An α-Helical Domain in Hemoglobin Regulated by the Proximal Histidine†

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ABSTRACT: When iron is substituted by zinc in zinc–hemoglobin, the protein shows a tetrameric structure in which the helical content is much less than in iron–hemoglobin. The unfolding reveals the presence of a special α-helical domain regulated by the reciprocal interactions of the metal atom of the heme and the proximal histidine.

Removal of heme produces in hemoglobin a loss of tetrameric structure and of α-helical content. Both these characteristics can be restored upon recombination of apohemoglobin with proto-, meso-, deutero-, and hematoheme (Antonini et al., 1964; Fabry et al., 1968). Substitution in the various heme of iron with copper and manganese does not modify these results (Fabry et al., 1968). Iron-free protoporphyrin IX can also restore the tetrameric structure and the helical content in apohemoglobin (Fabry et al., 1968; Sebban et al., 1980).

In view of these data, it was concluded that the metal in the heme has no influence on the structure of the protein.

It may be noted that in all of the compounds investigated the conformation of the heme is essentially planar. The proximal histidine is at liberty either of establishing a coordination bond with the metal or of forming weak interactions with the inner nitrogen atoms of the metal-free porphyrin.

We felt that it was of interest to investigate what is the effect, on the conformation of hemoglobin, of a nonplanar conformation of the heme. In deoxyhemoglobin where the heme is not planar, the consequent repositioning of the heme and of the proximal histidine is accompanied by modifications of the tertiary structure of hemoglobin subunits (Baldwin & Chotia, 1979), which result in a stronger tetrameric structure of the protein (Kellett, 1971) and represent the R to T conformational transition of the system. If the interaction of the proximal histidine and the heme plays a central role in determining these conformational changes, large modifications of their contacts should result in a new structural attitude of the system.

The tetrahedral geometry of the ligand distribution around zinc makes the structure of Zn-heme[1] nonplanar (Falk, 1964). The metal may be able of a fifth coordination bond; however, according to Falk & Phillips (1964) when a fifth ligand is forced on the atom, it loses one of the coordination bonds with the pyrrole nitrogens of the porphyrin. Also, the fifth coordination bond would be strongly displaced from the normal to the plane of the porphyrin ring.

Crystallographic analyses reported by Hoard (1975) of pyridylzinc tetrapyridylporphyrin and of pyridylzinc octaethylporphyrin confirm the out of the plane position of Zn and the distortions produced by the metal on the conformation of the porphyrin. The pyrrole nitrogens are alternatively tilted up and down (ruffled), and the fifth coordination bond with pyridine is weak and tilted in the octaethyl derivative. Apparently Zn can form a strong bond with a molecule of water in the fifth position.

Zn-heme recombines with apohemoglobin (Leonard et al., 1974). When this occurs, the proximal histidine either does not form a bond with the metal and is displaced by the volume that it occupies or forms a bond and is displaced by the special geometry of the bond itself. On the basis of the analyses reported by Hoard (1975), Dr. M. F. Perutz suggests the possibility that in Zn–Hb a water molecule remains coordinated to the zinc on the side where it protrudes from the heme, adding to the distortion of the proximal histidine.

In any case a reciprocal repositioning of the heme and the proximal histidine is very likely to occur in Zn–Hb.

The effect of Zn-heme on the structure of hemoglobin is dramatic, the protein remains tetrameric; however, the α-helical content is drastically reduced, suggesting the presence of an α-helical domain regulated by the reciprocal positioning of the heme and the proximal histidine.

Materials and Methods

Apohemoglobin was prepared from human hemoglobin by the method of Teale (1959). Its concentration was determined spectrophotometrically by using $E = 0.85$ mL mg^{-1} cm^{-1} at 280 nm in 0.1 M NaOH.

Apohemoglobin in 0.2 M phosphate buffer at pH 7.0 was recombined with 1.5 stoichiometric equivalents of Zn-heme dissolved in 0.01 M NaOH. The mixture was left overnight in the cold, and then it was filtered through Sephadex G-25 equilibrated with 0.01 M phosphate buffer at pH 6.4 and absorbed on a CM-cellulose column equilibrated with the same buffer. A gradient elution was performed with equal volumes on 0.01 M phosphate buffer at pH 6.1 and 0.04 phosphate buffer at pH 7.5. The main fraction was collected and stored in the cold, in the dark.

