Specialized Functional Domains in Hemoglobin: Dimensions in Solution of the Apohemoglobin Dimer Labeled with Fluorescein Iodoacetamide†

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ABSTRACT: The fluorescence characteristics of 8-anilinonaphthalene-1-sulfonic acid (ANS) coupled to apohemoglobin and to apohemoglobin labeled with fluorescein iodoacetamide (FIA) at β-93 have been compared. The quenching of emission of ANS produced by FIA was measured both with steady-state and with time-resolved techniques. In this system the emission of ANS in the β-heme pockets was totally quenched by FIA at β-93. Steady-state measurements indicated a 57% efficiency of energy transfer between ANS in the α-heme pockets and FIA at β-93. Time resolution showed that the initial (unquenched) lifetime of ANS was 18.2 ns. In the presence of FIA two new components were generated with lifetimes of 2.0 and 6.6 ns. Assuming a random orientation of the probes, the distances inferred from these measurements were near 4.6 and 3.6 nm for the time-resolved and near 48 Å for the steady-state measurements. In the tridimensional model of hemoglobin the distance between the iron atom of the α_1 chains and the SH group of the β_1 chains at position 93 is 3.6 nm in oxyhemoglobin and 4.1 nm in deoxyhemoglobin. To these distances 0.5–1.0 nm may be added to allow for the dimensions of the probes. Thus it appears that removal of the heme fails to produce any important enlargement of the molecule. On the contrary, the data suggest a slight shrinking of apohemoglobin, which may be consistent with a collapse of the heme pocket when heme is removed. The rest of the molecule does not seem to be greatly affected. These observations suggest that the modification of the secondary structure of hemoglobin upon removal of heme is an event that specifically regulates the conformation of the $\alpha_1\beta_2$ interface, which is broken in apohemoglobin. This gives further support to the hypothesis that in hemoglobin domains of secondary structures specifically store and transmit to functional interfaces information with regard to the state of the heme.

In a previous paper (Kowalczyk & Bucci, 1983) we have explored the size in solution of a single β subunit of apoHb, measuring the efficiency of energy transfer between pyridoxamine 5-phosphate coupled to the valine at β_1 and ANS combined with the heme pockets of apoHb.[1] The results indicated that in spite of the substantial loss of far-UV ellipticity, produced by the removal of heme in apoHb, the β subunits conserved the general shape and size that they have in crystalline hemoglobin.

This paper presents a continuation of the investigation, still based on fluorescence spectroscopy; however, this time a different pair of probes was used, namely, FIA and ANS, and rather than phase fluorometry, pulse fluorometry was used for measuring lifetimes. We meant to compare independent sets of data as a test for their validity; also, the geometry of labeling and the spectral characteristics of the new probes allowed us to explore the intersubunit distance between the β-93 cysteine and the α-heme pocket in the $\alpha_1\beta_1$ dimer of apoHb.

Assuming that the donor–acceptor pair had random reciprocal orientations, the results indicated that the distance in consideration was very similar to that measured in the tridimensional model of crystalline hemoglobin.

These results seem to confirm that removal of heme does not modify the general shape of the hemoglobin subunits, the only gross effect being the dimerization of the system.

Materials and Methods

Human hemoglobin was prepared as previously described (Oton et al., 1981) and purified following the procedure of Huisman & Dozy (1965). This procedure also eliminated organic phosphates from the hemoglobin solutions.

Labeling of the β-93 cysteine of (carbonmonoxy)hemoglobin with FIA was performed with a 2-fold excess of FIA over the β-93 residues in 0.1 M phosphate buffer, pH 7.0, for 60–90 min at 4 °C. The hemoglobin concentration was 2–5 g/dL. The labeled protein was purified by chromatography on CM-cellulose using a linear gradient formed by 0.01 M phosphate buffer at pH 6.0 and 0.03 M phosphate buffer at pH 7.5. The labeled protein was eluted before the residual nonlabeled hemoglobin and was electrophoretically pure on cellulose acetate.

