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Structural Rearrangements Due to Ligand Binding and Haem Replacement in Myoglobin and Leghaemoglobins

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(Received July 30, 1976/June 1, 1977)

Structural rearrangements in sperm whale myoglobin and leghaemoglobins caused by changes in the spin or oxidation state of the iron as a consequence of ligand binding have been measured by difference spectroscopy in the ultraviolet. When compared with the high-spin acetate complex, ligands which cause a transition to the low-spin state also cause large perturbations of tyrosine(s) remote from the haem pocket in myoglobin but only minor perturbations of tryptophan(s) in leghaemoglobin. This may indicate a weaker coupling between events at the haem site and conformational changes in the protein in leghaemoglobins. The absorption spectra of various haem-liganded forms of the two proteins as well as the binding of the dye rose Bengal to the two apoproteins are consistent with weaker interactions between the haem and apoprotein and a more solvent-exposed haem pocket in leghaemoglobin compared with myoglobin.

Leghaemoglobins are plant haemoproteins which resemble animal myoglobins with respect to their molecular weight, monomeric state and their ligand-binding (especially oxygen-binding) properties. Their high oxygen affinity is presumed to be central to their biological function in the root nodules of legumes (see *e.g.* [1]). Most of the reported structural, spectroscopic and ligand-binding studies have been on the major leghaemoglobin component from soybean (*Glycine max*, cr Lincoln). It has recently been shown that this plant produces several leghaemoglobins of differing amino acid sequence but similar overall conformation [1,2], that these characteristics are probably general in *Leguminosae* (Thulborn *et al.*, unpublished) and that the most readily discernable differences between leghaemoglobins of widely different origin and amino acid sequence, is their antigenicity [3]. Comparing the leghaemoglobins with sperm whale myoglobin reveals interesting similarities and differences [2,4]. For the purposes of the present paper, the most pertinent conclusion was that the haem moiety may be more loosely held in the leghaemoglobins than in myoglobin and there is less resistance to changes in spin state on ligand binding in the former case. This is consistent with the observations that soybean leghaemoglobin binds nicotinic acid with much higher affinity [5,6] and its haem moiety is more readily exchangeable [7] than in myoglobin. Evidence has also been adduced that the haem pocket in the plant globin is not as non-polar as that in myoglobin [8,9].

The purpose of the studies described here was to seek evidence of structural changes occurring in both types of haem proteins arising from changes in spin state. The reason for our interest in such changes is the observation by Perutz [10] that high-spin to low-spin transitions in tetrameric haemoglobin are accompanied by deep-seated molecular rearrangements but little is known of corresponding changes in monomeric systems. In order to compare them with myoglobin and to generalise from the results, several leghaemoglobins and a variety of ligands (including nicotinate) and both oxidation states were used. Changes in absorption spectra in the visible, Soret and near-ultraviolet were measured, usually by the difference technique. Finally, the dye rose Bengal was used as a spectroscopic indicator of the polarity of the haem pockets in the two types of proteins.

MATERIALS AND METHODS

Materials

The dye rose Bengal (3′4′5′6′-tetrachloro-2,4,5,7-tetraiodofluorescein disodium salt) was obtained from Fluka. Chlorohemin was lot 8071 from Nutritional Biochemicals Corp. and was recrystallised according to Fischer [11]. Leghaemoglobins were extracted and purified as already described [2,4]. Sperm whale

Abbreviation. CD, circular dichroism.

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myoglobin was batch 10 from Seravac or batch 52348 from Koch-Light.

Potassium ferricyanide, potassium cyanide, potassium cyanate, sodium acetate, sodium azide, sodium dithionite and sodium fluoride were AR grade from British Drug Houses. Nicotinic acid was AR grade from Townson and Mercer and imidazole from Sigma. Other chemicals were AR grade and glass-distilled water was used throughout.

