Biophysical Chemistry, 37 (1990) 287–292
Elsevier

BPC 01486

A cooperative hemoglobin with directly communicating hemes

The *Scapharca inaequivalvis* homodimer

Emilia Chiancone [a], Daniela Verzili [a], Alberto Boffi [a], William E. Royer, Jr [b] and Wayne A. Hendrickson [b]

[a] *CNR Center of Molecular Biology, Department of Biochemical Sciences, University 'La Sapienza', 00185 Rome, Italy and* [b] *Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biophysics, Columbia University, New York, NY 10032, U.S.A.*

Received 27 February 1990
Accepted 12 March 1990

Hemoglobin; Cooperativity; Ligand binding; Subunit interaction

The unique functional properties of the homodimeric hemoglobin (HbI) extracted from the Arcid blood clam *Scapharca inaequivalvis* are discussed in the light of the unusual assembly of this protein. At variance with vertebrate hemoglobins, in *S. inaequivalvis* HbI, the heme-carrying E and F helices form the subunit interface and bring the heme groups almost into direct contact. This creates a new pathway for transferring information about the ligation state of the heme from one subunit to the other which allows cooperativity in the binding of heme ligands to be displayed by a homodimer. The tight coupling between the two subunits and the two heme groups also manifests itself in other reactions that are cooperative in *S. inaequivalvis* HbI, but not in human hemoglobin, namely, the cleavage of the proximal histidine–heme iron bond and the modification of specific residues located at the subunit interface.

1. Introduction

Hemoglobins are the most widespread respiratory pigments in the animal kingdom. The heme iron can combine reversibly with molecular oxygen, since it is protected from oxidation and kept in the ferrous state by the globin moiety. In order to fulfill this role, all globins share a similar chain folding, the characteristics of the heme pocket and the linkage to the heme iron by a bond to the N_δ of the proximal histidine [1].

In higher vertebrates globins are assembled into the familiar $\alpha_2\beta_2$ tetramer, a highly sophisticated molecular machinery that meets the oxygen requirements of the organism by varying the oxygen affinity depending upon oxygen concentration, a phenomenon that gives rise to cooperative oxygen binding. The oxygen affinity is modulated further by other factors of the ever-changing physiological milieu, such as pH, organic phosphates and carbon dioxide concentration [2]. In essence, the structural basis for the cooperative binding of oxygen and other heme ligands lies in the capability of the molecule to switch between at least two distinct quaternary structures, one characteristic of the deoxygenated protein and endowed with a low ligand affinity (the T structure) and the other characteristic of the oxygenated protein and endowed with a high affinity for ligands (the R structure). The switch between the two quaternary structures has been described in great detail [3,4]; it is accompanied by changes in the tertiary structure around the heme and is associated with changes in the number of salt bridges and hydro-

Correspondence address: E. Chiancone, CNR Center of Molecular Biology, Department of Biochemical Sciences, University 'La Sapienza', 00185 Rome, Italy.

gen bonds at the subunit interfaces, particularly at the level of the $\alpha_1\beta_2$ contact. The latter changes lead to a destabilization of the tetrameric molecule in the oxygenated protein and to its dissociation into $\alpha\beta$ dimers at low concentrations [5,6]. The structural constraints necessary for cooperativity are such that $\alpha\beta$ dimers and homotetramers like HbH, which is built of four β chains, are noncooperative [7].

In this scenario the high cooperativity displayed by the homodimeric hemoglobin (HbI) extracted from the erythrocytes of *Scapharca inaequivalvis* [8] and other Arcid clams [9–11] came as a surprise. It contradicted both prerequisites necessary for cooperative ligand binding in vertebrate hemoglobins, namely, the presence of two types of globin chain and of a tetrameric molecule. HbI, the minor hemoglobin component of Arcid erythrocytes, binds oxygen with an affinity of 8 Torr and a strong cooperativity as indicated by a Hill coefficient, n, of 1.5; its oxygen binding properties are unaffected by changes in salt concentration, buffer composition and pH over the range 5–9. The other component, HbII, accounts for about 65% of the hemoglobin; it is a heterotetramer characterized by an oxygen affinity of 9 Torr (at neutral pH) and a cooperativity corresponding to $n = 2.0$. At variance with HbI, HbII displays a slight anion-linked acid Bohr effect [12]. It is noteworthy that in both hemoglobin components the manifestations of cooperativity are the same as in vertebrate hemoglobins. Thus, at equilibrium, cooperativity is primarily entropic in origin with oxygen as ligand [13], while kineti-