Heme was removed according to the procedure of Teale (1959), and amino acid analyses were performed with a Beckman 120c amino acid analyzer. The yield of (carboxymethyl)cysteine indicated a stoichiometry of at least 0.9 mol of FIA/31 000 daltons. It should be stressed that, because of the formation of lactones, the yield of (carboxymethyl)-cysteine tends to be underestimated by this procedure (Bradbury & Smith, 1973).

The extinction coefficient of apoFIA at 495 nm was estimated from the absorption spectrum of the protein by measuring its concentration with the Lowry method using standards of apoHb and serum albumin (Lowry et al., 1951). On this basis the extinction coefficient was measured to be

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[1] Abbreviations: apoHb, heme-free hemoglobin; apoFIA, heme-free hemoglobin labeled with FIA; FIA, fluorescein iodoacetamide; ANS, 8-anilino-1-naphthalenesulfonate; apoANS, complex of heme-free hemoglobin with ANS; TNS, 6-*p*-toluidinylnaphthalene-2-sulfonic acid.

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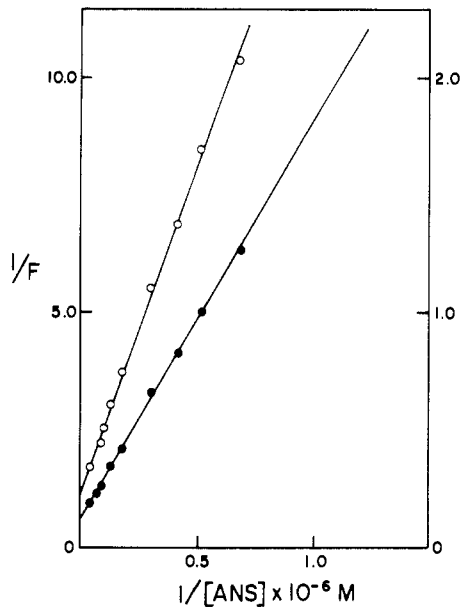

FIGURE 1: Double-reciprocal plots of titration of apoHb (○) and apoFIA (●) with ANS: protein concentration 0.5×10^{-6} M per subunit in 0.1 M phosphate buffer, pH 7.0, at 4 °C; excitation at 360 nm and emission at 450 nm. The ordinate axis on the right is for apoHb (○).

very near $\epsilon = 77\,000$ in 0.1 N NaOH at 495 nm. This compared very well with the extinction coefficient $\epsilon = 75\,000$ found by Cheung et al. (1982) at alkaline pH. It was taken as further evidence of the labeling of apohemoglobin with 1 mol of FIA per dimer (31 000 daltons).

Fluorescence lifetimes were measured with an ORTEC-9200 nanosecond fluorometer as previously described (Oton et al., 1981). A broad-band filter centered at 350 nm was used in excitation and an interference filter (Baird Atomic) centered at 460 nm with a bandwidth of 10 nm was used in the emission beam. In the absence of ANS the emission due to FIA was nondetectable. Deconvolutions of the fluorescence transients were performed with algorithms based either on the method of moments (Isenberg et al., 1973) or on the Marquardt procedure (Marquardt, 1963). The two methods gave practically the same results.

Steady-state fluorescence was measured with an SLM 8000 spectrofluorometer. The emission spectra were corrected by using standards of quinine and β-naphthol (Lippert et al., 1959).

Circular dichroism was measured on a JASCO-20 spectropolarimeter. Sedimentation velocity was performed on a Beckman Model E analytical ultracentrifuge equipped with schlieren optics.

Both FIA and ANS were purchased from Molecular Probes and were pure by thin-layer chromatography. All other reagents were analytical grade or better. Sodium phosphates were used to make the buffers.

