Structural and functional effects of selective chemical modifications of *Scapharca inaequivalvis* haemoglobins in relation to their unique assembly

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The structural and functional roles of lysyl and thiol groups in the dimeric (HbI) and tetrameric (HbII) haemoglobins from the mollusc *Scapharca inaequivalvis* have been assessed. In these haemoglobins a unique mode of assembly (the haem-carrying E and F helices form the intersubunit contact of the dimeric unit) is associated with co-operative oxygen binding. Extensive acylation is accompanied by significant haem oxidation. Modification of one or two lysyl residues per chain (corresponding to ~ 20% of the total residues) does not affect the structural and functional properties of both haemoglobins, in line with the proposal that the intersubunit contacts are rich in hydrophobic residues. The modification of the thiol groups does not influence the state of association in both HbI and HbII, despite the location of the cysteine residue common to all polypeptide chains in the vicinity of the major intersubunit contact. The effect on the functional properties depends on the size of the thiol reagent: *p*-chloromercuribenzoate and phenylmercuric acetate increase the oxygen affinity about 20-fold, but iodoacetamide and mercuric chloride have no effect. Moreover, electrophoresis experiments indicate that *p*-chloromercuribenzoate is bound in a co-operative fashion, the degree of co-operativity being much higher in the dimeric HbI. Thus, only in HbII are intermediates containing substoichiometric amounts of *p*-chloromercuribenzoate formed in significant amounts. Their oxygen binding properties show that reaction of only one thiol group/tetramer suffices to alter the oxygen affinity of the molecule.

INTRODUCTION

Arcid molluscs (e.g. *Scapharca inaequivalvis*, *Anadara broughtonii*, *Anadara trapezia*, *Anadara satowi*) contain in their red cells two haemoglobin components which are characterized by very similar structural and functional properties (Chiancone *et al.*, 1981; Furuta *et al.*, 1977; Como & Thompson, 1980a; Ohnoki *et al.*, 1973).

HbII is a tetramer constructed from two pairs of unlike chains, and HbI is a dimer made from a third chain type (Chiancone *et al.*, 1981; Furuta *et al.*, 1977; Como & Thompson, 1980a). The polypeptide chains have the typical myoglobin fold with an additional helical segment at the N-terminus, the pre-A helix, but are assembled in a unique way. Thus, the haem-carrying E and F helices are not on the surface of the molecule as in vertebrate haemoglobins, but form the intersubunit contact in the dimeric HbI; HbII is a dimer of heterodimers with one dimer related to the other by interaction between the A helices and the non-helical AB and GH corners (Royer *et al.*, 1985). The importance of the extensive contact between the E and F helices in the dimeric building block is brought out by the invariance of this part of the amino acid sequence in all known arcid haemoglobins. Moreover, in this region of the molecule, arcid haemoglobins differ from the vertebrate haemo-

globin and myoglobin chains owing to the presence of several additional hydrophobic residues which form a characteristic hydrophobicity pattern (Petruzzelli *et al.*, 1985). Accordingly, the stability of the basic dimeric unit of arcid haemoglobins towards dissociation is not affected by pH in the range 5–9 and by the presence of NaCl at high concentration (Gattoni *et al.*, 1983).

From a functional viewpoint the dimeric component shows co-operativity in oxygen binding ($n = 1.5$) and a constant oxygen affinity ($p_1 = 7.8$ mmHg) over the pH range 5.5–9. HbII likewise shows co-operativity in oxygen binding ($n = 2$); its oxygen affinity at neutral and alkaline pH values is slightly lower ($p_1 = 9.2$ mmHg) than that of the dimeric protein, but becomes higher at pH values below 6.5 owing to the presence of an acid Bohr effect (Chiancone *et al.*, 1981).

The uniqueness of assembly and hence of the molecular basis of co-operativity in arcid haemoglobins, warrants the search for amino acid residues which play key structural and/or functional roles in these haemoglobins. The present paper reports a study of the reactivity of thiol and ϵ -amino groups with various reagents and of the influence of these chemical modifications on the assembly and functional properties of the haemoglobins from *S. inaequivalvis*.

Abbreviations used: HbA, human haemoglobin; IAA, iodoacetamide; Mb, myoglobin; NEM, *N*-ethylmaleimide; PMA, phenylmercuric acetate; PMB, *p*-chloromercuribenzoate.