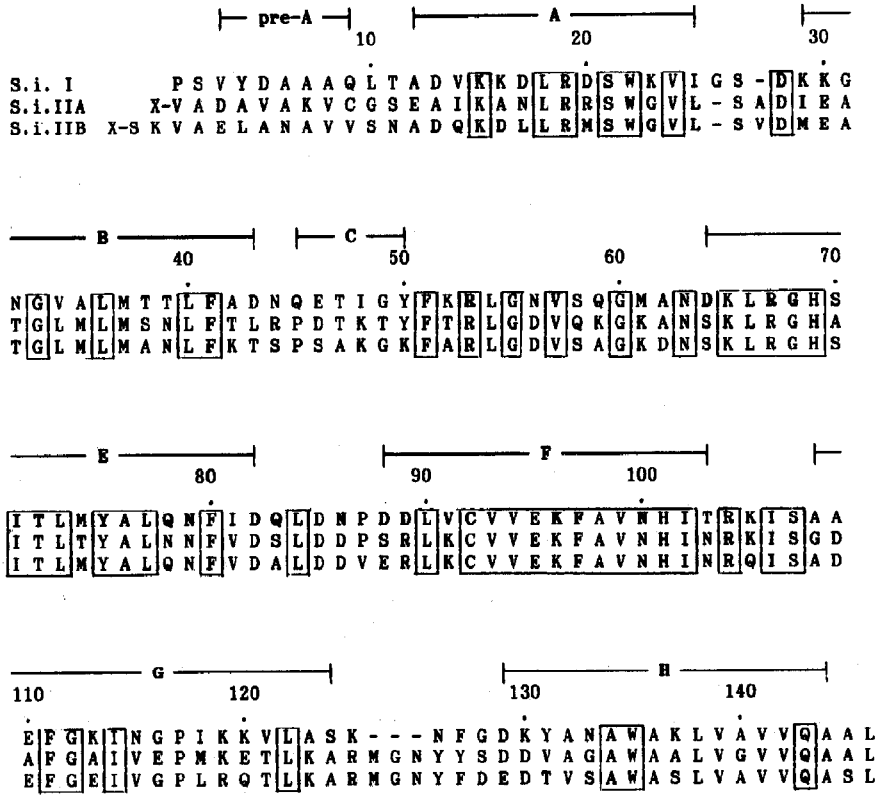

Fig. 1. Comparison of the amino acid sequences of the constitutive *S. inaequivalvis* hemoglobin chains. The numbering and the helix notation refer to the sequence of the homodimeric HbI [15]. The tetrameric HbII sequences are taken from ref. 31. Identical residues in the Arcid hemoglobin chains sequenced to date (see ref. 31) are indicated by boxes; residues involved in contacts across the interface of HbI [18] are in bold-face type.

cally it manifests itself in the accelerating time course of carbon monoxide binding and in the rates of oxygen dissociation [13].

2. Structural basis of cooperativity

Two observations suggested that the structural basis for the unusual presence of cooperativity in the Arcid hemoglobin dimer could reside in a distinct assemblage of the globin chains: (i) the conserved residues in the three constitutive Arcid globin chains are not distributed randomly, but are clustered at the level of the E and F helices (fig. 1) as if these regions were serving a special purpose in the molecule [15]; (ii) the two hemoglobins do not show antigenic cross-reactivity, indicating that the highly conserved E and F helices are not exposed to solvent as in vertebrate hemoglobins [16]. Soon thereafter, the low-resolution X-ray structures of the carbon monoxide derivatives of HbI and HbII showed that the E and F helices form the intersubunit contact in the dimeric protein and bring the heme groups into a spatial relationship totally different from that in vertebrate hemoglobins: the Fe-to-Fe distance is only 18.4 Å as compared with 25 Å for the $\alpha_1\beta_2$ pair. HbII is a dimer of heterodimers assembled like HbI, the contact between dimers being much less extensive than the intradimer one [17].

Details of the dimer interface that point to the existence of a direct pathway of communication between the two heme groups have been revealed very recently by the high-resolution structure of carbonmonoxy-HbI [18]. The interface has both hydrophilic and hydrophobic character and can be classified into four unique regions: an 'E-E' region near the center of the contact where the E helices of both subunits cross each other, two symmetrically related hydrophobic 'E-F' regions where the E helix of one subunit lies across the F helix of the other, and two symmetrically related salt bridges that connect Lys 30 (in the B helix) with Glu 89 (in the F helix) of the contralateral subunit. However, with respect to the existence of a new pathway of heme-heme communication, the most interesting part of the interface is the 'heme-F' one (fig. 2). This region is highly hydrophilic and involves both heme groups and the last two turns of the F helices. The two hemes are almost in

