Dimensions in Solution of Pyridoxylated Apohemoglobin†

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ABSTRACT: Human apohemoglobin was labeled at Val-β1 with pyridoxamine 5′-phosphate. Correlation times were evaluated from steady-state fluorescence anisotropy and lifetime measurements upon quenching with KI. Multiple correlation times were present in the system with a major component of 23.3 ns and a minor component of less than 0.1 ns. The initial depolarization produced by these fast motions occurred in a cone with a semiangle near 33°. The sedimentation velocity and circular dichroism spectra of pyridoxal 5′-phosphate labeled apohemoglobin were very similar to those of unlabeled apohemoglobin. Addition of 8-anilino-1-naphthalenesulfonate did not modify these parameters. Energy transfer of fluorescence was measured between the label, pyridoxamine 5′-phosphate, positioned at Val-β1, as donor, and 8-anilino-1-naphthalenesulfonate, bound inside the heme pocket, as acceptor. A quantum yield of 0.21 was measured for labeled apohemoglobin with a standard of pyridoxamine 5′-phosphate. Quenching of the lifetime and of the emission of the donor in the presence of acceptor was measured at 390 nm upon excitation at 313 nm. From these parameters, and on assumption of a random orientation of the fluorophores, an average distance of about 25 Å was estimated between the two probes. Numerical correction for 85% saturation of the donor with acceptor produced distances near 23 Å for the quenching of emission intensity and near 19 Å for the quenching of lifetimes. In the tridimensional model of deoxyhemoglobin, the distance between Val-β1 and the nearest iron is about 22 Å. Transfer to acceptors positioned in the α subunits was negligible. Taking into account the dimensions of the probes, it appears that removal of heme and the consequent loss of helical structure of the system did not produce an expansion of the β subunits.

Removal of heme from hemoglobin results in an apparent loss of α-helical content of the system, which (using the parameters of Chen et al., 1974) goes from 76% to about 58% (Bucci & Kowalczyk, 1982). Also, it is known that the system becomes dimeric (Antonini & Brunori, 1971). All of these parameters return to normal upon recombination of apohemoglobin with heme (Antonini & Brunori, 1971). The effect of the loss of secondary structure on the general folding of the protein subunits and on their tertiary structure is not known.

Results obtained in this laboratory suggest that in the hemoglobin system there are α-helical domains regulated by the heme and characterized by low conformational energy. In fact, their unfolding does not modify the stability of the protein as a whole, suggesting that they have allosteric relevance (Franchi et al., 1982; Bucci & Kowalczyk, 1982).

Evidence obtained from tritium-exchange experiments indicates the presence of helical segments that "breathe" rapidly in and out of their structure (Englander & Rolfe, 1973; Malin & Englander, 1980). This conformational freedom of the system is consistent with the internal flexibility detected in experiments of fluorescence depolarization (Oton et al., 1981; Sassaroli et al., 1982).

It is possible that the loss of helical structure upon removal of heme represents only a new average in the conformational fluctuations of the system with only small modifications of its tertiary structure. These considerations prompted attempts to investigate the diameters in solution of the apohemoglobin molecule. These have been approached by inserting fluorescent probes in the protein, from which to obtain correlation times and which are capable of functioning as donor–acceptor pairs for measurements of energy transfer of fluorescence.

The interpretation of energy-transfer experiments is always made difficult and open to criticism by the practical impossibility of determining what is the reciprocal orientation of the probes used as donors and acceptors. We faced this challenge by using two different donor–acceptor pairs, namely, pyridoxal 5′-phosphate (donor) and ANS[1] (acceptor) and ANS (donor) and fluorescein (acceptor). In both cases, the hypothesis of random orientation of the probes was supported by the observation that their dipoles were rotating in cones with average semiangles in excess of 30°. Also, this assumption produced consistent results with the two different pairs of probes; therefore, it was taken as the basis for the interpretation of the data.