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MATERIALS AND METHODS

Haemoglobin solutions of HbI and HbII were prepared from the red cells of the mollusc *S. inaequalis* haemolysed with distilled water. The two haemoglobin components were separated as reported previously (Chiancone *et al.*, 1981). Human haemoglobin (HbA) has been prepared by the method of Rossi Fanelli *et al.* (1961). Sperm whale myoglobin (Mb) was purchased from Sigma Chemical Co. Haemoglobin concentrations (in haem) were determined at 578 nm assuming $\epsilon = 14300 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for HbI and $\epsilon = 14200 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for HbII in the oxygenated form. Oxygen equilibria were measured at 20 °C by the method of Rossi Fanelli & Antonini (1958). Absorbance measurements were carried out with a Cary 219 spectrophotometer.

The acetylation reaction was carried out on the oxy- or carbomonoxy-derivatives at 4 °C by the method of Riordan & Vallee (1964). Acetic anhydride was added with a microsyringe to $\sim 5 \times 10^{-4} \text{ M}$ -haemoglobin solutions, in 0.1 M-NaHCO₃, under stirring. The pH was maintained constant at 8.5 by addition of 0.1 M-NaOH; the solutions were then dialysed against 0.1 M-phosphate buffer, pH 7. The succinylation reaction was carried out in the same manner and succinic anhydride (solid) was added in small amounts. The total amounts of acetic or succinic anhydride added varied from 100 to 600 mg/g of protein (molar ratio 15 to 90/protein monomer). Titration of free amino groups was performed with trinitrobenzenesulphonate (TNBS) by the method of Habeeb (1966). The molar absorption coefficient of trinitrophenylated amino groups determined for human haemoglobin ($\epsilon = 10800 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 335 nm) was used also for HbI and HbII.

The reactions of thiol groups with *p*-chloromercuribenzoate (PMB), phenylmercuric acetate (PMA) and HgCl₂ were carried out at 4 °C in 0.1 M-phosphate buffer, pH 7, by the procedures of Stefanini *et al.* (1972); the reaction with *N*-ethylmaleimide (NEM) and iodoacetamide (IAA) was carried out as described by Means & Feeney (1971). In order to assess the completeness of the reaction with the various reagents, unreacted thiol groups were titrated with PMB (Boyer, 1954).

Electrophoresis experiments were performed on starch gels (Poulik, 1957). Sedimentation velocity experiments were carried out on a Spinco model E ultracentrifuge at 48000 or 56000 rev./min and at 20 °C. Deoxygenation was obtained by addition of a few grains of sodium dithionite to the haemoglobin solutions. Sedimentation coefficients were determined from the maximum ordinate of the schlieren peaks and were reduced to s_{20} by standard procedures.

Differential gel filtration experiments were performed as described by Chiancone *et al.* (1981). A column (50 cm $\times$ 0.8 cm) of Bio-Gel P-100 (Bio-Rad Laboratories) or Sephadex G-75 (Pharmacia) was used at a constant flow rate of 10 ml/h and a temperature of 7–10 °C.

RESULTS

Modification of amino groups

The acetylation and succinylation reactions were carried out on HbI, HbII and on HbA as a control; depending on the conditions used they yielded modified proteins with a percentage of blocked amino groups (lysyl residues and *N*-terminus) which varied from 7% to

55% (one to eight amino groups modified per polypeptide chain). It may be recalled that the *N*-terminus is proline in HbI and is acetylated in both polypeptide chains of HbII (Como & Thompson, 1980a,b; Furuta *et al.*, 1977; Fisher *et al.*, 1984; Petruzzelli *et al.*, 1985). As shown in Table 1, there is always a significant oxidation of the haem iron when the degree of acylation is higher than 20%. The two molluscan haemoglobins appear to be more susceptible to oxidation than is HbA.

Physicochemical characterization of acylated proteins.

Upon electrophoresis the modified molluscan proteins move as bands of variable heterogeneity and with a higher anodic mobility than those of the native proteins. Acetylated derivatives always give more homogeneous bands than the succinylated ones. Moreover, heterogeneity decreases with increasing degree of acylation (Bucci *et al.*, 1963). In particular, 30% acetylation suffices to yield relatively homogeneous preparations of HbII, HbA and Mb, but not of HbI, in agreement with the higher number of lysyl residues of the last protein (Fig. 1).