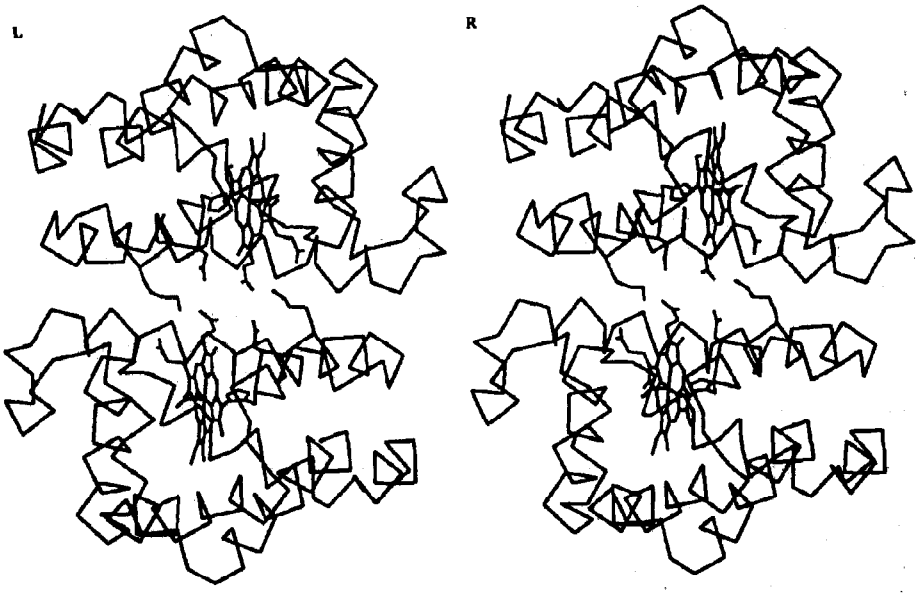

Fig. 2. Stereo diagram of *S. inaequivalvis* carbonmonoxy-HbI plotted down the molecular diad. Included with the α-carbon plot are the two heme groups and side chains for the proximal His 101, Lys 96 and Asn 100 of each subunit. Note the intimate involvement of one propionate group from each heme in the heme-F region of the interface near the center of the diagram.

direct contact with each other with the propionate groups of one pyrrole ring adopting rather extended conformations that bring oxygen atoms from different hemes within 4.0 Å from each other. More extensive interactions of these propionate oxygens occur with two residues in the F helix of the neighboring subunits, Lys 96 and Asn 100, that are within 1–1/2 helical turns from the proximal His 101.

The network of interactions at the heme-F region provides a direct connection for transferring information between the two heme groups. Indeed, the high-resolution structure of the deoxy derivative shows that striking changes occur in the heme environment, while the changes in quaternary structure are rather subtle [19]. In deoxyhemoglobin, Lys 96 no longer interacts with the same propionate of the heme group in the neighboring subunit, but forms a salt bridge with the other propionate; meanwhile, the bridging propionates adopt folded conformations and come to be linked by pairs of hydrogen bonds from Asn 100 in place of bridging water molecules in the carbon monoxide structure. Moreover, Phe 97, which lies outside the subunit in the 'E-F' patch of the interface in the CO derivative, packs inside against the heme in the deoxy form. The structural and electronic features at the heme group in deoxy-HbI resemble those of the α-chains in the human hemoglobin tetramer in that the coordination of the proximal histidine to the metal ion is distorted and sterically hindered. This is indicated by a number of spectroscopic markers such as the unusually high ellipticity of the Q_0 band [8], the small hyperfine shift of the $N_\delta H$ group [20] and the broad $g_\perp$ signal of the protein reconstituted with Co-porphyrin [21].

3. Manifestations of cooperativity

The structural transition that occurs upon ligand binding is reflected in specific changes in the pattern of exchangeable resonances between 9.5 and 13 ppm, namely, in the region of the spectrum where proton NMR signals of inter- and intrasubunit hydrogen bonds of human hemoglobin occur. In comparison with human hemoglobin, however, the hydrogen-bonded resonances which relate to liganded and unliganded HbI have an unusual characteristic: their intensity corresponds to less than a single proton peak, indicating that the relevant hydrogen bonds are dissociated in part on the NMR time scale and hence that the interactions are weak [21,22].