Results

Binding of ANS by Apohemoglobin Free and Labeled with FIA. Solutions 0.5×10^{-6} M in subunits of apohemoglobin were titrated with ANS up to a concentration of 10^{-4} M. The fluorescence increase was monitored at 450 nm upon excitation at 350 nm and was corrected for the reduced transmission of the samples. Dissociation equilibrium constants were computed from double-reciprocal plots of fluorescence intensity against the concentration of ANS, as shown in Figure 1 (Klungsoyr, 1971). It was estimated that in all samples the amount of free ANS was at least 95% of the total.

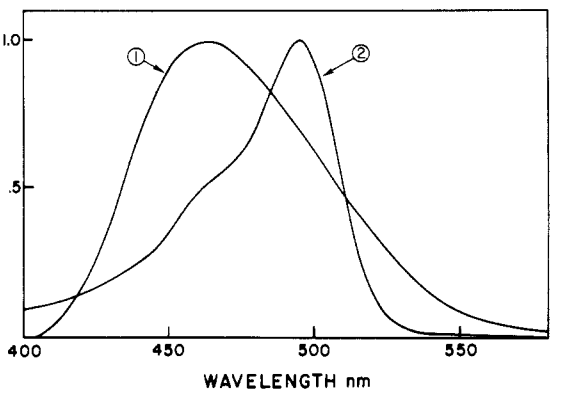

FIGURE 2: (1) Normalized corrected emission spectrum of apoHb–ANS complex upon excitation at 350 nm. (2) Normalized absorption spectrum of apoFIA. A 0.1 M phosphate buffer, pH 7.0, at 4 °C was used.

As shown in Figure 1, both in the presence and in the absence of the label, the plots were linear, indicating that the two binding sites were equivalent. The constants calculated in the presence and in the absence of the label were indistinguishable, with a value of $K = 1.15 \pm 0.5 \times 10^{-5}$ M. These data were in very good agreement with measurements reported by Stryer (1965). They indicated that preferential binding of ANS to either apohemoglobin subunit was nondetectable, both in the presence and in the absence of FIA.

Sedimentation Velocity and Circular Dichroism of ApoFIA. At concentrations near 3 mg/mL in 0.05 M phosphate buffer at pH 7.0 and temperatures between 16 and 18 °C, the sedimentation coefficient of apoFIA in both the presence and absence of ANS was near $s_{20,w} = 2.4$ S, indicating the presence of a dimeric system as in normal apohemoglobin (Chu & Bucci, 1979).

Under the same conditions, at 4 °C, the far-UV CD spectrum of apoFIA was indistinguishable from that of normal apoHb both in the presence and in the absence of ANS.

Quenching of ANS Fluorescence by FIA. Figure 2 shows the absorption spectrum of apoFIA and the corrected emission spectrum of the ANS–apohemoglobin complex. From these, the overlap integral J was computed (Fairclough & Cantor, 1978):

$$J = \frac{\int_0^\infty f(\lambda)\, \epsilon_A(\lambda)\, \lambda^4\, d\lambda}{\int_0^\infty f(\lambda)\, d\lambda}$$

where $f(\lambda)$ is the corrected emission spectrum of the donor in arbitrary units, $\epsilon_A(\lambda)$ is the molar extinction coefficient of the acceptor in M^{-1} cm^{-1}, and λ is the wavelength in nanometers. Assuming a value of 0.92 for the quantum yield of apoANS (Stryer, 1965) and following the procedure suggested by Fairclough & Cantor (1978), the distance R_0 at which 50% energy transfer occurs between the randomly oriented probes was $R_0 = 5.08 \pm 0.05$ nm.

Figure 3 shows the emission spectra of apoANS, apoFIA, and the complex of ANS with apoFIA. It shows that the quenching of fluorescence produced by FIA did not modify the shape of the emission spectrum of apoANS. Therefore, the quenching produced by the label was computed by integrating the emission of apoANS in the presence and absence of FIA between 420 and 450 nm. Excitation was at 350 nm.