Concentration Estimations

Absorption spectra for the reduced pyridine haemochromes of soybean, serradella and lupin leghaemoglobins were identical with those for sperm whale myoglobin. Since the spectra for other complexes of these proteins were much more variable than this, the formation of pyridine haemochromes was used as a measure of haemoprotein concentration as in previous studies [2,4].

Formation of Liganded Components

Most liganded complexes of leghaemoglobin and myoglobin were formed simply by titration with the appropriate ligand until no further spectral changes could be observed. The deoxyferrous and oxyferrous complexes were formed as described previously [4] and the percentage of oxyferrous and ferric forms was assayed as described in the same paper.

Formation of Apoproteins

Apoproteins of myoglobin and leghaemoglobins were made from the haem proteins by the acetone/HCl method of Rossi-Fanelli *et al.* [12]. Acetone (BDH) was redistilled from potassium permanganate and Drierite. Glass-distilled water was de-ionised using a Bio-Rad mixed-bed ion-exchange resin. If the removal was carried out at -20 °C the products usually had less than 1% of the original haem content as judged from the A_{400}/A_{280} ratio and were recovered in about 70% yield. Their concentrations and those of the haem proteins were determined as previously described [2,4]. Molar absorption coefficients at 280 nm in phosphate pH 7.5 were 17900 and 16300 M^{-1} cm^{-1} for soybean apoleghaemoglobin and sperm whale apomyoglobin respectively.

Absorption Spectra and Difference Spectra

Absorption spectra were measured with a Cary 14 spectrophotometer. Difference spectra were usually run on this instrument using conditions similar to those reported by Nicola and Leach [13] except that a four-cell arrangement was usually used to allow compensation for the addition of absorbing ligands to the sample cell.

Circular Dichroic Spectra

The instruments and procedures used for measuring CD spectra were those described by Nicola *et al.* [4].

RESULTS

Effect of Spin and Oxidation State on Soret and Visible Absorption Spectra

Spectral data for soybean leghaemoglobin a and the main lupin component have been reported by a number of workers [1,9,14–16] and these have been supplemented by data for the aquoferric, fluoroferric and nicotinoferric as well as the oxyferrous and deoxyferrous soybean derivatives [4]. In the present work, Soret and visible absorption spectra have been measured for the acetate, cyanate, azide and imidazole derivatives of ferric soybean leghaemoglobin. All of these spectra are best discussed by grouping the derivatives as follows: high-spin ferrous = deoxy; low-spin ferrous = oxy, pyridine and nicotinate; high-spin ferric = acetate and fluoride; low-spin ferric = cyanide, imidazole and nicotinate; mixed-spin ferric = aquo, cyanate, azide.

The spectra for soybean leghaemoglobin a are very similar to those reported for lupin leghaemoglobin [9]. They are also quite similar in general form to those for sperm whale myoglobin [17] with corresponding complexes showing the same spin-state tendencies. However, there are differences in the peak absorption intensities and their wavelengths. Table 1 shows that the Soret band for all classes of leghaemoglobin spectra consistently occurs at shorter wavelengths (blue shift) than for corresponding complexes of myoglobin independent of the spin or oxidation state of the iron. Also the mixed-spin complexes for leghaemoglobin appear to be further towards the low-spin state than those for myoglobin (not shown).

Binding of Nicotinic Acid

The binding of nicotinic acid with relatively high affinity to both ferrous and ferric leghaemoglobin is of some interest because this ligand does not bind to myoglobin except at much higher concentrations [18] and because it has been postulated that it may play a regulatory role *in vivo*. This hypothesis and the binding of nicotinic acid to soybean leghaemoglobin a has been discussed in detail by Appleby *et al.* [1,5,6]. We have extended these observations to show that the high-affinity binding of nicotinic acid is probably a general phenomenon for leghaemoglobins. Thus, leg-

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haemoglobins from serradella, snake bean and lupin all showed changes in absorption spectra for their ferrous forms (reduced with sodium dithionite) when titrated with nicotinic acid in 0.01 M phosphate buffer, pH 7.0, which were qualitatively similar to those of the soybean protein [5,6]. The structural consequences of nicotinic acid binding are best seen using difference spectra. Fig. 1 shows such a set of spectra for the titration of ferric soybean leghaemoglobin a in the near-ultraviolet (Fig. 1A) and visible (Fig. 1B) regions.