A further aspect of the structural transition between the liganded and unliganded states concerns the presence of ligand-linked differences in the state of association of HbI. In this respect, the extrusion of Phe 97 from the heme pocket into the interphase upon oxygenation is likely to provide a significant contribution, since it entails a concomitant increase in the hydrophobicity of the E-F contact region which in deoxy-HbI contains an extensive, ordered water network. The initial characterization of the stability of the dimeric structure carried out by determinations of sedimentation velocity did not bring out significant differences in the sedimentation coefficient of oxy- and deoxy-HbI which corresponds to the value expected for a dimeric hemoglobin [8]. In a sedimentation velocity study still in progress, experimental Schlieren patterns have been compared with simulated ones computed for rapidly equilibrating self-associating solutes with concentration-dependent sedimentation and diffusion coefficients [23]. Again ligand-linked differences in the state of association of the protein appear to be small, at variance with the behavior of human hemoglobin, where oxygenation leads to a substantial dissociation of the $\alpha_2\beta_2$ tetramer [5,6].

The ligand-linked structural differences apparent in the high-resolution X-ray structure result in a different reactivity of specific residues located at the subunit interface such as Cys 92 (F4) whose rate of binding to mercurials depends on the state of oxygenation of the protein (Verzili et al., unpublished results). A further unexpected characteristic of this reaction is its marked cooperativity [24], a finding which indicates that alterations in a localized portion of the E-F contact are transmitted readily to the symmetrically related region.

Another reaction that is noncooperative in vertebrate hemoglobins displays cooperativity in *S. inaequivalvis* HbI, namely, the cleavage of the bond between the N_δ of the proximal histidine and

the heme iron that occurs at low pH in both the deoxygenated and NO derivatives [25,26]. In human hemoglobin this bond plays a crucial role in the transmission of quaternary conformational changes as it represents the only structural link between the protein and the heme group. Its cleavage can be accounted for by a single proton titration, assigned to protonation of the proximal imidazole [27]. In HbI the same phenomenon displays cooperativity and hence indicates that transmission of conformational changes from the heme to the subunit interface may occur via an additional pathway, the natural candidate being the heme-F interface [26].

How fast can this direct heme-heme communication take place as compared to the time required for the R-to-T structural relaxation? An answer to this question can be found in the unusual characteristics of the nanosecond geminate recombination with oxygen which shows a dependence of the rate on the extent of photolysis [28]. In this type of experiment, after the heme-ligand bond has been broken by an appropriate flash, the ligand molecule recombines at the same site rather than escaping to the exterior. In human hemoglobin the rates of geminate recombination have half-times on the nanosecond time scale, whereas the R-to-T structural transition takes place in the microsecond time regime. It follows that the geminate recombination phenomenon can be regarded as an intrasubunit event, which requires, for example, that the rate of geminate recombination be independent of the extent of photolysis [29,30]. In HbI the rate of geminate rebinding with oxygen does show a dependence on the amount of protein photolysed, indicating that interheme communication occurs in the nanosecond time range at a much faster rate than the R-to-T transition which takes place in microseconds [14]. However, the rate of geminate rebinding increases with fractional photolysis whereas one would expect the opposite dependence, namely, more T-like behavior and presumably slower ligand binding at the heme with increasing fractional breakdown.

Lastly, it is of interest to combine the three-dimensional structural information available for *S. inaequivalvis* HbI with the recently obtained sequence data on the tetrameric component HbII [31]. Such an analysis (fig. 1) shows that about one third of the residues involved in contacts across the dimer interface are changed in the tetramer chains. The substitutions of Lys and Asp 89, which form a salt bridge across the dimer interface, are intriguing as they allow the identification of residues that are likely to direct the assembly of the two hemoglobin components. Thus, in the tetramer a salt bridge across the interface of the dimeric building block can still be formed, but with a sign reversal, since Lys 30 is substituted with an Asp in both tetramer chains, while Asp 89 is substituted with an Arg residue. Lys 96 and Asn 100, the crucial residues of the heme-F region that interact with the propionate groups of the neighboring subunit, are conserved in both tetramer chains, indicating that direct transfer of information between adjacent heme groups also occurs in the dimeric building module of HbII. It is noteworthy that in HbII the presence of two such modules endows the protein with a sensitivity to allosteric effectors which is lacking in HbI. HbII undergoes an anion-linked polymerization of the native tetrameric structure which depends on the binding of other protein ligands, namely, oxygen and protons and results in changes of both oxygen affinity and cooperativity [12].

