subunit are then propagated to the other subunits of the $\alpha_2\beta_2$ tetramer through the so-called $\alpha_1\beta_2$ dimer interface. At this interface, the consequence of ligand binding is the breakage of the bonds characterizing the deoxy structure. As a result, the transition to the ligand-bound structure is rendered possible. The assignment of the low ligand affinity state (T state) and the high ligand affinity one (R state) to these two structurally and energetically distinct conformers provides the link between the tridimensional structure and thermodynamics.

A variety of thermodynamic models have been proposed to describe the experimentally observed equilibrium ligand binding properties of vertebrate hemoglobins, and in particular, of human hemoglobin (HbA). All assume that the hemoglobin tetramer can exist in at least two distinct quaternary structures [4–7]. However, more realistic linkage schemes must explicitly take into account other effects, such as the dissociation of hemoglobin into dimers [9], and the fact that heterotropic contributions (e.g. from Bohr protons, chloride ions, diphosphoglygerate) to the observed cooperativity are tightly linked to homotropic ones. Hence, a detailed modeling is required to single out their specific energetic contribution [5,7].

In this framework, the cooperative homodimeric *Scapharca* HbI represents a challenging system from both the mechanistic and the thermodynamic point of view. The unique mode of assembly points to a direct communication between the two heme groups. Moreover, the lack of quaternary transitions and heterotropic modulators indicates that classical two state thermodynamic models do not provide an adequate description of the observed cooperativity. In fact, the three parameters L, K_R and K_T that describe homotropic cooperativity cannot be univocally defined for homodimers, whose behavior can be accounted for by two parameters only.

2. Structural basis of heme–heme interaction

As for human hemoglobin, the X-ray crystallographic studies on deoxy- and liganded-HbI reveal the details of the structural rearrangements that occur upon ligand binding. The α-carbon skeletons relative to the deoxy- and liganded species are almost perfectly superimposable, indicating that no significant ligand-linked quaternary changes take place [1,2]. The major ligand-linked structural rearrangements may be classified as tertiary ones. They involve the movement of Phe 97 (position F4), which is tightly packed against the proximal histidine (His 101) in the deoxy derivative, but is extruded into the dimer interface in the liganded species (Fig. 1a), where it disrupts a network of well-ordered water molecules [8–10]. Thus, 6 out of the 17 structured water molecules that characterize the deoxy interface are absent in the liganded species (Fig. 2). The displacement of Phe 97 is also accompanied by the sinking of the two hemes towards the bottom of their respective pockets, a movement which brings the two iron atoms further apart by 0.6 Å. As a consequence, a rearrangement of the interactions, established by the heme propionate