Table 1. Relationship between acylation of ϵ -amino groups and haem oxidation in the haemoglobins from *S. inaequalis* and in human haemoglobin

Protein	-NH ₂ blocked (%)	Hb oxidized (%)
Succinylation		
HbA	7	0
	30	5
	55	32
HbII	8	17
	13	12
	24	18
	30	60
	70	75
HbI	33	31
Acetylation		
HbII	17	0
	30	20
	34	50
HbI	16	0
	25	8

Fig. 1. Electrophoretic pattern of normal and acetylated *S. inaequalis* haemoglobins

Starch gel electrophoresis was carried out with the discontinuous buffer system of Poulik (1957); degree of acetylation was 30% in all proteins. From left to right: HbI, acetylated HbI, HbII, acetylated HbII, HbA, acetylated HbA, Mb, acetylated Mb. Arrow indicates the position of the origin.

Sedimentation velocity analyses of the reacted proteins in the oxygenated form show that acylated HbI sediments as a single, fairly symmetrical peak of sedimentation velocity similar to that of the unreacted protein. In contrast, the schlieren patterns of both acetylated and succinylated HbII show two components, a major one whose s_{20} value (~ 4.0 S) does not differ significantly from that of unreacted HbII and a minor one of sedimentation velocity around 1.8 S. The relative amount of the slow moving component decreases upon addition of dithionite, suggesting that it may be ascribed to oxidized material. At variance with the native protein, which undergoes a reversible polymerization into an 8–10 S component (Chiancone *et al.*, 1981), acylated HbII does not polymerize after deoxygenation. Moreover, polymerization is substantially decreased in the deoxygenated deionized protein.

Functional characterization of acylated proteins. The oxygen equilibrium properties of acylated HbI, HbII and HbA were studied on those preparations which contained a maximum of 20% oxidized material (see Table 1). Under these conditions acylation does not affect the functional properties of either HbI or HbII, but increases the oxygen affinity and decreases co-operativity in oxygen binding by HbA (Fig. 2). In a sample of succinylated HbI where the oxidized material amounted to $\sim 30\%$ a slight increase in oxygen affinity and decrease in co-operativity were observed at pH 6.3.

Modification of thiol groups

Sequence analysis (Petruzzelli *et al.*, 1985; Furuta & Kajita, 1983; Como & Thompson, 1980*a,b*; Fisher *et al.*, 1984) has shown that the three constituent polypeptide chains of arcid haemoglobins possess a cysteine residue in the same position of the EF corner (92 in HbI, 93 and 94 in the so-called α and β chains of HbII, respectively); in addition, the α chain of HbII has another cysteine in position 9. All the thiol groups are titratable with PMB (Chiancone *et al.*, 1981; Furuta *et al.*, 1980). Differential

Fig. 3. Electrophoretic patterns of the tetrameric haemoglobin from *S. inaequalvis* after reaction with PMB

Starch gel electrophoresis was carried out with the discontinuous system of Poulik (1957). From left to right: 1, unreacted HbII, 2, HbII reacted with 1 mol of PMB/tetramer, 3, with 2 mol of PMB/tetramer, 4, with 4 mol of PMB/tetramer and 5, fully reacted HbII (6 mol of PMB/tetramer).

gel filtration experiments indicate that reaction with PMB does not induce dissociation into subunits at concentrations as low as 0.03 mg/ml, in agreement with the sedimentation velocity data obtained by Furuta *et al.* (1980) on *A. broughtonii* haemoglobins reacted with PMB.

Fig. 2. pH dependence of the oxygen affinity and co-operativity of chemically modified *S. inaequalvis* haemoglobins

Data were obtained in 0.1 M-phosphate buffer at 20 °C; protein concentration: 6×10^{-5} M. HbA: $\square$, native protein; $\bullet$, 8% and $\blacktriangle$, 30% succinylation. HbII: $\square$, native protein; $\circ$, 17% and $\triangle$, 30% acetylation; $\bullet$, 13% and $\blacktriangle$, 24% succinylation. HbI: $\square$, native protein; $\circ$, 16% and $\triangle$, 25% acetylation; $\blacktriangle$, 33% succinylation.

Fig. 4. Electrophoretic pattern of the dimeric haemoglobin from *S. inaequalis* after reaction with PMB

Starch gel electrophoresis was carried out with a discontinuous phosphate buffer system, pH 7. From left to right: 1, unreacted protein; 2, HbI reacted with 0.5 mol of PMB/dimer; 3, HbI reacted with 1 mol of PMB/dimer; 4, fully reacted HbII (2 mol of PMB/dimer).