4. Concluding remarks

The structural and functional characterization of the homodimeric hemoglobin from *S. inaequivalvis* has shown for the first time that there is no unique assembly of globin folds associated with cooperative oxygen binding. At variance with vertebrate hemoglobins where cooperativity is restricted to the $\alpha_2\beta_2$ tetramer, in Arcid hemoglobins the minimum functional unit is a homodimer which displays strong homotropic interactions; however, due to the strong coupling between the two heme groups that are essentially in direct communication this basic cooperative unit is not sensitive to heterotropic effectors and hence represents a less refined molecular machinery than the vertebrate hemoglobin tetramer.

References

1 R.E. Dickerson and I. Geis, Hemoglobin (Benjamin/Cumming, Menlo Park, CA, 1983).
2 E. Antonini and M. Brunori, Hemoglobin and myoglobin in their reactions with ligands (North-Holland, Amsterdam, 1971).
3 M.F. Perutz, Proc. R. Soc. Lond. Ser. B, 208 (1980) 135.
4 J. Baldwin and C. Chothia, J. Mol. Biol. 129 (1979) 175.
5 E. Antonini and E. Chiancone, Annu. Rev. Biophys. Bioeng. 6 (1977) 239.
6 G.K. Ackers, Biophys. J. 32 (1980) 331.
7 M.F. Perutz, Annu. Rev. Biochem. 48 (1979) 327.
8 E. Chiancone, P. Vecchini, D. Verzili, F. Ascoli and E. Antonini, J. Mol. Biol. 152 (1981) 577.
9 S. Ohnoki, Y. Mitomi, R. Hata and K. Satake, J. Biochem. (Tokyo) 73 (1973) 717.
10 H. Furuta, M. Ohe and A. Kajita, J. Biochem. (Tokyo) 82 (1977) 1723.
11 P.F. Como and E.O.P. Thompson, Aust. J. Biol. Sci. 33 (1980) 643.
12 A. Boffi, P. Vecchini and E. Chiancone, J. Biol. Chem. 265 (1990) 6203.
13 M. Ikeda-Saito, T. Yonetani, E. Chiancone, F. Ascoli, D. Verzili and E. Antonini, J. Mol. Biol. 170 (1983) 1009.
14 E. Antonini, F. Ascoli, M. Brunori, E. Chiancone, D. Verzili, R.J. Morris and Q.H. Gibson, J. Biol. Chem. 259 (1984) 6730.
15 R. Petruzzelli, B.M. Goffredo, D. Barra, F. Bossa, A. Boffi, D. Verzili, F. Ascoli and E. Chiancone, FEBS Lett. 184 (1985) 328.
16 D. Verzili, G. Citro, F. Ascoli and E. Chiancone, FEBS Lett. 181 (1985) 347.
17 W.E. Royer Jr, W.E. Love and F.F. Fenderson, Nature 152 (1985) 277.
18 W.E. Royer Jr, W.A. Hendrickson and E. Chiancone, J. Biol. Chem. 264 (1989) 21052.
19 W.E. Royer Jr, W.A. Hendrickson and E. Chiancone, Science (1990) in the press.
20 T. Inubushi, T. Yonetani and E. Chiancone, FEBS Lett. 235 (1988) 87.
21 D. Verzili, R. Santucci, M. Ikeda-Saito, E. Chiancone, F. Ascoli, T. Yonetani and E. Antonini, Biochim. Biophys. Acta 704 (1982) 215.
22 I.W.M. Fung and C. Ho, Biochemistry 14 (1975) 2526.
23 D.J. Cox, Arch. Biochem. Biophys. 146 (1971) 181.
24 A. Boffi, M. Gattoni, R. Santucci, P. Vecchini, F. Ascoli and E. Chiancone, Biochem. J. 241 (1987) 449.
25 C. Spagnuolo, A. Desideri, E. Chiancone and F. Ascoli, in: Invertebrate oxygen carriers, ed. B. Linzen (Springer, Berlin, 1986) p. 101.
26 M. Coletta, A. Boffi, P. Ascenzi, M. Brunori and E. Chiancone, J. Biol. Chem. 265 (1990) 4828.
27 M. Coletta, P. Ascenzi and M. Brunori, J. Biol. Chem. 263 (1988) 18286.
28 E. Chiancone and Q.H. Gibson, J. Biol. Chem. 264 (1989) 21062.
29 J.S. Olson, R.I. Rohlfs and Q.H. Gibson, J. Biol. Chem. 262 (1987) 12930.
30 J. Hofrichter, E.R. Henry, H. Sommer, A. Deutsch, M. Ikeda-Saito, T. Yonetani and W.A. Eaton, Biochemistry 24 (1985) 2667.
31 R. Petruzzelli, A. Boffi, D. Barra, F. Bossa, F. Ascoli and E. Chiancone, FEBS Lett. 259 (1989) 133.