Volume 314, number 3, 481–485 FEBS 11921 December 1992

The homodimeric hemoglobin from *Scapharca* can be locked into new cooperative structures upon reaction of Cys^{92}, located at the subunit interface, with organomercurials

Gianni Colotti[a], Alberto Boffi[a], Daniela Verzili[a], Massimiliano Coletta[b] and Emilia Chiancone[a]

[a]*CNR Center of Molecular Biology, Department of Biochemical Sciences 'A. Rossi Fanelli', University 'La Sapienza', 00185 Rome, Italy* and [b]*Department of Molecular, Cellular and Animal Biology, University of Camerino, Via F. Camerini, 2, 62032 Camerino (MC), Italy*

Received 9 November 1992

In the cooperative, homodimeric hemoglobin from *Scapharca inaequivalvis*, HbI, the subunit interface is formed by the heme-carrying E and F helices and contains the only cysteine residue of the globin chain (Cys^{92}, F2) in an area which changes from hydrophilic to hydrophobic upon oxygenation. Binding of organomercurials to HbI is cooperative and entails major quaternary rearrangements. The reaction of Cys^{92} with *p*-chloromercuri-benzoate (PMB) and *p*-nitro-*o*-chloromercuriphenol (PN), a sensitive reporter of the cysteine microenvironment at neutral pH values, has been followed in stopped flow experiments. Kinetic evidence for the cooperativity of mercurial binding has been obtained and the rate of the corresponding conformational transition has been estimated. As expected PN, but not PMB, is able to monitor the oxygen-linked change of the cysteine microenvironment. The modification of Cys^{92} with PN has unique functional effects. In PN-reacted HbI cooperativity is maintained, albeit to a different extent, depending on the ligation state of the protein during mercaptide formation. It may be envisaged that PN locks the protein into new, cooperative, quaternary structures stabilized by hydrogen bonding interactions between the ionized nitrophenol moiety and the contralateral subunit.

Molluscan hemoglobin; Cooperativity; Chemical modification; Conformational change

1. INTRODUCTION

In the homodimeric hemoglobin, HbI, from the Arcid clam *Scapharca inaequivalvis* cooperativity in oxygen binding [1] arises from a molecular mechanism which differs from that operative in tetrameric vertebrate hemoblogins. This peculiarity reflects the unique mode of assembly of the globin chains: in HbI the heme-carrying E and F heliced do not face outwards, but form the subunit interface and bring the two heme groups in direct communication via a hydrogen bond network which differs in detail in the carbonmonoxy and deoxy derivatives [2,3]. At variance with vertebrate hemoglobins, oxygen binding is accompanied by only subtle quaternary movements of the two subunits relative to one another [3].

The two symmetry-related regions of the subunit interface where the most significant ligand-linked changes occur contain Cys^{92} (F2), the only cysteine residue of the polypeptide chain [4]. The reactivity of Cys^{92} therefore provides an ideal handle to both study and affect the interface. Indeed Boffi et al. [5] observed that sulfhydryl alter the oxygen binding properties of HbI and that the effect depends to a first approximation on the size of the reagent used. *p*-Chloromercuribenzoate (PMB), for example, brings about a significant increase in oxygen affinity accompanied by the loss of cooperativity. These functional properties reflect the drastic change in quaternary structure which causes the HbI crystals to crack upon reaction with PMB. Boffi et al. [5] also disclosed an unexpected property of the two Cys^{92} residues in oxy-HbI, namely that they bind PMB in a cooperative fashion.

The present paper reports a study of the reactivity of Cys^{92} with a chromophoric mercurial, *p*-nitro-*o*-chloromercuriphenol (PN), and of the oxygen binding properties of the modified protein. PN is a sensitive reporter group of the cysteine microenvironment at neutral pH values due to the ionization of its nitrophenol group [6]. In principle therefore PN should allow one to monitor the oxygen-linked changes in hydrophilicity around Cys^{92} attendant the movement of Phe^{97}. Thus, the X-ray structures [2,3] show that in the liganded derivative the phenyl group of Phe^{97} lies in the same hydrophobic patch of the interface that comprises Cys^{92}, but leaves the interface and packs against the proximal His^{101} upon deoxygenation.

The kinetics of Cys^{92} modification by PN depends on the state of oxygenation of the protein, provides evidence for cooperativity in mercurial binding and for the

Correspondence address: E. Chiancone, CNR Center of Molecular Biology, c/o Dept. of Biochemical Sciences 'A. Rossi Fanelli', University 'La Sapienza', 00185 Rome, Italy. Fax: (39) (6) 4440062.

related conformational change. Most unexpectedly, PN-reacted HbI maintains cooperativity in oxygen binding albeit to a different extent depending on the ligation state of the protein during the cysteine modification reaction. This characteristic has been explained in terms of the capacity of PN to lock HbI into new cooperative structures by establishing hydrogen bonding interactions across the subunit interface by means of the charged nitrophenol moiety.