This paper reports part of the investigation, that based on the usage of PLP and ANS as donor and acceptor, respectively. In fact, PLP can be positioned on each of the β1 valines of hemoglobin (Benesch et al., 1972), and ANS is known to recombine specifically and with high affinity with the heme pocket of apohemoglobin and apomyoglobin (Stryer, 1965).

The investigation was conducted by using phase fluorometry, which, with the instrumentation available to us, was more accurate than pulse fluorometry for measuring lifetimes near 3 ns or less. The energy transfer was negligible for distances exceeding the diameters of a single β subunit.

The results show that if a random reciprocal orientation of the probes is assumed, their distance in apohemoglobin is close to and not larger than that measurable in the model of the crystal structure of hemoglobin between the iron of the heme and the valine at β1 of the same subunit. Also, the correlation times associated with the emission of PLP were those expected from a spherical, compact αβ dimer.

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[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonate; PLP, pyridoxal 5′-phosphate; PAP, pyridoxamine 5′-phosphate; APOPLP, apohemoglobin labeled with PLP in position β; apohemoglobin, heme-free hemoglobin; DPG, 2,3-diphosphoglycerate; CD, circular dichroism.

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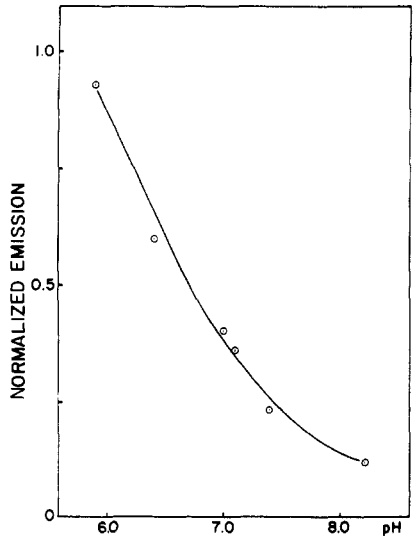

FIGURE 1: Fluorescence intensity as a function of pH for APOPLP, in 0.2 M phosphate buffer at 4 °C.

Materials and Methods

Pyridoxamine 5′-phosphate (PAP) was purchased from Sigma Chemical Co.; ANS was obtained from Molecular Probes Inc. All other reagents were analytical grade or better. ANS was pure in thin-layer chromatography.

Human hemoglobin was prepared from washed red cells hemolyzed in the presence of chloroform. The deoxy derivative was coupled to pyridoxamine 5′-phosphate as described by Benesch et al. (1972). For purifying the reacted material, a column of CM-cellulose was used with a linear gradient formed by equal volumes of 0.02 and 0.2 M phosphate buffers at pH 6.8. The second peak was collected and analyzed for its phosphorus content (Ames & Dubin, 1960). If necessary, this fraction was chromatographed a second time under the same conditions, only the elution gradient was made less steep by using lower concentrations of phosphate in the second buffer. The preparations were considered satisfactory when pure in cellulose–acetate electrophoresis, with a phosphorus content between 1.9 and 2.0 mol of P/mol of tetrameric hemoglobin. In these preparations, oxygen binding equilibria performed at 20 °C in 0.02 M phosphate buffer at pH 7 with a Hemoscan apparatus (SLM-AMINCO) gave values of log $P_{1/2}$ near 0.9 and of n in the Hill plots near 2.1. Heme was removed from this protein by the procedure described by Rossi-Fanelli et al. [reported by Antonini & Brunori (1971)].