In these experiments a 20-fold molar excess of ANS was added to apohemoglobin, with and without the label, at 0.5 μM concentrations in 0.1 M phosphate buffer, pH 7.0, at 4 °C. The absorbance of ANS at both excitation and emission

Table I: Lifetimes of ApoFIA-ANS Complex in 0.1 M Phosphate Buffer, pH 7.0, at 4 °C[a]

α_1	τ_1	y_1	α_2	τ_2	y_2	α_3	τ_3	y_3	τ_{av}[b]
0.51 ± 0.06	2.0 ± 0.10	0.82	0.33 ± 0.02	6.6 ± 1.0	0.16	0.14 ± 0.05	18.5 ± 2.0	0.02	5.79 3.82[c]

[a] ApoFIA is 10^{-5} M per subunit; ANS is 10^{-5} M. [b] $\tau_{av} = \Sigma_i \alpha_i \tau_i / \Sigma_i \alpha_i$. [c] Average of τ_1 and τ_2.

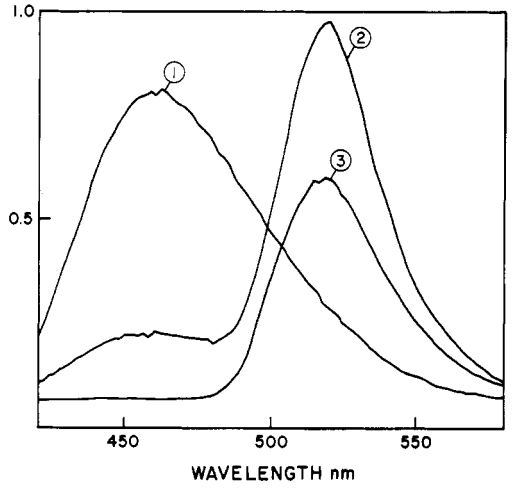

FIGURE 3: Recorder tracings of (1) emission spectrum of apoHb–ANS complex, (2) emission spectrum of apoFIA–ANS complex, and (3) emission spectrum of apoFIA. ApoHb and apoFIA are 0.5×10^{-6} M in subunits; ANS is 10^{-5} M; 0.1 M phosphate buffer, pH 7.0, at 4 °C. All spectra were normalized to that of the apoFIA–ANS complex.

wavelengths was less than 0.05 and was disregarded. In our samples, the presence of acceptor produced a 78% quenching of the emission of apoANS.

A value of $R_0 = 5.08$ nm implies that the distances for which the transfer efficiency varies from 95 to 5% are 3.1 and 8.3 nm, respectively. In the crystal of hemoglobin the distance between the SH group at β-93 and the iron of the heme in the same subunit is near 1.3 nm. Therefore, unless this distance nearly triples, the emission of ANS from the heme pockets of the β subunits should be totally quenched. Taking this into consideration, the transfer efficiency E between ANS in the α-heme pocket and FIA at β-93 was computed from

$$E = 1 - F/(0.5F_0)$$

where F and F_0 are the emission intensities of apoANS in the presence and absence of acceptor, respectively.

On this basis a transfer efficiency of 0.57 ± 0.03 was computed, which using

$$R = (1/E - 1)^{1/6} R_0$$

produced a distance $R = 4.82$ nm between apoANS in the α-heme pockets and FIA at β-93.

Quenching of Lifetime of ANS in the Presence of FIA. All of the experiments were conducted in 0.1 M phosphate buffer, pH 7.0, at 4 °C on at least three different preparations of apoHb and apoFIA. The decay of apoANS was essentially monoexponential with a value of $\tau = 18.2 \pm 0.2$ ns. Often a second component, approximately 2% of the total emission, was detected, with a lifetime of 1 ns or less. Because of its low amplitude, its presence was neglected.