2. MATERIALS AND METHODS

The dimeric HbI was isolated from the red cells of *S. inaequivalvis* and purified as described previously [1]. Hemoglobin concentrations (in heme) were determined at 578 nm on the basis of $E = 14{,}300$ $M^{-1} \cdot cm^{-1}$ for the oxygenated derivative.

PMB and PN were commercial products of Sigma (St. Louis, MO, USA) and Eastman Kodak (Rochester, NY, USA), respectively. PMB and PN solutions were prepared by dissolving 5 mg of mercurial in 0.1 M NaOH and diluting to 5 ml with 0.1 M phosphate buffer at pH 7.0. PMB concentration was determined at 232 nm in 0.1 M phosphate buffer, pH 7.0 by using the extinction coefficient $E = 16{,}900$ $M^{-1} \cdot cm^{-1}$ [7]. PN concentration was determined in 0.1 M NaOH, using the extinction coefficient $E = 17{,}400$ $M^{-1} \cdot cm^{-1}$ at 405 nm [6]. Polyacrylamide gel electrophoresis experiments were carried out according to Davis [8].

Oxygen equilibria were measured at 20°C in a tonometer with a side-arm which allowed addition of the mercurial either before or after deoxygenation of the protein. The amount of mercurial added corresponded to 1.1–1.3 equivalents of reagent per sulfhydryl group. The kinetics of mercaptide formation was followed in rapid mixing experiments performed in Tris-HCl buffer at 20°C using either a Durrum instrument connected to an OLIS data acquisition system or a Hi-Tech SFA-11 apparatus (dead time 0.4 s) connected to a Hewlett-Packard HP 8452 A diode array spectrophotometer. By taking advantage of the multiwavelength facility of the latter instrument, concomitant with mercaptide formation possible variations in the state of oxygenation and/or oxidation were monitored at 578, 560 and 542 nm. Mercaptide formation was followed at 250 nm [7] for PMB and at 468, 470 and 472 nm for PN [6].

The kinetic data were analyzed in terms of a reaction scheme consisting of two irreversible second order steps, given the stability of the mercaptide bond, and a first order conformational transition:

$$P + L \xrightarrow{k_1} PL \quad (1)$$

$$PL \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} PL^* \quad (2)$$

$$PL^* + L \xrightarrow{k_3} PL_2 \quad (3)$$

$$PL + L \xrightarrow{k_4} PL_2 \quad (4)$$

where P is the protein, L the mercurial and PL* the monoligated fast-reacting species. The solution of the system of differential equations was approximated with the Euler method and the data were fitted to the theoretical curves by a least squares method (Nelder–Meade algorithm). All calculations were carried out with the Matlab program on a Microvax computer.

3. RESULTS

Firstly, gel electrophoresis experiments were carried out to establish whether PN like PMB binds to oxy-HbI in a cooperative fashion. Indeed, when the protein is treated with substoichiometric amounts of PN, essentially no band of intermediate mobility is evident in addition to those corresponding to the unreacted and fully reacted protein (data not shown). Similar results have been reported for PMB-reacted HbI [5].

Thereafter, mercaptide formation was followed in rapid mixing experiments performed on both oxy- and deoxy-HbI. Due to the strong absorbance of PN in the visible region the experiments were carried out using 1.0–1.2 equivalents of mercurial/heme. The ligand-linked change in the cysteine microenvironment apparent in the X-ray data [2,3] is reflected in the timecourse and signal amplitude of the reaction. The timecourse shows a distinct lag phase only in the oxygenated protein and the signal amplitude is significantly smaller in oxy- than in deoxy-HbI (Fig. 1A). Under similar experimental conditions the reaction of Cys^{92} with PMB is independent of the state of ligation of the protein (Fig. 1B).

In a further series of rapid mixing experiments performed with PN as a function of reagent concentration (with oxy-HbI and PN at a 1:1.2 molar ratio) the halftimes of mercaptide formation were observed to change roughly as expected for a second order reaction.