The visible difference spectra show the progressive decrease in the charge-transfer band intensities at 494 and 629 nm and the increase in the α and β band intensities at 530 and 556 nm respectively. This is typical for a high-spin to low-spin transition.

The changes which occur concomitantly in the near-ultraviolet, (Fig. 1A), are perhaps more interesting. These changes run parallel with those in the visible and, in fact, some of this difference absorption is due to spectral changes in the haem. Strickland *et al.* [19] however, have shown that neither the haem nor the haem undecapeptide of cytochrome *c* (which contains no aromatic amino acids) display any fine structure bands in the haem absorption region between 250 and 310 nm, even at liquid nitrogen temperatures, so the haem cannot be the origin of the sharp difference peaks seen in Fig. 1(A). The peaks below 280 nm may well arise from the transfer of nicotinic acid from an aqueous environment into the non-polar haem pocket, since the parent spectrum of nicotinic acid shows peaks at 270, 264 and 257 nm. However, the negative difference peak at 294 nm and probably the one at 286 nm must arise from a change in the environment of a tryptophan residue and this change is proportional to the extent of binding of nicotinate. Such negative difference peaks, superimposed on an increasingly positive haem background, suggests that the tryptophan(s) become more exposed to aqueous solvent as the titration proceeds.

Table 1. *Wavelengths of the main Soret peak for various complexes of sperm whale myoglobin, soybean leghaemoglobin a and lupin leghaemoglobin a*

Complex type	Ligand	Wavelength for		
		sperm whale Mb [17]	soybean Lb	lupin Lb [9]
		nm		
Low-spin ferric	CN	423	417	416
High-spin ferric	F	406	403	403
Mixed-spin ferric	H_2O	410	403	404
Low-spin ferrous	O_2	418	412	—
High-spin ferrous	—	434	427	421

'Form' Difference Spectra in the Near-Ultraviolet

'Form' difference spectra (*i.e.* the difference spectra for various liganded complexes against the pure high-spin acetate complex) were recorded in the near-ultraviolet for both sperm whale myoglobin and soybean leghaemoglobin a (see Fig. 2). As mentioned before, haem itself shows no fine structure bands between 250 and 310 nm as evidenced by the difference spectrum for low-spin ferric haem cyanide *versus* high-spin ferric haem chloride. On the other hand, difference