As expected on the basis of these results, and given the irreversibility of the reaction of thiol groups with PMB, HbI and HbII fully reacted with PMB give rise to a single electrophoretic band (Figs. 3 and 4). Moreover, when HbI and HbII are treated with substoichiometric amounts of the reagent (i.e. less than 2 mol of PMB/dimer and 6 mol of PMB/tetramer), stable partially reacted species are formed. The behaviour of HbII is depicted in Fig. 3: in the presence of PMB at concentrations less than 4 mol/tetramer, five electrophoretic bands are present whose intensity depends on the amount of PMB. This pattern can be ascribed to the distribution of PMB among four thiol groups of similar reactivity; on such basis the five bands of lane 3 correspond to HbII reacted with 0, 1, 2, 3, 4 mol of PMB/tetramer. This interpretation, which assigns a similar reactivity to only four out of the six thiol groups of HbII, i.e. to the invariant cysteine residues, is supported by the presence of only one band in HbII reacted with 4 mol of PMB (lane 4); this band has a lower anodic mobility than the band of the fully reacted HbII.

Fig. 4 shows a similar experiment carried out on HbI. In the protein treated with substoichiometric amounts of PMB (lane 2), in addition to the bands corresponding to the unreacted and the fully reacted protein there is an intermediate band of very low intensity.

Functional characterization of proteins after reaction with PMB. Complete reaction of the thiol groups with PMB produces a dramatic increase in oxygen affinity and

Fig. 5. Oxygen equilibrium curves of *S. inaequalis* HbII after reaction with various amounts of PMB

Data were obtained in 0.1 M-phosphate buffer, pH 7.0, at 20 °C; protein concentration, 6×10^{-5} M: $\square$, native protein; $\circ$, $\bullet$, HbII reacted with 1 mol of PMB/tetramer; $\triangle$, equimolar mixture of unreacted and fully reacted HbII; $\blacksquare$, HbII reacted with 4 mol of PMB/tetramer; $\blacklozenge$, fully reacted HbII.

Fig. 6. Oxygen equilibrium curves of *S. inaequalis* HbI after reaction with various amounts of PMB

Data were obtained in 0.1 M-phosphate buffer, pH 7.0, at 20 °C; protein concentration, 6×10^{-5} M: $\square$, native protein; $\bullet$, HbI reacted with 0.66 mol of PMB/dimer; $\blacklozenge$, fully reacted HbI.

a loss of co-operativity in both HbI and HbII (Figs. 5, 6). A similar effect of PMB has been observed also in the haemoglobins from *A. broughtonii* (Furuta *et al.*, 1980).

Fig. 6 also shows that the Hill plots of HbI samples treated with 1 mol of PMB/tetramer are biphasic. These samples (see Fig. 3, lane 2) contain to a first approximation only two species in roughly equal

Table 2. Oxygen binding properties of the haemoglobins from *S. inaequalvis* reacted with thiol group reagents

0.1 M-Phosphate buffer, pH 7.0. Temperature: 20 °C.

Protein	Reagent	$\log p_{\frac{1}{2}}$	$n_{\frac{1}{2}}$
HbI	—	0.89	1.5
	PMB	-0.41	1.01
	PMA	0	1.1
	NEM	-0.15	1.4
	IAA	0.81	1.4
	HgCl ₂	0.86	1.2
HbII	—	0.96	2.0
	PMB	-0.35	1.02
	PMA	-0.39	1.1
	NEM	0.3	1.19
	IAA	0.92	1.66

amounts: unreacted haemoglobin and haemoglobin reacted with 1 mol of PMB/tetramer; the more extensively reacted species are present in barely discernible quantities. Very similar Hill plots are obtained with equimolar mixtures of unreacted and fully PMB-reacted HbII. This similarity allows one to assign the low affinity phase to unreacted haemoglobin and the high affinity one to the reacted protein, and in turn indicates that HbII containing 1 mol of PMB/tetramer has already acquired the functional properties of the fully reacted protein. In accordance with this finding, the oxygen equilibrium properties of HbII reacted with 4 mol of PMB/tetramer are indistinguishable from those of the protein containing 6 mol of PMB/tetramer.