The kinetics of mercaptide formation suggested that PN gives rise to a different product depending on the ligation state of HbI during the cysteine modification reaction. On this basis, oxygen equilibrium experiments were performed in which the chromophoric mercurial was added to the protein either before or after deoxygenation. The oxygen affinity of the two products thus obtained is similar ($\log P_{1/2} \simeq 0.35$), whereas cooperativity differs significantly due to an alteration in both asymptotes of the binding curve. The Hill coefficient is 1.28 or 1.12, respectively, when the protein is modified in the oxygenated or deoxygenated state (Table I). In contrast to PN, but in line with the kinetic data of Fig. 1B, PMB upon reaction with oxy- or deoxy-HbI induces the same variation in oxygen affinity ($\log P_{1/2} \simeq -0.2$) and cooperativity ($n = 1.0$).

4. DISCUSSION

The present work describes the kinetic features of the cooperative binding of organomercurials to the *S. inaequivalvis* HbI homodimer and provides the estimate of the rate at which the corresponding conformational transition occurs. In addition it shows that binding of one specific organomercurial, PN, to Cys^{92} locks HbI into new cooperative quaternary structures characterized by distinct oxygen binding properties relative to the native protein.

The cooperative binding of organomercurials by the two symmetry-related Cys^{92} residues of the *S. inaequivalvis* homodimer was detected by Boffi et al. [5] by means of gel electrophoresis experiments on oxy-HbI

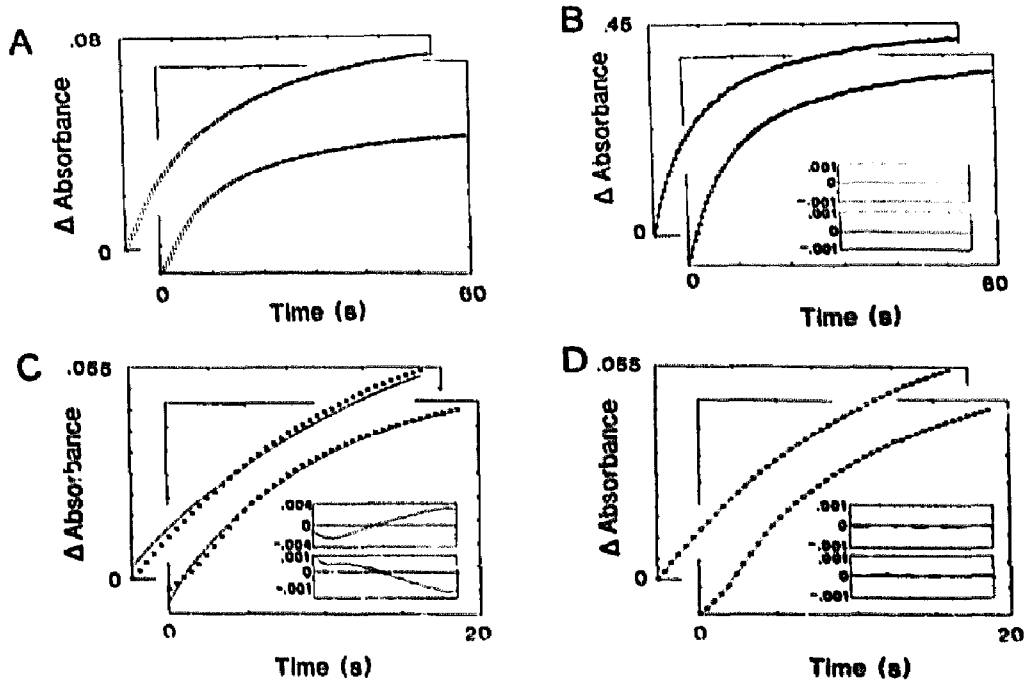

Fig. 1. Time course of the reaction of oxy- and deoxy-Hbl with PN (A,C,D) and PMB (B). In each panel and inset the top trace refers to deoxy- and the bottom trace to oxy-Hbl. Experiments were performed with a Hi-Tech apparatus on solutions of Hbl and mercurial at the same concentration (34 μM for the PN reactions and 30 μM for the PMB reactions, after mixing), in 0.1 M Tris-HCl buffer, pH 7.5, at 20°C. Observation wavelength: 470 nm for PN and 255 nm for PMB. The lines in (B) and (D) were calculated using the rate constants given in Table II. The lines in (C) using only steps (1) and (4) of the scheme given in Section 2 with k_1 = 2,350 $M^{-1}\cdot s^{-1}$ and k_4 = 9,900 $M^{-1}\cdot s^{-1}$ for oxy-Hbl and k_1 = 2,950 $M^{-1}\cdot s^{-1}$ and k_4 = 7,000 $M^{-1}\cdot s^{-1}$ for deoxy-Hbl.