Table I shows the lifetimes detectable in the fluorescence decay of ANS added to apoFIA. Simulations based on a single exponential failed to give satisfactory fits to the experimental data. Best fits were obtained by assuming three exponential components (Figure 4). The values of χ^2 were near 0.16, and the plot of the residuals and of the autocorrelation function

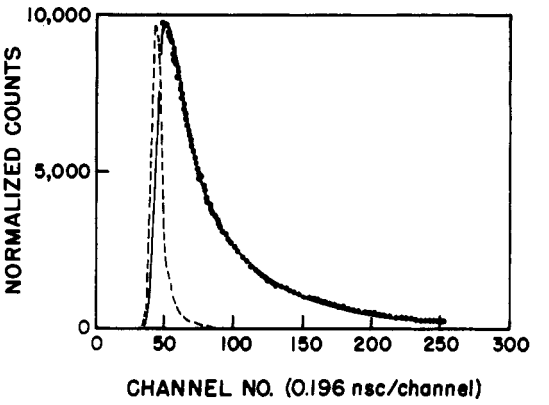

FIGURE 4: Normalized fluorescence decay of apoFIA–ANS complex in 0.1 M phosphate buffer, pH 7.0, at 4 °C: protein concentration 10^{-6} M per subunit; ANS 10^{-5} M. The dashed line is the lamp pulse. The continuous line is the fit to the data obtained with the Marquardt algorithm performing a three-component analysis. The lifetimes are reported in Table I.

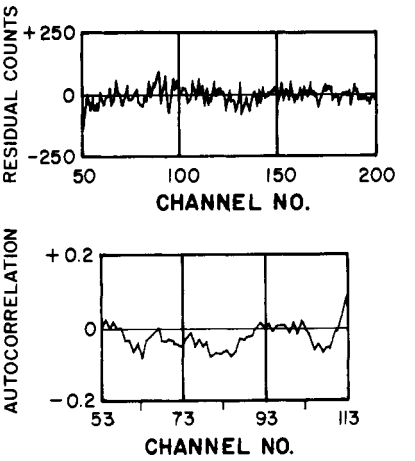

FIGURE 5: Residuals and autocorrelation function of the analysis shown in Figure 4.

Table II: Computed Distances[a]

approach	R_1	R_2	(R_3)	R_{av}
3-component simulation	3.58	4.63	(00)	4.47[b]
from av of τ_1 and τ_2				4.07[b]
decrease of emission intensity				4.83

[a] R_i are the distances in nanometers computed either from the lifetimes τ_i listed in Table I or from the quenching of the emission intensity. [b] From the values of τ_{av} in Table I.

indicated nearly perfect fits (Figure 5). Attempts to fit the data with four components failed because similar exponents were assigned to two of the four components.

In Table I are also listed the weight fractions y of the various components estimated by correcting their preexponentials (α_i) for the quenching of their lifetimes (τ_i) using the equation

$$y = \frac{\alpha_i \tau_0 / \tau_i}{\sum \alpha_i \tau_0 / \tau_i}$$

where τ_0 is the original lifetime of the donor in the absence of quencher.

Table II reports the distances computed following the procedure suggested by Fairclough & Cantor (1978) from the various lifetimes and their averages as reported in Table I.

Discussion

The presence of a short component in the lifetime of ANS and other substituted naphthalenes either bound to proteins or in water/organic solvent mixtures has been already noticed. Only in pure organic solvents does the fluorophore show a monoexponential lifetime decay (Robinson et al., 1978a,b). Also in our experiments in the absence of the acceptor, there was a short component. It is possible that it was the signal, poorly resolved numerically, of the relaxation of the environment around the excited state, as reported by Gafni et al. (1972) and Lakowicz & Cherek (1981) for apohemoglobin and TNS. It is also possible that the dye carried with itself, or found, water molecules inside the heme pockets, which could be responsible for the short lifetime component.

The small overlap between absorption and emission of apoHb-ANS and the distance of at least 3.6 nm between the heme pockets of apoHb exclude the possibility of a detectable self-quenching of ANS. Anderson et al. (1970) failed to notice ANS–ANS transfer in apoANS. As mentioned above, the ANS–FIA pair is useful for exploring transfer distances between 3.1 and 8.3 nm. Therefore, only the intersubunit distance between the α-heme pocket and FIA at β-93 was investigated in our experiments.