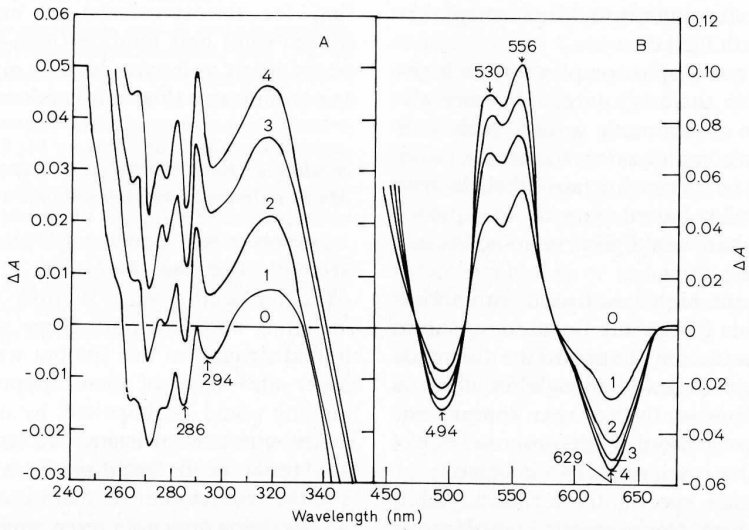

Fig. 1. *Difference spectral titration of soybean ferric leghaemoglobin a with nicotinic acid (A) in the near-ultraviolet and (B) in the visible.* Leghaemoglobin concentration was 33.4 μM in 0.01 M potassium phosphate buffer, pH 5.4, and each addition (1,2,3,4) increased the concentration of nictonic acid by 11.6 μM. Difference spectra were performed with a four-cell arrangement that allowed blanking of both protein and ligand. Saturation was achieved at a final concentration of 58 μM nicotinic acid (not shown)

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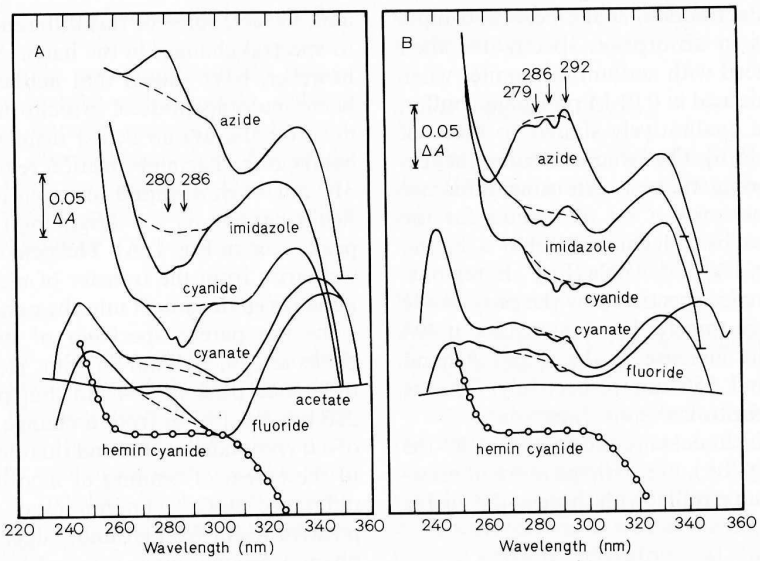


Fig. 2. *Form difference spectra for (A) sperm whale ferric myoglobin and (B) soybean ferric leghaemoglobin a liganded complexes.* For (A) ΔA of 0.05 corresponds to a $\Delta\varepsilon$ of 1.9 mM^{-1} cm^{-1}; for (B) ΔA of 0.05 corresponds to a $\Delta\varepsilon$ of 2.3 mM^{-1} cm^{-1}. All complexes were measured against the corresponding acetate complex as reference. The difference spectra are labelled according to the bound ligand. Some of these difference spectra have been offset vertically for clarity and the baseline position for each marked with a dash through the curve on the long-wavelength side. The dashed curves between 270 and 295 nm are inferred background absorptions due to the haem. For reference, the curve (O——O) for cyanoferric haem (haemin cyanide) *versus* haemin chloride is also shown. All samples were in 0.1 M sodium acetate buffer, pH 5.2, and liganded complexes were formed by titration with solid ligand until no further spectral changes were observed. Leghaemoglobin a concentration was 22 μM and myoglobin concentration 26 μM at 20 °C. A four-cell arrangement was used

spectra of both myoglobin and leghaemoglobin low-spin ferric complexes *versus* the high-spin acetate complex do show fine structure difference bands in this spectral region and these must reflect changes in the environments of the aromatic amino acids upon such a transition. However, there is a difference between the responses of myoglobin and leghaemoglobin to a change in the sixth ligand.