reacted with PMB. The study of the kinetics of Cys^{92} modification by PN and PMB has confirmed and extended these observations. The kinetic behavior of the two mercurials differs strikingly in particular with respect to the capacity to monitor the oxygen-linked change in the Cys^{92} microenvironment revealed by the X-ray data [2,3]. Thus, the progress curve describing the reaction of Cys^{92} with PMB (Fig. 1B) is similar in oxy- and deoxy-Hbl. In contrast, when PN is used, the time course of Cys^{92} modification and the signal amplitude depend markedly on the state of oxygenation of the protein (Fig. 1A). The smaller amplitude observed in oxy-Hbl indicates that in this derivative the nitrophenol group undergoes a smaller change in ionization upon mercaptide formation. In turn this observation is in accordance with the existence of Cys^{92} in a hydrophobic patch in CO-liganded Hbl and a hydrophilic environment in the deoxygenated protein [2,3]; the CO liganded structure is practically identical to the oxygenated one (Royer, personal communication).

Table I

Oxygen binding properties of Hbl reacted with organomercurials

	n	log $P_{1/2}$	No. of exps.
Native Hbl	2.47 ± 0.02	0.84 ± 0.04	3
PN-Hbl (reacted as oxy)	1.28 ± 0.09	0.34 ± 0.04	4
PN-Hbl (reacted as deoxy)	1.12 ± 0.05	0.37 ± 0.04	4
PMB-Hbl (reacted as oxy)	1.00	−0.21	1
PMB-Hbl (reacted as deoxy)	1.00	−0.25	1

Experimental conditions: temperature, 20°C; 0.1 M Tris-HCl buffer at pH 7.5.

The time course of mercaptide formation provides the kinetic evidence for cooperativity in mercurial binding. The fit presented in Fig. 1C shows that a simple scheme consisting of two consecutive irreversible reactions in which the second mercurial molecule binds faster than the first one (steps (1) and (4) of the scheme given in Section 2) does not describe the data satisfactorily. On this basis, additional steps have been introduced: step (2) which corresponds to the conformational transition reflected in the cracking of the Hbl crystals upon mercurial binding, and step (3) which describes the binding of mercurial to the fast reacting species, PL*, generated in step (2). Table II. lists the values of the kinetic constants obtained for the binding of PN and PMB to oxy- and deoxy-Hbl: representative

fits are shown in Fig. 1D,B. Table II brings out that the second mercurial molecule binds roughly tenfold faster than the first one as indicated by the ratio k_3/k_1. This ratio is slightly higher in the case of PN and does not show a significant dependence on the state of oxygenation of the protein. The value of k_4 appears to be significantly lower than k_1 in all derivatives. The corresponding process (step 4) contributes to the observed time-course differently depending on the relative populations of PL and PL*. Thus, when the relative concentration of PL* is high, as for oxy-HbI reacting with PN, the contribution of step (4) is nog significant and the time-course is characterized by a distinct lag phase. In the other reactions studied, where both PL and PL* are populated, the contribution of step (4) is significant and manifests itself in the disappearance of the lag phase.

Concerning the parameters of the reversible organomercurial-induced conformational transition, the ratio k_2/k_{-2} determines the concentration of PL* and in turn influences the sigmoidicity of the progress curve. Indeed, binding of PN to oxy-HbI is characterized by the highest value of the k_2/k_{-2} ratio.

The occurrence of the organomercurial-induced conformational change implies information transfer between distant, symmetrically related regions of the subunit interface; this communication in turn is rendered possible by the multiplicity of interactions across the interface and by the relative rigidity of the E and F helices apparent in the X-ray structures [2,3]. The organomercurial-induced transition takes place in seconds, as indicated by k_2, and thus is several orders of magnitude slower than the R-to-T transition of hemoglobins which occurs on a microsecond time scale [9]. The difference in rate of these two processes may reflect in part the distance of the residues involved (in HbI the heme groups are practically in direct contact, whereas the Cys^{92} residues are separated by some 25 Å) and in part the type of structural rearrangements brought about the oxygen and mercurial binding. Whereas oxygen binding entails rather subtle movements of one subunit relative to the other [2,3], the presence of a bulky residue at the subunit interface may lead to major alterations at the interface.