The concentration of Zn–Hb was determined spectrophotometrically by using $\epsilon = 8.37 \times 10^{-3}$ M^{-1} cm^{-1} at 550 nm (Leonard et al., 1974). Sedimentation velocity was measured with a Beckman Model E ultracentrifuge by using the schlieren optics.

Spectropolarimetric measurements were performed with a JASCO-20 instrument at 15 °C. Evaluation of the relative amounts of secondary structure was performed by using the procedure suggested by Chen et al. (1974) as already reported (Fronticelli-Bucci & Bucci, 1975).

Zn-heme was obtained from Mid-Century, Inc. The product was obtained by crystallization and was chromatographically pure.

Spectrofluorometric measurements were performed with an SLM 4800 instrument.

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[1] Abbreviations: Zn–Hb, the product of recombination of apohemoglobin with zinc protoporphyrin IX; Zn-heme, zinc protoporphyrin IX; apoHb, heme-free hemoglobin; Tris, tris(hydroxymethyl)aminomethane.

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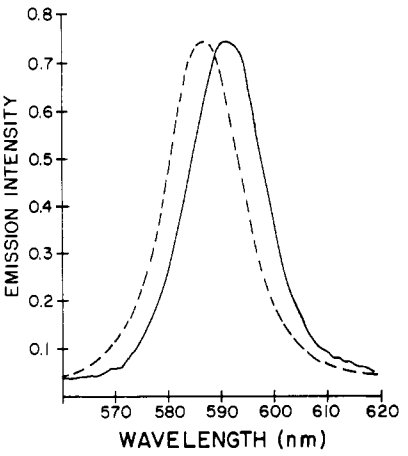

FIGURE 1: Normalized, noncorrected, emission spectra of (---) Zn-heme and (—) Zn–Hb, upon excitation at 425 nm in 0.05 M phosphate buffer at pH 7.2, at 4 °C. The shape of the spectra was independent of excitation wavelength.

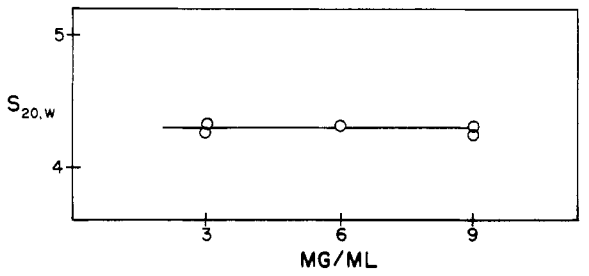

FIGURE 2: Concentration dependence of the sedimentation velocity of Zn–Hb in 0.05 M phosphate buffer at pH 7.0 at 15 °C.

Results

Figure 1 shows the fluorescence emission spectra of free Zn-heme and Zn–Hb. The hydrophobic environment of the heme shifts the emission maximum of Zn–Hb to a longer wavelength.

Figure 2 shows the sedimentation velocity of Zn–Hb at concentrations between 3 and 9 mg/mL in 0.05 M Tris buffer at pH 8.0. In all cases the schlieren diagram showed the presence of single symmetrical peaks. The sedimentation constant is independent of concentration. The value of $s_{20,w}$, near 4.2, is very similar to that of normal hemoglobin in similar conditions, indicating the presence of a tetrameric molecule.

Figure 3 shows the CD spectra of Zn–Hb, apohemoglobin, and normal (carbon monoxy)hemoglobin. Zn–Hb is the compound with the lowest optical activity at all wavelengths.

Figure 4 and Figure 5 show the computer simulations performed for estimating the relative amounts of secondary and nonordered structures in apohemoglobin and Zn–Hb. The simulations assume average lengths of five and eight amino acids for the helical segments. Longer lengths give essentially the same results as the eight-residue assumption. It appears that the best simulation for Zn–Hb is obtained by assuming an average length of five residues. The best simulation for apohemoglobin is obtained by assuming an average length of eight residues. In this case a five-residue assumption totally fails to give a simulation.

Table I reports the relative amounts of secondary structures obtained with the various simulations. It includes the values for (carbon monoxy)hemoglobin as routinely obtained in our laboratory.