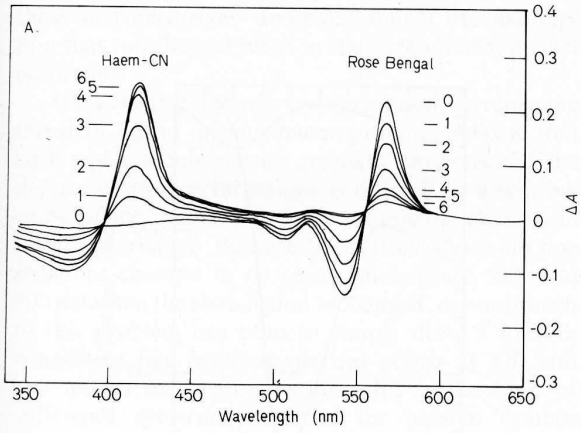
On formation of a low-spin complex from a high-spin one in myoglobin there is a large difference absorption attributable to aromatic amino acids (see especially the cyanide complex *versus* the acetate) with peaks at 280 and 286 nm. These are most likely to arise from the exposure of a buried tyrosine to aqueous solvent since tryptophan would give rise to additional peaks at higher wavelengths.

Replacement of one high-spin ligand for another in myoglobin (fluoride or cyanate for acetate) shows a much smaller aromatic contribution to the difference spectrum. The azide complex of myoglobin which is mixed-spin but predominantly low-spin appears not to follow the expected pattern if the proposed background absorption that we have drawn is correct.

The form difference spectra for leghaemoglobin (Fig. 2B) show much smaller aromatic contributions than do those for myoglobin. The wavelengths of the difference peaks are, however, indicative of a change in the environment of tryptophan rather than of tyrosine, as was observed for the nicotinate *versus* aquoferric complex in Fig. 1A. Since tryptophan difference spectra are expected to be three times larger than for tyrosine, given the same change in environment [13, 20], this means that the change in the tryptophan(s) environment in leghaemoglobins is minor compared to that for the tyrosine(s) in myoglobins. Cyanate (mixed-spin) and fluoride (high-spin) gave aromatic peaks nearly as intense as those given by cyanide (low-spin), imidazole (low-spin) and azide (mixed-spin).

Binding of Rose Bengal to the Apoproteins of Myoglobin and Leghaemoglobin

Coulson and Yonetani [21] have used the anionic, aromatic dye rose Bengal as a sensitizer in photo-oxidation studies on a number of apohemoproteins including apomyoglobin. They showed that this dye bound strongly at one site but weakly at a number of other sites in all of these apoproteins and that this binding could be impaired by addition of stoichiometric amounts of haem. We have used rose Bengal as a probe of the haem pocket in apomyoglobin and apoleghaemoglobin. Its main usefulness for this purpose is that it does not present any of the complications with spin and oxidation states that make it difficult to interpret unambiguously many of the changes that occur in haemoproteins.



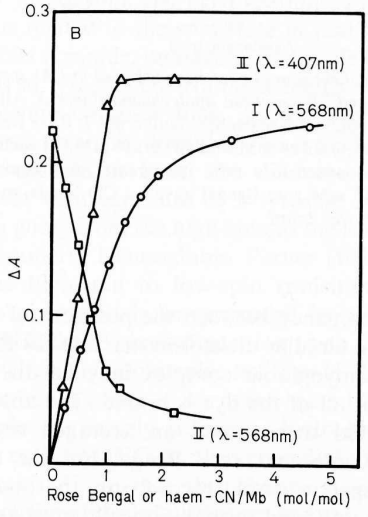


Fig. 3. *Difference spectra produced when soybean apoleghaemoglobin a saturated with rose Bengal is titrated with cyanoferric haem.* (A) The curve labelled 0 is the difference spectrum for leghaemoglobin · rose Bengal *versus* free rose Bengal. Curves 1 – 6 are the difference spectra produced by additions of cyanoferric haem (concentration of haem increasing by 0.9 μM on each addition). Apoleghaemoglobin a was 3.5 μM in sodium bicarbonate (50 mg/l adjusted to pH 7.5). A four-cell arrangement was used. (B) Plot of the binding data in (A). Curve I is a plot of the size of the difference peak at 568 nm when apoleghaemoglobin a is titrated with rose Bengal (representing binding of the dye). Curves II show that when the result of curve I is titrated with cyanoferric haem the difference peak at 568 nm decreases in size while the difference peak at 407 nm (representing haem binding) increases in proportion