Fluorescence lifetimes were measured with a SLM 4800 phase fluorometer, by using modulation frequencies of 30 MHz and a reference standard of 1,4-bis[2-(5-phenyloxazolyl)]-benzene (POPOP) (Lakowicz et al., 1980). Steady-state fluorescence anisotropy was measured in the same instrument, by using Glan prism polarizers and evaluating the instrumental correction factors from measurements obtained with horizontally polarized light in excitation. Fluorescence spectra were obtained on an SLM 8000 spectrofluorometer. 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 measured with a Model E Beckman ultracentrifuge using schlieren optics. Spectrophotometric measurements were performed on a Cary 14 instrument. Protein concentration was measured spectrophotometrically with $\epsilon = 14\,000$ M^{-1} cm^{-1} per heme at 540 nm for the CO derivative of hemoglobin and $E = 0.85$ mL mg^{-1} at 280 nm for apohemoglobin in 0.1 M NaOH. Coupling of

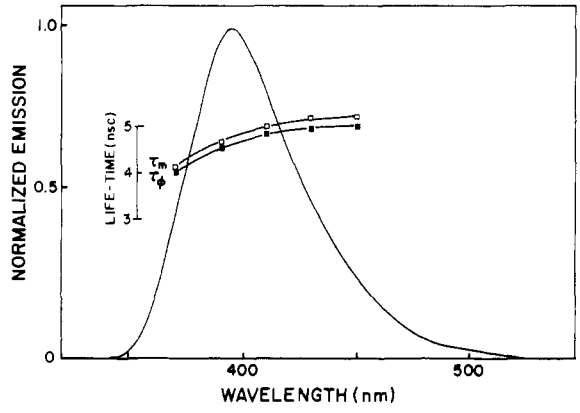

FIGURE 2: Corrected emission spectrum of APOPLP upon excitation at 313 nm. Phase and modulation lifetimes at several wavelengths across the spectrum are shown. In 0.02 M phosphate buffer at pH 6.5, at 4 °C.

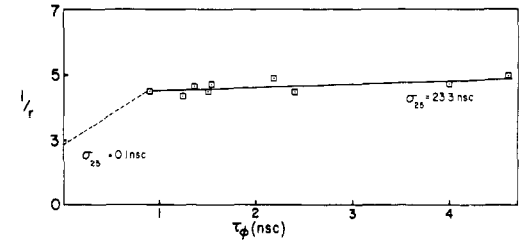

FIGURE 3: Lifetime-resolved anisotropy of APOPLP upon quenching with KI. The estimated values of correlation times (σ_{25}) corrected for water at 25 °C are shown. Protein concentration near 2 mg/mL, in 0.02 M phosphate buffer at pH 6.5, at 4 °C.

PLP did not modify appreciably these quantities.

Results

Fluorescence Characteristics of APOPLP. Figure 1 shows the pH dependence of the intensity of fluorescence of APOPLP. The increase at lower pH is much more pronounced than that obtained with solutions of PAP. It suggests the presence of positively charged groups around the label, which are increasingly protonated below pH 8.0. It is consistent with the positioning of the label in the "DPG pocket" of hemoglobin, as expected (Arnone et al., 1977).

Figure 2 shows the corrected emission spectrum of APOPLP upon excitation at 313 nm. Phase and modulation lifetimes across the spectrum are there reported. The emission spectrum was pH independent. The similar values of phase and modulation lifetimes and their low sensitivity to wavelength are consistent with the essential homogeneity of the system.

Lifetime-Resolved Anisotropy of APOPLP. The lifetime dependence of the anisotropy of the system was measured by quenching the emission of APOPLP with KI. The total concentration of K^+ ions was kept at 0.2 M by using a compensatory amount of KCl. In a few samples, the total concentration of K^+ was increased to 0.36 M without appreciable modification of the results. The anisotropy of PAP at −14 °C in glycerol was taken as the maximum anisotropy of the system (r_0).