Discussion

As already noted by Leonard et al. (1974) and Hoffman (1975) and confirmed by us, the spectrophotometric titration of apohemoglobin with Zn-heme has a stoichiometry of one

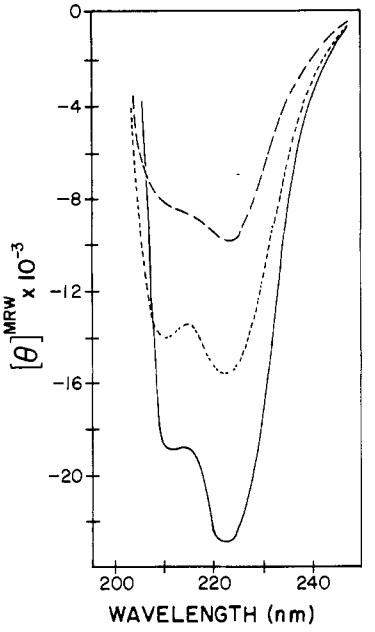

FIGURE 3: Circular dichroism spectra of (--) Zn–Hb, (···) apohemoglobin, and (—) (carbon monoxy)hemoglobin in 0.05 M phosphate buffer at pH 7.0 at 15 °C.

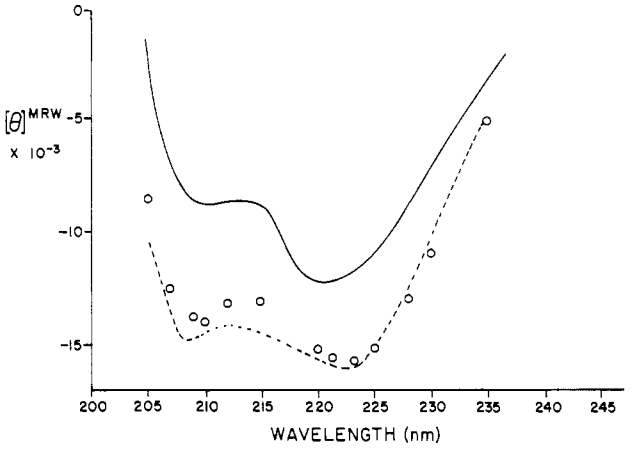

FIGURE 4: Analysis of the CD spectrum of apohemoglobin shown in Figure 3. Computer simulations were performed by assuming various lengths for the helical segments, and the parameters are listed in Table I. The open circles are experimental points. (—) 5 residues in length; (---) 8 residues in length.

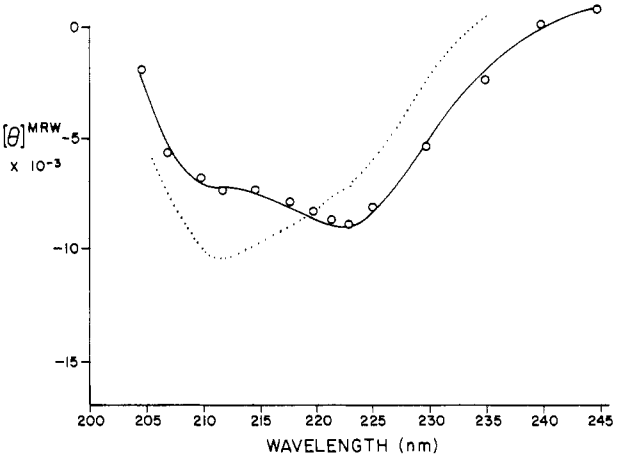

FIGURE 5: Analysis of the CD spectrum of Zn–Hb shown in Figure 3. Computer simulations were performed by assuming various lengths for the helical segments, and the parameters are listed in Table I. The open circles are experimental points. (—) 5 residues in length; (···) 8 residues in length.

Table I: Relative Amounts of Secondary Structure Estimated with the Procedure of Chen et al. (1974)

protein	av no. of residues[a]	% α helix	% β conformation	% nonordered structure
apoHb	8	58	0	42
Zn-Hb	5	55	4	41
Zn-Hb	8	28	30	42
HbCO	8	76	0	24

[a] Average number of residues in the helical segments.

heme per chain. Our samples of Zn–Hb are chromatographically pure, give single symmetrical peaks in the schlieren diagrams of the analytical ultracentrifuge, and show a value of $s_{20,w}$ near 4.2, practically independent of protein concentration. The fluorescence emission spectrum is sharp and very similar to that reported by Leonard et al. (1974). Is shows the expected shift toward longer wavelength in respect to that of free Zn-heme. All of these characteristics indicate that Zn–Hb is a homogeneous protein with size and shape very similar to those of normal tetrameric hemoglobin.