In Fig. 3A is shown a typical haem competition experiment after rose Bengal had been bound to soybean apoleghaemoglobin a. The cyano complex of free haem was used because this is known to be monomeric in solution. The difference absorption band due to the binding of cyano-haem is well separated from that due to the bound rose Bengal and

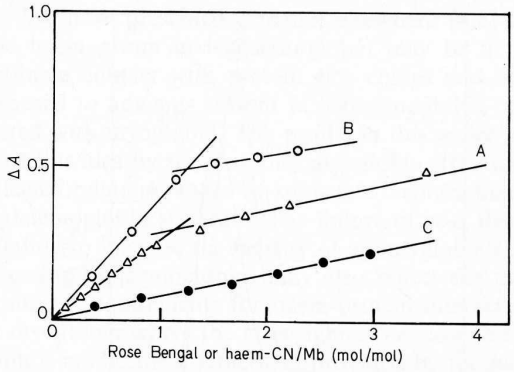


Fig. 4. *Plot of the binding data for rose Bengal and cyanoferric haem to sperm whale apomyoglobin followed by difference spectroscopy.* Curve A is the size of the difference peak at 407 nm when the apoprotein is titrated with cyanoferric haem. Curve B is the size of the difference peak at 568 nm when the apoprotein is titrated with rose Bengal. Curve C is the size of the difference peak at 568 nm when the result of curve A is titrated with rose Bengal. Apomyoglobin concentration was 6.9 μM in 3 ml sodium bicarbonate (50 mg/l, pH 7.5) and consecutive 1, 3 or 6 μl additions of stock rose Bengal (2.91 mM) or cyanoferric haem (2.69 mM) were made each time

it can be clearly seen that as the cyano-haem binds to the protein there is a stoichiometric release of bound rose Bengal. The binding of rose Bengal to apoleghaemoglobin a and the competition with haem is plotted in Fig. 3B. This shows that rose Bengal binds to only one high-affinity site in apoleghaemoglobin a and since it is stoichiometrically released from this site by haem this is probably the haem pocket (though we cannot rule out a strong anticooperative linkage between two different sites).

Fig. 4 shows the results of a similar binding study of rose Bengal to apomyoglobin. In confirmation of the conclusions of Coulson and Yonetani [21] it can be seen that there is one high-affinity site for rose Bengal in apomyoglobin but there are, in addition, a number of weaker binding sites. This is shown by curve (C) which indicates that binding of the dye will still occur even when the pocket is occupied by the haem.

It is clear from these data that, if the rose Bengal concentration is held below that of the apoprotein of either myoglobin or leghaemoglobin, the dye will be bound only at the haem site and the spectral properties of such a complex between 400 and 600 nm will reflect the haem environment. Such spectra are shown for apomyoglobin and apoleghaemoglobin in Fig. 5. The spectrum of rose Bengal is shifted by about 20 nm to higher wavelengths when incorporated into these apoproteins and this is consistent with the transfer from aqueous solution to a non-polar environment as evidenced by the spectral changes that occur when rose Bengal is transferred from water to ethanol. Thermal perturbation difference spectra properties of these complexes are described by Nicola and Leach [8].