HbI treated with substoichiometric amounts of PMB (0.6 mol of PMB/dimer) likewise displays a biphasic oxygen equilibrium curve (Fig. 6). This sample (see Fig. 4, lane 2) contains in roughly equal amounts unreacted HbI and HbI reacted with 2 mol of PMB/dimer; the latter species is responsible for the high affinity phase.

In a further series of experiments, the effect of other thiol reagents on the oxygen equilibrium properties of the two molluscan haemoglobins was tested. The results compiled in Table 2 show that PMA and NEM produce an increase in oxygen affinity as does PMB, whereas HgCl₂ and IAA have no effect.

DISCUSSION

The present results on selective chemical modifications of *S. inaequalvis* HbI and HbII provide important information on the nature of the intersubunit contacts in these unique haemoglobins. The characteristic hydrophobicity pattern at the level of the haem-carrying E and F helices, that form the major intersubunit contact, and the extreme stability of the dimeric component in concentrated NaCl and over the pH range 5–9, have led to the suggestion that salt bridges and/or hydrogen bonding play a less relevant role in the packing of the dimeric building block than is the case in vertebrate haemoglobins (Petrucelli *et al.*, 1985). As to the contact between two dimers in the tetramer, similar conclusions have been drawn on the basis of the stability of HbII towards dissociation into dimers in high salt concentrations (Gattoni *et al.*, 1983).

The absence of significant changes induced by acylation on the state of association and on the oxygen equilibrium of HbI and HbII confirms this picture. Thus, the behaviour of acylated HbI and HbII indicates that there are no reactive lysyl residues essential for the stabilization of the two types of intersubunit contacts and for the establishment of the functional properties specific to the two arcid haemoglobins. New information on the ligand-linked polymerization of HbII is provided by the sedimentation behaviour of the acylated protein. In native HbII removal of oxygen causes polymerization of the tetramer into higher-molecular-mass species. Moreover, the dependence of polymerization on temperature, pH, ionic strength and nature of the buffer indicates that the deoxy-polymers are stabilized by hydrophobic and electrostatic interactions and that the latter comprise the specific binding of buffer components (Chiancone *et al.*, 1981). The primary role played by the electrostatic contributions is confirmed by the finding that modification of 1–2 lysyl residues/polypeptide chain (which represents a modification of 10–25% of the lysine residues) suffices to abolish polymerization of deoxy-HbII. It may be envisaged that these reactive lysyl residues relevant to the self-association of deoxy-HbII form an 'acceptor pocket' for anions in the deoxygenated derivative, but not in the oxygenated one, and that in turn the bound anions favour the interaction between deoxygenated tetramers. In line with this picture, polymerization is substantially decreased in the deionized protein and does not occur if, before deoxygenation, HbII is blocked in a high affinity conformation by reaction with PMB (Furuta *et al.*, 1981).

Perhaps the most interesting observation of the present study concerns the effect which thiol reagents have on the structural and functional properties of *S. inaequalvis* haemoglobins. As mentioned above, arcid haemoglobin chains contain an invariant cysteine residue located in a critical position at the EF corner; in addition, the so-called α chain of the tetrameric component has another cysteine in position 9 (R. Petrucelli, personal communication). In accordance with the behaviour of the haemoglobins from *A. broughtonii* (Furuta *et al.*, 1980), in *S. inaequalvis* HbI and HbII the reaction of all thiol groups with PMB alters the functional properties dramatically: the oxygen affinity increases 20-fold owing to a change in both asymptotes of the binding curve, and co-operativity is abolished. The data compiled in Table 2 clearly show that these effects are not common to all thiol reagents. Their influence on the functional properties of *S. inaequalvis* HbI and HbII becomes progressively smaller as the size of the reagent decreases. It may be envisaged that the presence of bulky substituents at the EF corner alters the relative positions of the E and F helices, thereby changing the conformation of the molecule at the intersubunit contacts without affecting the state of association. As a result the reacted haemoglobins have a different quaternary structure with respect to the native proteins in both the liganded and unliganded state and the ligand-linked quaternary transition cannot take place. The observation that the crystals of carbomonoxy HbI and HbII crack upon reaction with PMB (W. E. Love & W. E. Royer, personal communication) is in accordance with this picture.