Correlation times (σ) were estimated from the slopes of the curves obtained by plotting the reciprocal anisotropy (r) against the quenched lifetimes (τ), according to

$$\frac{1}{r} = \frac{1}{r_0} + \frac{\tau}{\sigma} \qquad (1)$$

The data are shown in Figure 3. The curve does not extrapolate to the measured value of $1/r_0$, indicating the presence of fast motions of the probe. An upper limit of correlation

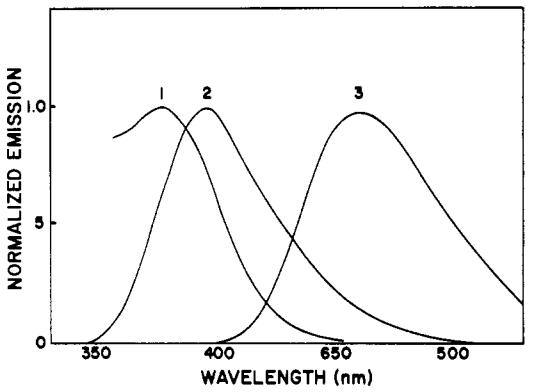

FIGURE 4: (1) Normalized absorption spectrum of ANS bound to APOPLP, obtained by difference spectrophotometry. (2) Normalized corrected emission spectrum of APOPLP. (3) Normalized emission spectrum of ANS bound to APOPLP. Excitation at 313 nm. In 0.02 M phosphate buffer at pH 7.0 at 4 °C.

time for these fast rotations was obtained from the slope of a line arbitrarily drawn joining the measured value of $1/r_0$ to the nearest experimental points in the graphs. All of the experiments were performed at 4 °C; therefore, correlation times at 25 °C (σ_{25}) were obtained by correcting for the viscosity of water.

The value of σ_{25} = 23.3 ns (with 95% fiducial limits near ±4.5 ns) obtained from the main portion of the curve is consistent with the rotation of a dimeric hemoglobin species. This datum is supported by values of correlation times measured with pulse fluorometry, to be reported elsewhere.

The correlation time obtained from the arbitrary limiting slope of the curve was near σ_{25} = 0.1 ns, indicating a fast mobility of the probe around its point of attachment to the protein. The semiangle θ of the cone of rotation of the probe was estimated to be 33° by using

$$\frac{r_\infty}{r_0} = \frac{3\cos^2\theta - 1}{2} \tag{2}$$

where r_∞ is the anisotropy obtained from the extrapolation to the ordinate axis of the main portion of the curve in Figure 3.

Fluorescence Energy Transfer between APOPLP and ANS. Figure 4 shows the emission spectrum of APOPLP superimposed on the absorption spectrum of ANS added to APOPLP. It also shows the emission spectrum of ANS added to APOPLP. The presence of PLP coupled to the protein modified the intensity but not the shape of the emission spectrum of ANS.

The absorption spectrum was obtained by difference spectrophotometry, by adding small amounts of ANS to a relatively high concentration of APOPLP (5–8 mg/mL) so as to have a negligible amount of free ANS in solution. The spectrum both qualitatively and quantitatively resembles very closely the one reported by Stryer (1965) for ANS and apomyoglobin. On this basis, the overlapping integral was computed with ϵ = 6800 M^{-1} cm^{-1} for ANS bound to APOPLP. The quantum yield of APOPLP was measured to be 0.21 under standards of PAP (Chen, 1965). From these values and the procedure described by Fairclough & Cantor (1978), the distance R_0, at which 50% energy transfer occurs between randomly oriented probes, was computed to be 25.5 ± 0.3 Å.

Apohemoglobin is an $\alpha_1\beta_1$ dimer (Antonini & Brunori, 1971); therefore, in APOPLP, the transfer of energy between PLP and ANS occurred along two possible distances. One was the intrasubunit distance between PLP at β1 and ANS in the β heme pocket. In the crystal of hemoglobin, the distance between the α carbon of the β1 valine and the iron of the heme is very near 20 Å in oxyhemoglobin and 23 Å in deoxyhemoglobin, for an average of 21.5 Å. The other transfer is between PLP at β1 and ANS in the α heme pocket in the $\alpha_1\beta_1$ dimer. In the hemoglobin crystal, this distance is 30 Å in oxyhemoglobin and 33 Å in deoxyhemoglobin, for an average of 31.5 Å.