The tetrametric structure also indicates a complete saturation of apohemoglobin with Zn-heme. In fact partially saturated apohemoglobins are distinctly less than tetrameric and nonhomogeneous at least before the purification of the various intermediate of saturation (Cassoly, 1981).

The mere inspection of the CD spectra in Figure 3 immediately shows that the structure of Zn–Hb is very different from that of apohemoglobin and of (carbon monoxy)-hemoglobin; in fact, recombination with Zn-heme decreases the optical activity of apohemoglobin. A similar effect of Zn-heme on apomyoglobin was also reported (Andres & Atassi, 1970).

Figure 4 shows that for apohemoglobin the simulation based on an average length of eight residues of the helical segments is by far the most probable. For Zn–Hb the curve simulated assuming a length of eight residues does not fit accurately the data, and the values so obtained for the relative amounts of secondary structures imply a drastic conformational change, with the appearance of a substantial amount of β conformation. This seems to be hardly compatible with the persistence in Zn–Hb of a compact tetrameric structure. Instead the simulation based on five residues length corresponds very well to the data and results in more acceptable values for the secondary structures of the molecule. The simulation strongly suggests that in Zn–Hb a few helical segments are considerably shortened.

The interaction of Zn-heme with the proximal histidine is expected to produce a severe distortion of the position of that residue. This suggests that the F helix is one of the segments affected by the presence of zinc. However, the unfolding of the F helix alone is not enough to explain the loss of helical content of the system, and additional helical segments must be unfolded. The same reasoning applies to possible interactions of Zn-heme with the distal histidine of the E helix.

These findings clearly show that the conformation of hemoglobin subunits is very sensitive to the stereochemistry of the metal present in the heme, supporting the hypothesis based on crystallographic analyses (Perutz, 1970).

Whatever is the truth with regard to the conformation of Zn–Hb, it appears that the unfolding produced by the presence of Zn does not disturb the stability of the rest of the molecule, which remains compact and tetrameric. This implies that the unfolded portion does not contribute much to the free energy of folding of the whole system, so defining an independent domain with low conformational energy in the structure of hemoglobin.

It is interesting to note that the interaction of Zn with the proximal histidine does not disturb the tetrameric structure of Zn–Hb at concentrations of a few milligrams per milliliter, while at the same concentrations apohemoglobin is dimeric (Chu & Bucci, 1979). This suggests that the unfolding of the $\alpha_1\beta_2$ interface produced by removal of heme is due to the absence of the porphyrin ring and that a different structural domain is affected. This hypothesis is consistent with the observation that recombination of apohemoglobin with iron-free protoporphyrin IX reconstitutes the tetrameric structure of the system (Fabry et al., 1968). It appears that there is a detailed distribution of functions between the porphyrin ring and the metal atom of the heme in regulating the conformation of hemoglobin subunits.

A variety of structural domains are present in the hemoglobin system. An additional heme-dependent domain was identified by Franchi et al. (1982) in the isolated β subunits. Also, the librational motions demonstrated in hemoglobin and in the β subunits (Bucci et al., 1980; Oton et al., 1981) indicate the presence of domains in motion with respect to each other. These librational motions are regulated by the presence of ligands (Sassaroli et al., 1982), showing that they too are heme dependent.

More and more hemoglobin appears to be a system whose average conformation is a dynamic equilibrium of complex fluctuations involving several structural domains. The heme orchestrates these equilibria, consistent with the hypothesis already formulated (Sassaroli et al., 1982) that the conformational fluctuations of the system have allosteric significance.