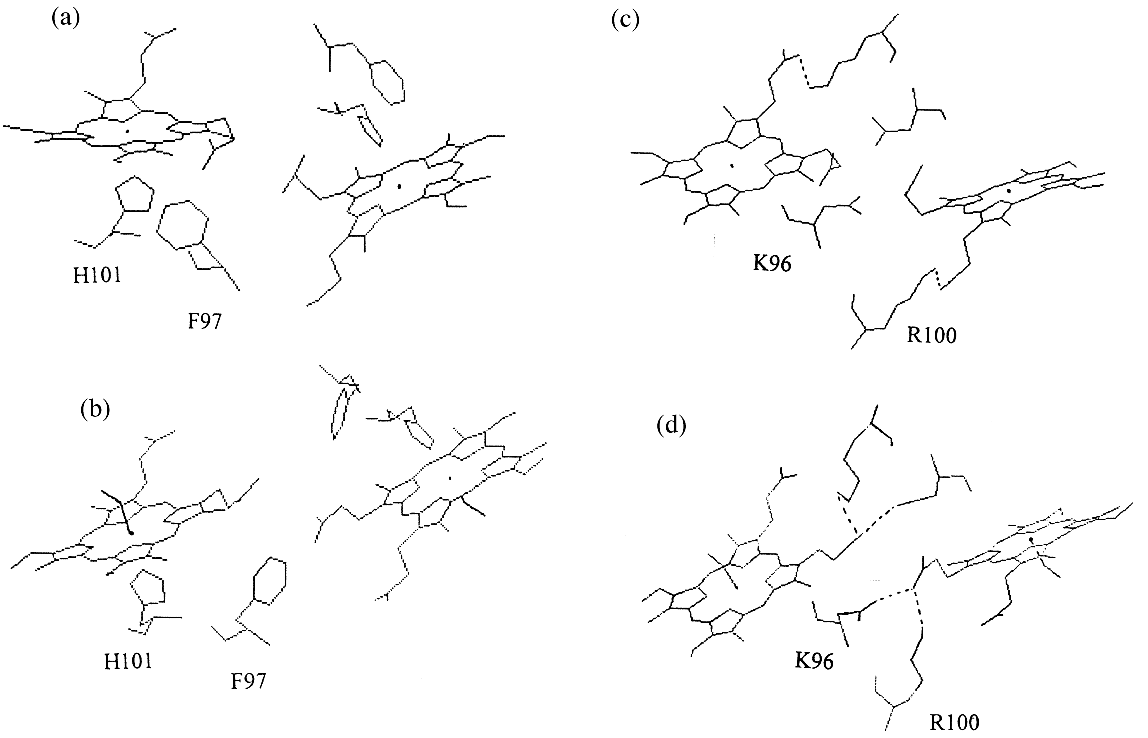

Fig. 1. Ligand-linked structural changes in *S. inaequivalvis* HbI. The heme pocket regions are viewed down the molecular dyad in the deoxy (a, c) and oxy (b, d) derivatives (Protein Data Bank codes are 4SDH and 1HB1, respectively). Panels a and b depict the ligand-induced movement of Phe 97 from the heme pocket into the interface; panels c and d depict the interactions established by the heme propionates with amino acids of the contralateral subunit. Ordered water molecules at the subunit interface are not shown.

groups, occurs (Fig. 1b). Thus, the hydrogen bond with Asn 100 of the contralateral subunit, made by one of the two heme propionates in the deoxy derivative, is no longer present in the liganded species, where Asn 100 is replaced by a water molecule. The rupture of this interaction is paralleled by a shift in the hydrogen bonding to Lys 96 from one heme propionate to the other. The structural events at the basis of cooperative oxygen binding appear, therefore, to be localized within a well-defined symmetrical region at the subunit interface that includes the two heme pockets.

The time frame of the ligand-linked structural changes has been set by means of time-resolved optical absorption and resonance Raman studies. Laser-photolysis of carbonmonoxy HbI in the nanosecond time regime, followed by transient optical absorption in the Soret region, revealed that relaxation from the photolyzed to the deoxy species is a single process that takes place within a few microseconds after photolysis [11]. Consis-

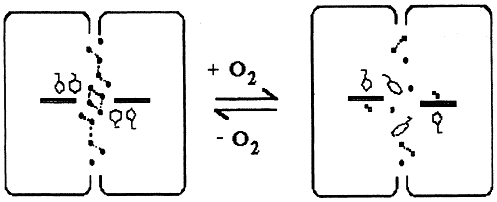

Fig. 2. Ligand-linked water release from the *S. inaequivalvis* HbI interface. The scheme indicates the movement of Phe 97 and the six water molecules that are released upon ligand binding to deoxy-HbI.

tent with these data, the Raman active vibrations relative to the stretching of the iron–histidine bond, the bending of the heme propionates, and the pyrrole ring out-of-plane modes all relax concertedly with a half time of approximately 1 μs after photolysis; the typical time scale of tertiary conformational transitions [12]. In HbI, these processes are not followed by the slower quaternary relaxation that occurs within hundreds of microseconds in HbA [13]. In summary, the picture which emerges from both the structural and dynamic data indicates that HbI exists in two distinct tertiary conformations which interconvert in a single step process.