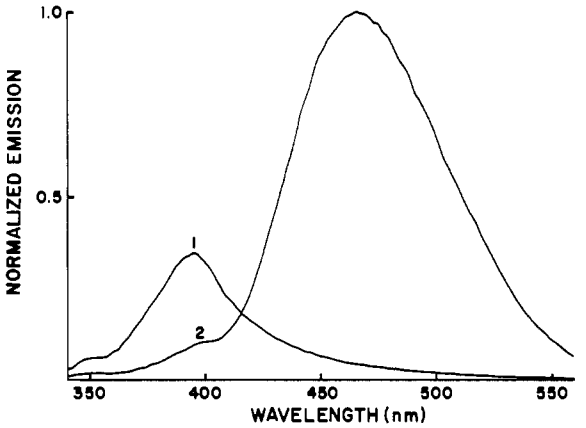

FIGURE 5: Quenching of emission of APOPLP produced by 10^{-4} M ANS, upon excitation at 313 nm. Protein concentration 0.16 mg/mL, in 0.02 M phosphate buffer at pH 7.0, at 4 °C. (1) Before and (2) after addition of ANS.

We assessed the relative weight of these transfers on the apparent average distance between donor and acceptor, following the treatment developed by Gennis & Cantor (1972) for the case of a single donor and multiple acceptors. The average transfer efficiency (E_{av}) was given by

$$E_{av} = \frac{\sum_i (R_0/R_i)^6}{1 + \sum_i (R_0/R_i)^6} \tag{3}$$

where R_i is the distance from the donor of the ith acceptor. From this, the apparent average distance (R_{av}) was evaluated by using the familiar equation

$$R_{av} = R_0(1/E - 1)^{1/6} \tag{4}$$

These simulations showed that for R_i's differing by about 10 Å, R_{av} is equal to the shorter distance within 4% or less. This implies that in our experiments, we were investigating only the intrasubunit distance between PLP at β1 and ANS in the β heme pocket.

Lifetimes and fluorescence intensity of APOPLP were measured at 390 nm where the fluorescence of ANS was negligible. Excitation was at 313 nm. Protein concentration was 2×10^{-5} M (per dimer) and ANS was 10^{-4} M. All experiments were conducted in 0.02 M phosphate buffer at pH 7.0.

From data reported by Stryer (1965), it is possible to estimate that under these conditions, apohemoglobin is 80–90% saturated with ANS. Higher concentrations of ANS could not be used because of its absorption both at 313 and 390 nm.

Figure 5 shows the quenching produced by ANS on the fluorescence of APOPLP. After correction for the absorption of ANS at the excitation and emission wavelengths, we estimated that, in the presence of 10^{-4} M ANS, 49% of the emission of APOPLP was quenched. It should be noted that because of the incomplete saturation of the donor with acceptor, the quenching was incomplete, producing an overestimation of the distance between the probes.

The lifetimes of the sample were also modified. In the absence of ANS, the average of phase and modulation lifetimes

Table I: Distances between PLP and ANS in APOPLP, Estimated from Various Parameters

parameter	av distance (Å)	max and min distance estimated from the standard deviations of the parameters
fluorescence intensity of quenched samples	25.3	24.5, 26.3
phase lifetime of quenched samples	25.9	24.8, 26.8
fluorescence intensity corrected for 85% saturation of the donor[a]	23.7	22.1, 25.5
lifetime τ_2 evaluated from eq 7	19.2	17.8, 22.5

[a] Estimated from $Q_c = 1 + (Q_E - 1)/\alpha$, where Q_c is the corrected quenching, Q_E is the original experimental quenching, and α is the fractional saturation of the donor with acceptor.

gave $\tau = 3.46 \pm 0.21$ ns. In the presence of ANS, we obtained a modulation lifetime $\tau_m = 2.81 \pm 0.22$ ns and a phase lifetime $\tau_\phi = 1.81 \pm 0.23$ ns.