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Mechanism for the Increase in Solubility of Deoxyhemoglobin S due to Cross-Linking the β Chains between Lysine-82β_1 and Lysine-82β_2†

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ABSTRACT: In a previous publication [Walder, J. A., Walder, R. Y., & Arnone, A. (1980) *J. Mol. Biol. 141*, 195–216], we showed that bis(3,5-dibromosalicyl) fumarate reacts selectively with oxyhemoglobin at the 2,3-diphosphoglycerate binding site to cross-link the β chains of the tetramer between Lys-82β_1 and Lys-82β_2. This chemical modification markedly increases the solubility of deoxyhemoglobin S and is therefore of potential clinical value in the management of sickle cell disease. By crystallographic analysis of the cross-linked derivative in the deoxy form, we showed that this modification causes Lys-82β and the neighboring β-chain residues (specifically Phe-85β and Leu-88β which form the acceptor site for Val-6 within the deoxyhemoglobin S fiber) to be pulled inward toward the central cavity of the tetramer. No alterations in the structure were observed in the region of the mutation site in hemoglobin S, residue 6β. These results suggested that there is a direct relationship between the magnitude of the perturbation of the acceptor site and the increase in the solubility of deoxyhemoglobin S which results from cross-linking the two lysine residues. To test this hypothesis, we have compared the structures of deoxyhemoglobin A cross-linked by bis(3,5-dibromosalicyl) succinate (C_4), bis(3,5-dibromosalicyl) glutarate (C_5), and bis(3,5-dibromosalicyl) adipate (C_6) and the solubilities of the corresponding derivatives of deoxyhemoglobin S. As the length of the bridging group is increased, there is a progressive decrease in the movement of Lys-82β toward the central cavity and in the accompanying perturbation of the acceptor site for Val-6. Correspondingly, the solubility of the C_4-cross-linked derivative is increased by the largest amount (nearly 50%), and as the cross-link is increased in length, the solubility decreases, approaching that of native deoxyhemoglobin S. To complete the analysis of the stereochemistry of the reaction pathway, we have determined the effect of the cross-link on the structure in the liganded quaternary state by using CO-β_4 as a model of normal liganded $\alpha_2\beta_2$ tetrameric hemoglobins. The results of these studies indicate that the fumaryl group is able to span the two lysine residues in oxyhemoglobin without perturbing the structure of the protein. The structural correlations arrived at in this work provide important constraints for the design of new antisickling compounds.

The molecular basis for sickle cell disease is a single point mutation within the hemoglobin molecule; glutamic acid at the sixth position of the β chains is replaced by valine. This mutation has relatively little effect on the functional properties of hemoglobin but does markedly reduce its solubility in the deoxy form (Perutz et al., 1951). Under physiological conditions, deoxyhemoglobin S initially precipitates from solution in the form of extended helical fibers. Within the erythrocyte, these fibers tend to align parallel to one another to form large aggregates which distort the morphology of the cell, giving rise to a variety of abnormal shapes, most characteristically the sickled form. These abnormally shaped cells are less deformable than the normal erythrocyte and are responsible for the vaso-occlusive complications of the disease.

Knowledge of the pathogenesis of sickle cell disease has led to many efforts to design reagents that would interfere with the polymerization of deoxyhemoglobin S either by covalently modifying the protein (Cerami & Manning, 1971; Roth et al., 1972; Benesch et al., 1974; Hassan et al., 1976; Lubin et al., 1975; Nigen & Manning, 1977) or by binding noncovalently to some specific site on the hemoglobin molecule (Schoenborn, 1976; Kubota & Yang, 1977; Ross & Subramanian, 1977; Noguchi & Schechter, 1977, 1978; Votano et al., 1977; Schechter, 1980; Poillon, 1980, 1982). The initial observation by Klotz & Tam (1973) that aspirin acetylates amino groups on hemoglobin provided the impetus for the investigation of a large number of related acylating agents as potential antisickling compounds (Zaugg et al., 1975, 1980; Walder et al., 1977, 1979; Wood et al., 1981). Recently, we described in detail the properties of a series of these derivatives which we have shown form a new class of affinity reagents that react with hemoglobin at the 2,3-diphosphoglycerate (DPG)[1] binding site (Walder et al., 1980). The prototype of these reagents, bis(3,5-dibromosalicyl) fumarate, reacts selectively with hemoglobin in the liganded quaternary state to cross-link the β chains between Lys-82β_1 and Lys-82β_2, spanning the DPG binding pocket (Scheme I). As a result of this modification, the solubility of deoxyhemoglobin S is increased by about 36%

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[1] Abbreviations: DPG, 2,3-diphosphoglycerate; $NaDodSO_4$, sodium dodecyl sulfate; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane.

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