3. Thermodynamics and structure

The equilibrium oxygen binding properties of HbI have been investigated in detail as a function of pH, temperature, salt and protein concentration. HbI exhibits cooperative oxygenation with $n = 1.5$ and a constant oxygen affinity (at any given temperature) under all of the experimental conditions tested [14]. The lack of heterotropic effectors has allowed a detailed thermodynamic characterization of each oxygenation step, without the need for corrections for the contribution of non-heme ligands. In order to gain insight into the nature of cooperative oxygen binding, the thermodynamic properties were described in terms of the simple Adair reaction scheme. As in tetrameric vertebrate hemoglobins, in HbI the ΔH_i values are non-uniform with respect to each oxygenation step, and correspond to -11.4 and -7.4 kcal mol^{-1} for the first and the second step, respectively. The associated entropy changes are likewise non-uniform (-44.7 and -27 cal mol^{-1} K^{-1}, respectively, for the first and the second step) and nearly exactly compensate the enthalpy changes. Thus, the cooperative effect is dominated by a favorable difference in entropy, which outweighs the unfavorable enthalpy changes. The enthalpy–entropy compensation is a common phenomenon in tetrameric hemoglobins where it has been attributed, independently of the thermodynamic model used, to the stepwise free energy changes associated to the release of Bohr protons which accompanies each oxygenation step [4,5]. This interpretation evidently does not hold for HbI. Long before the availability of the high-resolution crystallographic structures of HbI, Ikeda-Saito et al. [14] suggested that the enthalpy–entropy compensation in HbI could arise from a different interaction with solvent of the liganded and unliganded forms. In particular, ligand binding would be associated to a movement of the solvent from the protein hydration shell to the bulk. The release of water molecules would thus produce the entropic force driving the cooperative phenomenon. Interestingly, the idea of the direct participation of water as an allosteric mediator is becoming increasingly popular to explain part of the heterotropic cooperativity in HbA [15,16]. However, direct measurements that bring out such a contribution are lacking, and the crystallographic structures of HbA do not show preferential hydration of the *R* or the *T* state.

The high-resolution crystallographic structures of oxy and deoxy HbI [8,9] provide a spectacular confirmation of Ikeda-Saito's first suggestion. Thus, 6 out of 17 tightly coordinated water molecules in the interface and two molecules in the heme pocket are lost upon oxygenation. These water molecules were proposed to be the effectors that accompanied the ligand-linked conformational transition in HbI [10,17], thereby challenging the thermodynamic description of the cooperative effect solely in terms of homotropic contributions. The cooperative oxygenation in HbI should be more properly described in terms of heterotropic rather than homotropic cooperativity, provided that the ligand-linked release of water molecules can be expressed in terms of a mass law equation defining the specific binding of such molecules [6]. These considerations prompted the following estimate of the energetic contributions underlying the ligand-induced release of water. The entropic cost for immobilizing a single water molecule from the liquid on a macromolecular surface has been estimated by Dunitz on the basis of the measured hydration entropy of salts [18]. The upper limit was ~ 7 cal

mol^{-1} K^{-1}, which corresponded to a free energy contribution of approximately 2 kcal mol^{-1} at 298 K. It applied to those water molecules, which were firmly bound in salt crystals, where all the translational and rotational degrees of freedom of the water molecule are quenched. The entropy cost was probably slightly less than 7 cal mol^{-1} K^{-1} for water molecules that were hydrogen bonded to a single amino acid residue, and still possess at least one degree of rotational motion. In this framework, a significant increase in entropy must accompany the ligand-induced release of the six well-ordered interfacial water molecule of HbI. Assuming that these molecules are all released upon the second binding step, the entropy increase could well correspond to the observed difference $\Delta S_1 - \Delta S_2 = +17$ cal mol^{-1} K^{-1}. It should be stressed that the water molecules that are lost upon the conformational transition accompanying ligand binding in HbI are not part of the generic 'hydration shell' of the protein, as they are characterized by a significantly lower B factor with respect to water molecules distributed on the protein surface [15,16]. The highly ordered water molecule network in the subunit interface must be considered an integral part of the protein structure that plays an active role in the ligand-linked conformational transition.

A discussion on the enthalpy changes associated to oxygenation along the same lines is rendered difficult by the possibility that the intrinsic heat of oxygen binding to the two heme molecules can be non-uniform, per se [5].

Lastly, it is of interest that the oxygen binding isotherms measured on single crystals of HbI are characterized by a cooperative behavior ($n = 1.45$) similar to that observed in solution [19]. This comparison confirms that the cooperative behavior in HbI is not based on quaternary effects, and in turn provides a beautiful correlation between crystallographic data and HbI function in solution. It should be recalled that HbA crystals, grown under similar conditions as HbI, shatter upon oxygenation, since lattice interactions do not accommodate the T–R quaternary transition [20].

4. Conclusions

The body of the available structural, dynamic and thermodynamic data indicates that in HbI, the mechanism of the heme–heme interaction is based on the strong direct coupling of the two heme groups. Thus, cooperative ligand binding in HbI is not associated to quaternary rearrangements, but only to limited tertiary changes that are confined within the heme pockets and the subunit interface. The preferential hydration of the intersubunit contact region in the deoxy species with respect to the liganded one, coupled to the analysis of the oxygen binding isotherms, indicates that HbI may represent the first unequivocal example of a protein in which the conformational transition is water-linked.

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