In the quenched samples, the difference between phase and modulation data was very probably due to the incomplete saturation of APOPLP with ANS. In fact, in these samples two lifetimes were present, the original lifetime of the unquenched donor and that of the quenched APOPLP.

As shown by Spencer & Weber (1959) in two-component systems, the phase lifetime is less than the average of the two components. Therefore, in our experiments the phase lifetime was better approaching the lifetime of the quenched donor, still producing an underestimation of the quenching with a consequent overestimation of the distance between the probes.

At frequency f, when two components are present, the average demodulation M

$$M = \frac{1}{[1 + (2\pi f\tau)^2]^{1/2}} \tag{5}$$

and the average phase shift ϕ

$$\phi = \tan^{-1}(2\pi f\tau_\phi) \tag{6}$$

are correlated to the true lifetimes τ_1 and τ_2 by

$$\begin{aligned}(1+\gamma)M\cos\phi &= \frac{1}{1+(2\pi f\tau_1)^2} + \gamma\frac{1}{1+(2\pi f\tau_2)^2}\\ (1+\gamma)M\sin\phi &= \frac{2\pi f\tau_1}{1+(2\pi f\tau_1)^2} + \gamma\frac{1}{1+(2\pi f\tau_2)^2}\end{aligned} \tag{7}$$

where γ is the relative intensity of the emission of the second component when that of the first is taken as unity. Thus, the total emission is $1 + \gamma$.

In our case, τ_1 was known, because it was the lifetime of the unquenched donor. Therefore, eq 7 could be solved for τ_2 and γ of the quenched component. The fraction y of APOPLP saturated with ANS can be evaluated by correcting for the amount of quenching with

$$y = \gamma(\tau_2/\tau_1)/[1 + \gamma(\tau_2/\tau_1)] \tag{8}$$

We obtained $\tau_2 = 0.54$ and $y = 0.85$. The value of y was very close to that expected from the data reported by Stryer (1965). This consistency gave support to the numerical analysis above described.

Table I lists the various distances evaluated from these data with

$$R = R_0(1/E - 1)^{1/6} \tag{9}$$

where E is the efficiency of energy transfer defined either by the intensity of emission or by the values of the lifetimes in the equation

$$E = 1 - P'/P \tag{10}$$

where P and P' are the chosen parameters in the absence and presence of acceptor, respectively.

Sedimentation Velocity and Circular Dichroism Measurements. These measurements were performed in 0.05 M phosphate buffer at pH 7.0 at 5 °C for CD and near 15 °C for the sedimentation runs. At concentrations of a few milligrams per milliliter, both in the presence and absence of ANS, the sedimentation velocity of APOPLP was independent of protein concentration and gave values near $s_{20,w} = 2.6$ Å, as usually obtained for normal untreated apohemoglobin. At concentrations near 0.1 mg/mL, the far-UV CD spectrum of APOPLP, both in the presence and absence of 10^{-4} M ANS, was indistinguishable from that of normal untreated apohemoglobin.

Discussion

It is relevant to stress that the pH dependence of the fluorescence intensity of APOPLP was consistent with the expected position of the probe on the surface of the protein. Indeed, it could be used to monitor the ionization of the positive groups present in the DPG pocket of apohemoglobin.

Measurements of circular dichroism indicated that labeling and addition of ANS did not modify the structure of apohemoglobin. Consistent with this proposition, quenching with KI showed the presence of a correlation time close to what is expected from the rotational behavior of a compact, quasi-spherical $\alpha\beta$ dimer (Oton et al., 1981). The experiments also showed that the probe had a considerable degree of freedom and moved in a cone with an average semiangle close to 33°.

Under the assumption of a random orientation of donor and acceptor, Table I lists the distances that can be estimated from the available parameters. It should be remembered that the distances obtained from fluorescence intensity and phase lifetime of the quenched samples (first two lines of the table) are very probably overestimations.