The presence of FIA at positions other than β-93 was improbable. In fact, the amino acid and spectrophotometric analyses of apoFIA were all consistent with a stoichiometry of 1 mol of FIA per $\alpha\beta$ dimer. In native hemoglobin, for which the labeling was performed, only the β-93 residue is readily available to alkylating agents (Antonini & Brunori, 1971). Also, in the alkylated samples, before purification, indications of overreaction were absent. On this basis we believe that, if additional positions were labeled in apoFIA, they had negligible relevance to the data here presented.

Inspection of Tables I and II reveals several phenomena. One is that probably our samples of apoFIA contained a small fraction of nonlabeled apoHb. This is probably the origin of the third decay component (τ_3), identical with that of nonquenched ANS. As shown in Table I the relative amount of nonlabeled apoHb was near 2%. It was probably produced by the manipulations necessary to remove the heme. The low amount made it unnoticeable in the various analyses, and its presence was revealed only when quenching increased its relative weight in emission. The presence of nonquenched fluorescence may result in overestimation of the R_{av} listed in Table II, especially that obtained from the emission intensity.

Another phenomenon apparent in Tables I and II is that the emission of ANS quenched by FIA was heterogeneous and produced at least 2 lifetimes. The shortest one (τ_1) gave a distance near 3.6 nm; the second quenched lifetime (τ_2) gave a distance near 4.6 nm. Thin-layer chromatography ruled out the presence of impurities in the reagents used for labeling. Also, the relative amounts of the two quenched components, as shown in Table I, are too large to be consistent with heterogeneity of labeling by FIA, which, as above discussed, was very improbable. This suggests the possibility that energy transfer may reflect the presence of two conformations of the protein, which relax into each other in times that are long relative to the detectable lifetimes.

In order to find a possible justification for this hypothesis, we have measured the distance between the iron of the α chains and the SH group at β-93 across the $\alpha_1\beta_1$ interface, in tridimensional models of oxy- and deoxyhemoglobin. We found a distance of 3.6 nm in oxyhemoglobin and of 4.1 nm in deoxyhemoglobin. The dimensions of ANS and FIA add 0.5–1.0 nm to these distances. In this way the expected distance between ANS and FIA is anywhere between 4.1 and 5.1 nm for an average of 4.6 nm.

The similarities of these distances to those listed in Table II are impressive and tantalizing. It is tempting to suggest that removal of heme may produce a new equilibrium between the oxy and deoxy conformations of the hemoglobin system. The loss of far-UV ellipticity of apohemoglobin, as compared to hemoglobin, makes this statement very speculative. Nevertheless, the correspondence indicates a general consistency of the measured distances with the conformational attitudes of the system, supporting the hypothesis that apohemoglobin exists in two main conformations slowly relaxing into one another.

Besides these details, which at present rest on speculative grounds, the most significant evidence emerging from the data of Table II is that the values of the R_{av}'s, which are probably overestimations, fail to show any major unfolding and expansion of the molecule of apohemoglobin. In fact, there is a suggestion that the molecule shrinks slightly.

The hypothesis of a random transfer of energy between ANS and FIA is basic to the estimation of the distances presented in Table II. This is supported by the observation that the label in apoFIA freely rotates in a cone with a semiangle near 30°. This evidence comes from measurements of the time decay of anisotropy to be reported elsewhere. The same experiments showed that the limiting anisotropy of apoANS was low, near 0.2, probably due to the broad-band excitation filter used, as here described for the lifetime measurements. The limiting anisotropy of FIA was near 0.35. According to Haas et al. (1978) this anisotropy of the probes would produce an uncertainty of about $\pm 10\%$ in the distances estimated assuming a random transfer. Even if a full 10% were added to the distances reported in Table II, an expansion of the molecule can be excluded. On the basis of this probable error it also appears that the difference of 1.0 nm between R_1 and R_2 in Table II is too large (about 30% of the reported values) to be explained by an isomerism of the position of the probes on the polypeptide chain, in the absence of a conformational isomerism of the protein.