The structural rearrangement induced by reaction of HbI with organomercurials (e.g. PMB and phenylmercuric acetate, PMA) usually results in the loss of cooperativity in oxygen binding [5]. In this light, the capacity of PN to stabilize new, cooperative states of the protein is really unique. The PN–HbI complexes are characterized by distinct oxygen binding properties, in particular by a different degree of cooperativity, depending on whether modification has been carried out in the oxy- or deoxy-state (Table I). The unique effect of Cys^{92}-bound PN, as compared to bound PMB and PMA, may be ascribed to the presence of a negative charge on its aromatic ring ortho to the mercaptide bond. It may be envisaged that Cys^{92}-bound PN locks the protein into new cooperative structures by means of hydrogen bonding interactions with amino acid side chain(s) on the neighboring subunit. Such interactions differ in detail when the mercaptide bond is formed in the oxygenated or deoxygenated protein due to the ligand-linked changes in the Cys^{92}. The most likely candidate for the interaction with the negatively charged Cys^{92}-bound PN is Arg^{67} whose amino groups are the only positively charged residues located within 5 Å from the sulfur atom. Moreover, they may form distinct hydrogen bonds with Cys^{92}-bound PN in the liganded and deoxygenated protein since they move about 0.5 Å away from the cysteine sulfur in the conformational transition that accompanies ligand binding [2,3].

In conclusion, the *S. inaequivalvis* HbI homodimer displays cooperativity in the reaction with non-heme ligands in addition to cooperative oxygen binding. Whereas the heme groups are in direct communication across the subunit interface, the former phenomenon, as exemplified by organomercurial binding, implies efficient information transfer between distant regions of the interface. The introduction of the bulky organomercurials at the interface alters the arrangement of the subunits in slightly different ways depending on the presence and position of a charge on the aromatic ring. The modified proteins thus obtained are usually devoid of cooperativity in oxygen binding. However, if the organomercurial is charged and capable of establishing new appropriate interactions across the interface, like PN, *new cooperative* quaternary structures of HbI can be stabilized that are characterized by distinct oxygen

Table II

Rate constants for the binding of organomercurials to HbI

	No. of exps.	k_1 ($M^{-1}\cdot s^{-1}$)	k_2 (s^{-1})	k_{-2} (s^{-1})	k_3 ($M^{-1}\cdot s^{-1}$)	k_4 ($M^{-1}\cdot s^{-1}$)
oxy-PN	9	5,500 ± 1,600	0.74 ± 0.16	0.018 ± 0.004	53,000 ± 17,000	750 ± 100
deoxy-PN	3	4,670 ± 950	0.50 ± 0.20	0.33 ± 0.03	56,000 ± 12,000	970 ± 110
oxy-PMB	1	11,000	0.25	0.04	80,000	1,500
deoxy-PMB	1	13,000	0.17	0.03	75,000	1,900

Values obtained from the data fitting procedure described in Section 2. Experimental conditions: temperature, 20°C; 0.1 M Tris-HCl buffer at pH 7.5.

binding properties relative to the native protein. The latter situation is reminiscent of a human hemoglobin mutant, Hb Ypsilanti [11], in which a new quaternary structure is stabilized by the interactions due to the mutated amino acid at the $\alpha 1$–$\beta 2$ interface.

Acknowledgements: This work was carried out by G.C. as partial fulfillment of the Ph.D. thesis. We wish to acknowledge grants 40% and 60% from MURST and a CNR grant from the Special Project 'Biotechnology Products for the Control of Cellular Communication' to E.C.

REFERENCES

[1] Chiancone, E., Vecchini, P., Verzili, D., Ascoli, F. and Antonini, E. (1981) J. Mol. Biol. 152, 577–592.
[2] Royer Jr., W.E., Hendrickson, W.A. and Chiancone, E. (1989) J. Biol. Chem. 264, 21052–21061.
[3] Royer Jr., W.E., Hendrickson, W.A. and Chiancone, E. (1990) Science 249, 518–521.
[4] Petruzzelli, R., Goffredo, B.M., Barra, D., Bossa, F., Boffi, A., Verzili, D., Ascoli, F. and Chiancone, E. (1985) FEBS Lett. 184, 328–332.
[5] Boffi, A., Gattoni, M., Santucci, R., Vecchini, P., Ascoli, F. and Chiancone, E. (1987) Biochem. J. 241, 499–504.
[6] McMurray, C.H. and Trentham, D.R. (1969) Biochem. J. 115, 913–921.
[7] Boyer, P.D. (1954) J. Am. Chem. Soc. 76, 4331–4337.
[8] Davis, B.J. (1964) Ann. NY Acad. Sci. 121, 404–427.
[9] Antonini, E., Ascoli, F., Brunori, M., Chiancone, E., Verzili, D., Morris, R.J. and Gibson, Q.H. (1984) J. Biol. Chem. 259, 6730–6738.
[10] Gill, S., Doyle, M.L. and Simmons, J.H. (1989) Biochem. Biophys. Res. Commun. 165, 226–233.
[11] Smith, F.R., Lattman, E.E. and Carter Jr., C.W. (1991) Proteins: Structure, Function and Genetics 10, 81–89.