As mentioned, in the model of crystalline hemoglobin, the distance between the α carbon of the valine at β1, where PLP is positioned, and the iron atom of the heme of the same subunit is close to 21.5 Å. If 1 or 2 Å are added at both ends of this distance, taking into account the dimensions of the probes, a distance close to 25 Å is obtained.

Thus, the average distances listed in Table I are either very similar or slightly shorter than those present in crystalline hemoglobin. It should be stressed that the more similar values resulted from overestimations.

A preferential binding of ANS to the heme pockets of the β subunits seems to be improbable. Stryer (1965) reports no indication of such preference, and the consistency between the binding constants estimated by us and measured by Stryer (1965) suggests that it is so also for APOPLP.

In Table I, we have included the variabilities that can be estimated from the upper and lower limits of the standard deviations of the parameters used for computing the transfer distances. For the quenching of steady-state fluorescence, which required corrections for the absorption of ANS, a deviation of ±10% was assumed.

The spans of variability listed in Table I still fail to indicate

an expansion of the molecule, consequent to the removal of heme. It is as if the loss of helical content, which on the basis of the spectral data of Chen et al. (1974) was estimated to go from 76% in hemoglobin to 58% in apohemoglobin (Bucci & Kowalczyk, 1982), did not modify the tertiary structure of the system.

The data in Table I are very consistent with the diameters measurable in the model of crystalline hemoglobin. Their validity is supported by the sedimentation velocity and correlation time of the APOPLP system, which were those expected from a compact quasi-spherical dimer.

Their validity is further supported by independent data of energy transfer obtained with ANS as donor in the heme pocket and fluorescein as acceptor positioned at $\beta 93$. These results, to be presented in a different paper, also indicate the persistence of the tertiary structure of hemoglobin in apohemoglobin.

The question may be posed whether the far-UV ellipticity of proteins is linearly dependent on their secondary structure components, as proposed by Chen et al. (1974). Discrepancies between far-UV ellipticity and X-ray structures are present in the literature. For example, lamprey hemoglobin and leghemoglobin both have ellipticities in the far-UV region about 70% that of hemoglobin or myoglobin (Sugita et al., 1968; Nicola et al., 1975), while in the crystals they both are about 80% helical, as hemoglobin and myoglobin are (Hendrikson & Love, 1971; Arutyunyan et al., 1980).

In the paper of Chen et al. (1974), the reported dependence of ellipticity on the length of the helical segments is per se an indication of nonlinearity. It is possible that distortions rather than melting of the helices decrease the CD signal, so that the loss of secondary structure in apohemoglobin is less than that estimated from the parameters of Chen et al. (1974). At present, these are only speculations, and the 18% loss of helical structure computed with the parameters of Chen et al. (1974) cannot be excluded.

It is reasonable to propose that in apohemoglobin the space left empty by the removal of heme is filled by the hydrophobic interactions of the amino acid side chains surrounding it. These interactions are probably the reason for the modification of secondary structure and may be interpreted as the result of local conformational fluctuations otherwise inhibited by the heme. They may produce a slight shrinking of the molecule, as hinted by the two last lines in Table I.

Our observations support the hypothesis formulated in the introduction of this paper, that removal of heme rather than modifying the general structure of the system allows a different averaging of local conformational fluctuations of the protein. It also supports the observation already formulated in several previous papers of ours (Bucci & Kowalczyk, 1982; Franchi et al., 1982; Sassaroli et al., 1982; Oton et al., 1981) that in hemoglobin there are helical domains regulated by the heme, whose stability is low and of little consequence for the structure of the system as a whole.

As previously noted (Franchi et al., 1982), the dimerization produced by the removal of heme stresses the functional relevance of these domains. In fact, it indicates that in this case the lost structure contributes to the conformation of the $\alpha_1\beta_2$ interface, which is the functional interface of the system, as proven crystallographically and functionally (Perutz, 1979; Pettigrew et al., 1982).

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