Most importantly, the data here reported are consistent with the results obtained with pyridoxal 5-phosphate and ANS as donor–acceptor pairs, in the same system (Kowalczyk & Bucci, 1983). In our opinion, this consistency provides the best support for the assumption of a quasi-randomness of the energy transfer in both sets of data. It is based on results, not speculation, and it is very improbable that a combination of errors would give such uniformity of data.

Also, the value reported for the frictional coefficient of apohemoglobin, which is very similar to that of hemoglobin (Rossi-Fanelli et al., 1958), and the correspondence between its sedimentation velocity and sedimentation equilibrium characteristics, as reported by Chu & Bucci (1979), are consistent with a compact globular form of apohemoglobin.

The persistence of the tertiary structure of hemoglobin in the heme-free system may appear inconsistent with the large loss of ellipticity in the far-UV region of the spectrum, indicative of changes in the secondary structure (Bucci & Kowalczyk, 1982). We have already discussed the possibility that a loss of far-UV ellipticity may not be linearly proportional to the α-helical content and that distortion rather than melting of helices may be in part responsible for the decreased ellipticity (Kowalczyk & Bucci, 1983).

Whatever the quantity of the helical loss is, our data indicate that removal of heme produces a modification of the secondary structure of the hemoglobin system, which has little effect on the general shape of the hemoglobin subunits and of the $\alpha\beta$ dimers. This modification of the secondary structure is regulated by the heme and affects specifically only the quaternary structure of the system, which is tetrameric in hemoglobin and becomes dimeric in apohemoglobin.

It is reasonable to propose that the hydrophobic interactions of the amino acid side chains surrounding the heme may produce a collapse of the heme pocket upon heme removal. This may indeed produce a slight, hardly detectable, shrinking of apohemoglobin. The results presented here and in a previous paper of ours (Kowalczyk & Bucci, 1983) suggest that the collapse of the heme pocket may be the only gross difference between the tertiary structures of apohemoglobin and hemoglobin. This collapse has hardly any effect on the structure of the molecules; however, it is not a small event in the system. In fact, the distortion that it forces on the secondary structures surrounding the heme pocket is transmitted to the $\alpha_1\beta_2$ interface, which is broken, so producing a dimerization of the protein. This illustrates how helical domains can amplify and transmit information on the status of the heme to the regulatory interfaces of the protein.

Experiments performed on the isolated β subunits (Franchi et al., 1982) suggest that the domain that connects the heme to the $\alpha_1\beta_2$ interface is at least in part situated in the 1–55 portion of the subunits. At present, we lack analogous information with regard to the α subunits.

Thus we begin to have evidence that the heme controls the conformation of the subunit interfaces through the action of specific domains of secondary structure. If it were possible to demonstrate that the reciprocal is also true, namely, that information from the interfaces can be transferred to the heme, this may complete the line of transmission of the heme–heme interaction between subunits.

Data obtained from NMR and Raman spectroscopy have already shown that the heme pocket is sensitive to amino acid substitution near the $\alpha_1\beta_2$ interface in mutant hemoglobins (Lindstrom & Ho, 1973; Nagai et al., 1980; Tsubanki et al., 1982; Wiechelman et al., 1976). We are currently obtaining data from Mössbauer spectroscopy, indicating that digestion of the terminal carboxyl residues in hemoglobin, which can be considered part of the $\alpha_1\beta_2$ interface, has an effect on the electronic structure of the iron atom (B. Balko, E. Bucci, R. L. Berger, L. J. Swartzendruber, and J. X. Montemarano, unpublished results).

Registry No. Heme, 14875-96-8.

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