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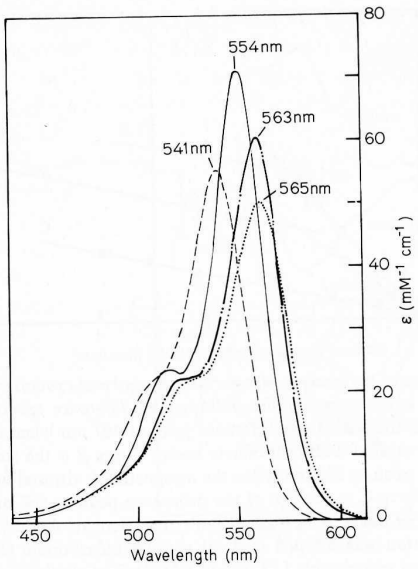

Fig. 5. *Absorption spectra of rose Bengal in (– – – –) water, (——) ethanol, (. . . . .) bound to apomyoglobin and (— · · —) bound to apoleghaemoglobin a.* Peak absorption wavelengths are indicated. For all spectra, rose Bengal concentration was 20 μM. Apomyoglobin and apoleghaemoglobin concentrations were also 20 μM in 0.1 M sodium phosphate buffer, pH 7.0

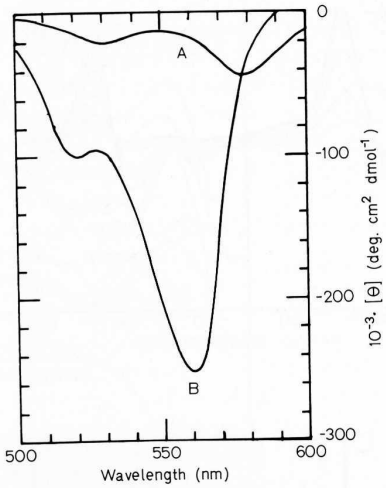

Fig. 6. *Circular dichroism of rose Bengal bound to (A) sperm whale apomyoglobin and (B) soybean apoleghaemoglobin a.* All samples were in 0.1 M potassium phosphate buffer, pH 7. Rose Bengal concentration was (A) 0.104 mM and (B) 0.134 mM. In each case the apoprotein was added in a ratio just greater than equimolar. A solution of 0.15 mM rose Bengal gave no CD bands on its own. Cell pathlength was 2 mm

CD Effects with Rose Bengal

The binding of rose Bengal had no significant effect on the far-ultraviolet CD spectrum of apomyoglobin but increased $[\theta]_{\bar{w}}$ at 220 nm of soybean apoleghaemoglobin from −16300 to −19300 deg. cm^2 dmol^{-1} at pH 7.05, suggesting that the dye stabilises apoleghaemoglobin in much the same way as does the haem group [4].

Fig. 6 shows the CD spectra for rose Bengal bound to apomyoglobin and apoleghaemoglobin a. A solution of rose Bengal free in solution gave no CD peaks but it showed strong negative CD bands when bound to apoleghaemoglobin and much weaker negative CD bands when bound to apomyoglobin. The wavelength positions of the CD peaks in these two complexes show the same trend as seen in corresponding absorption spectra: that is, the peaks are red-shifted on binding. It is not known in which direction the transition moments of rose Bengal point when the dye is bound in the haem pocket but these data are consistent with the postulate that the protein side chains in leghaemoglobin interact with transitions in both the x and y directions of the haem to give negative rotational strengths whereas in myoglobin there is a substantial cancellation of positive and negative strengths so that the CD spectrum can be slightly positive or negative depending on which component predominates [22].

The discrepancy between the positions of the absorption and circular dichroism maxima for the rose-Bengal · apomyoglobin complex may be due to the fact that not all of the dye is bound. The absorption spectra would then record an averaged result for bound and unbound rose Bengal but the circular dichroism spectra would record only the bound dye because the unbound form is optically inactive. Thus, if binding is incomplete, the distinction between the polarity of the leghaemoglobin and the non-polarity of the myoglobin pocket is accentuated because the true absorption maxima would be 578 nm for rose-Bengal · apomyoglobin (from circular dichroism) and 563 nm for rose-Bengal · apoleghaemoglobin (from circular dichroism and absorption). It would, however, decrease the large difference in ellipticity between the rose-Bengal · apomyoglobin and the rose-Bengal · apoleghaemoglobin complexes.