Within this general framework, *S. inaequalvis* HbI and HbII display several distinctive differences. Electro-

phoresis experiments show that in the homodimer HbI the two cysteines react with PMB in a fairly co-operative fashion, since upon treatment with substoichiometric quantities of the reagent very little of the singly reacted species is formed. In contrast, in the tetrameric HbII upon reaction of the four invariant cysteine residues with substoichiometric quantities of PMB significant amounts of intermediates are formed. This latter finding immediately suggests that HbII binds PMB with either no or little co-operativity. The existence of some co-operativity is clearly shown by the relative intensities of the electrophoretic bands of the intermediates containing 1, 2 or 3 mol of PMB/tetramer, which do not conform to those expected on the basis of a random binding reaction. Interestingly, in HbII the reaction of only one thiol group with PMB suffices to alter the oxygen affinity and co-operativity, and modification of the remaining thiols has no further effect. Another diversity in behaviour between the two haemoglobins, that may be related to small differences in the local environment of the invariant cysteine residues, concerns the effect of PMB and PMA. The two reagents, which differ by the presence of a carboxylate group, increase the oxygen affinity to the same degree in HbII, but not in HbI.

In conclusion, the data on the acylated proteins provide further evidence on the importance of hydrophobic interactions in the stabilization of the unique intersubunit contact of arid haemoglobins that involves the haem-carrying E and F helices. Moreover, the effects of the thiol reagents provide new information concerning the sensitivity of the functional properties to small structural alterations in the vicinity of these intersubunit contacts. Since such alterations are bound to be reflected directly in the position of the F helix, these data in turn strongly support the hypothesis of Royer *et al.* (1985), that in arid haemoglobins co-operativity may be triggered by the movement of the F helix upon binding of oxygen to one subunit as in vertebrate haemoglobins.

REFERENCES

- Boyer, P. D. (1954) *J. Am. Chem. Soc.* **76**, 4331–4337
- Bucci, E., Fronticelli, C., Bellelli, L., Antonini, E., Wyman, J. & Rossi Fanelli, A. (1963) *Arch. Biochem. Biophys.* **100**, 364–367
- Chiancone, E., Vecchini, P., Verzili, D., Ascoli, F. & Antonini, E. (1981) *J. Mol. Biol.* **152**, 577–592
- Como, P. F. & Thompson, E. O. P. (1980a) *Aust. J. Biol. Sci.* **33**, 643–652
- Como, P. F. & Thompson, E. O. P. (1980b) *Aust. J. Biol. Sci.* **33**, 653–664
- Fisher, W. K., Gilbert, A. T. & Thompson, O. P. (1984) *Aust. J. Biol. Sci.* **37**, 191–203
- Furuta, H. & Kajita, A. (1983) *Biochemistry* **22**, 917–922
- Furuta, H., Ohe, M. & Kajita, A. (1977) *J. Biochem. (Tokyo)* **82**, 1723–1730
- Furuta, H., Ohe, M. & Kajita, A. (1980) *Biochim. Biophys. Acta* **625**, 318–327
- Furuta, H., Ohe, M. & Kajita, A. (1981) *Biochim. Biophys. Acta* **668**, 448–455
- Gattoni, M., Verzili, D., Chiancone, E. & Antonini, E. (1983) *Biochim. Biophys. Acta* **743**, 180–185
- Habeeb, A. (1966) *Anal. Biochem.* **14**, 328–336
- Means, G. E. & Feeney, R. E. (1971) *Chemical Modification of Proteins*, pp. 217–220, Holden-Day Inc., San Francisco
- Ohnoki, S., Mitomi, Y., Hata, R. & Satake, K. (1973) *J. Biochem. (Tokyo)* **73**, 717–725
- Petrucelli, R., Goffredo, B. M., Barra, D., Bossa, F., Boffi, A., Verzili, D., Ascoli, F. & Chiancone, E. (1985) *FEBS Lett.* **184**, 328–332
- Poulik, M. D. (1957) *Nature (London)* **180**, 1477
- Riordan, J. F. & Vallee, B. L. (1964) *Biochemistry* **3**, 1768–1774
- Rossi Fanelli, A. & Antonini, E. (1958) *Arch. Biochem. Biophys.* **77**, 478–492
- Rossi Fanelli, A., Antonini, E. & Caputo, A. (1961) *J. Biol. Chem.* **236**, 397–400
- Royer, W. E., Jr., Love, W. E. & Fenderson, F. F. (1985) *Nature (London)* **316**, 277–280
- Stefanini, S., Chiancone, E., McMurray, C. H. & Antonini, E. (1972) *Arch. Biochem. Biophys.* **151**, 191–203