DISCUSSION

The blue shift of leghaemoglobin absorption spectra relative to corresponding myoglobin spectra, by analogy with solvent effects on other chromophores (see *e.g.* [13, 20]), probably indicates a more polar (aqueous) environment of the haem group in leghaemoglobin (see also [8]). This idea is also supported by the blue shift of absorption and CD spectra of the rose-Bengal · apoleghaemoglobin complex relative to

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those of rose-Bengal · apomyoglobin if our assumption that rose Bengal binds in the vacant haem pocket is correct.

Ultraviolet-difference spectra caused by replacing the sixth ligand in both haemoproteins indicate that such replacements perturb aromatic amino acids. The degree of such perturbations is difficult to assess because of the necessity to subtract out the changes in haem absorbance. Because haem itself shows no fine structure changes in its spectrum between 260 and 300 nm when the sixth ligand is changed, our approach to this problem has been to simply draw a smooth connecting line between spectral points at 270 and 300 nm as an approximation; this is the kind of difference spectrum obtained for haemin cyanide (low-spin) *versus* haemin chloride (high-spin) when protein perturbations are absent (Fig. 2).

For myoglobin, the perturbation of aromatic residues is related to the spin state in that the low-spin complexes (cyanide, imidazole, and azide) show larger changes in tyrosine environment relative to the high-spin acetate complex than do the other high-spin complexes (cyanate, fluoride). For cyanide and imidazole complexes at least, the difference spectra are consistent with exposure of a tyrosine residue (blue shift) in going from the high-spin to the low-spin state. For tetrameric haemoglobin Perutz [10] has shown that the high-spin to low-spin transition occurring upon oxygenation is accompanied by the expulsion of Tyr-H22 from a buried position to an exposed one. Sperm whale myoglobin also has Tyr-H22 in the same position and it seems to serve the same function as in haemoglobin; namely, it forms a hydrogen bond to a peptide carbonyl (FG-5), thus cross-linking the F and H helices [23], so that this change is probably the source of the observed difference spectra.

In contrast to this, the difference spectra for leghaemoglobin show (smaller) perturbations of tryptophan residues upon ligand exchange without the pronounced difference in response between high-spin and low-spin complexes. Even the bulky ligand, nicotinic acid, does not create a significantly larger perturbation to the structure than the other ligands.

There is a high degree of structural homology between myoglobin and leghaemoglobin revealed by sequence alignments [24–29] and the recent X-ray structure of lupin leghaemoglobin [30]. Pursuing this structural homology, neither myoglobin nor leghaemoglobin contain tyrosine or tryptophan residues in or very near to the haem pocket and this implies that ligand exchange and especially a high-spin to low-spin transition causes rather extensive structural rearrangements through the protein which involve these residues. These rearrangements may be more significant in myoglobin than in leghaemoglobin since the perturbation of the tyrosine residue is much greater than that of tryptophan in leghaemoglobin.

We have presented evidence elsewhere [4,8] that the haem group in leghaemoglobin may be in less intimate contact with protein side chains and more exposed to aqueous solvent in leghaemoglobin compared with myoglobin. The results in this paper support this idea by showing that myoglobin structure is affected relatively more by spin-state changes than is leghaemoglobin structure. The failure of rose Bengal binding to increase the helicity of apomyglobin (as it does for leghaemoglobin) may also reflect the more stringent requirements for haem-protein interactions in myoglobin where the fit is tighter (*i.e.* rose Bengal cannot mimic the interactions provided by the haem in native myoglobin but can in the more flexible leghaemoglobin pocket).

The concept of a more flexible, open haem pocket in leghaemoglobin helps to explain many of the properties of leghaemoglobin compared with myoglobin, in particular its ability to bind bulky ligands and its high oxygen affinity. The absence of Tyr-H22 in leghaemoglobin also means that a tyrosine is not transferred from a non-polar to an aqueous environment (an energy-requiring process) upon oxygenation (high-spin to low-spin transition) further accounting for the higher oxygen affinity of leghaemoglobin compared with myoglobin.

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