Haem-binding-site heterogeneity and haem Cotton effects of *Glycera dibranchiata* monomeric haemoglobins

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The five major components of the monomeric haemoglobin from *Glycera dibranchiata* were separated and characterized by absorption spectroscopy, isoelectric focusing, azide-binding affinities and nitrosyl autoreduction kinetics. The differences found among the components are discussed in terms of haem-pocket variations. In addition, the Fourier-transform i.r. spectra of pooled monomeric haemoglobin carbonyl (Hb_mCO) and the major component carbonyl are reported. The c.d. spectra of the carbonyl and azide derivatives of the five components are compared and found to be similar. The c.d. spectra of myoglobin(II) carbonyl [Mb(II)CO] and of apomyoglobin (apoMb) reconstituted with a symmetric synthetic iron porphyrin carbonyl, *meso*-tetra-(*p*-carboxyphenyl)porphinatoiron(II) carbonyl [TCPPFe(II)CO], are compared with the c.d. spectra of pooled Hb_mCO and its TCPPFe(II)CO analogue. Hb_mTCPPFe(II)CO shows a negative Soret c.d. band whereas MbTCPPFe(II)CO produces both a negative and a positive Soret c.d. band. Displacement of the symmetric porphyrin by 8-anilidonaphthalene-1-sulphonate and the resulting fluorescence emission are reported.

INTRODUCTION

A Cotton effect in c.d. is observed at an absorption frequency if displacement of electronic charge has a rotational as well as a linear component (Beychok, 1966). Cotton effects in haem proteins are of particular interest with regard to the sign and magnitude of c.d. bands since information about the haem-binding site is reflected in the dichroic band (Hsu & Woody, 1971).

The haemoglobin of the marine invertebrate *Glycera dibranchiata* includes both polymeric and monomeric components (Vinogradov *et al.*, 1970), the latter being polydisperse with respect to cation-exchange chromatography. The medium-resolution crystal structure (Padlan & Love, 1974) of the major monomeric component reveals the alteration E7 His→Leu in comparison with most other haemoglobins and with myoglobins. Substitution of this functionally significant E7 distal residue by a residue with a hydrocarbon side chain is unusual and thus Hb_m is of considerable interest.

As early as 1973, differences in the signs of c.d. bands were reported between the haem protein of *G. dibranchiata* and other haem proteins, that of *G. dibranchiata* having a pronounced negative Soret dichroism (Harrington *et al.*, 1973). This dichroic band may be assigned to porphyrin π – π^* transitions coupled with π – π^* transitions of the globin aromatic side chains (Hsu & Woody, 1971).

The PPIXFe moiety may assume two orientations within the active site of a protein, thus producing conformers that differ by 180° rotation about the α – γ axis of the haem plane. Several haem proteins, including native myoglobin, have been shown to exist in two such

conformations, which are distinguishable by n.m.r. (LaMar *et al.*, 1978).

During the pursuit of this work, the c.d. studies of three of the components of *G. dibranchiata* were reported, with two groups concluding that ‘a reversed haem orientation corresponds to a reversed—in sign—dichroic band in the Soret region’ (Santucci *et al.*, 1988). Invocation of haem geometric effects on the sign of c.d. bands reflects the conclusion of earlier studies on lamprey haemoglobin, which showed ligand-induced reversal of the Soret c.d. band (Lampe *et al.*, 1972). Still another group concluded that for reversed orientation in sperm-whale myoglobin there appears to be almost complete cancellation of the rotational strengths arising from interactions between π – π^* transitions of the haem group and aromatic amino acid residues (Light *et al.*, 1987).

Free PPIXFe(II)L₂ in solution has the haem plane as the only symmetry element (apart from *E*; LaMar & Walker-Jensen, 1979). Thus, when the axial co-ordination sites are inequivalently occupied, these species are racemic. Upon non-random incorporation into a globin, PPIXFe(II) will exist in an enantiomeric excess and display intrinsic chirality. Synthetic porphyrins such as TCPPFe have at least three mutually orthogonal symmetry planes, only one of which is necessarily destroyed by incorporation into a globin, whose pocket provides axial inequivalence; there is then no distinction among haem±90° or 180° rotational orientations, and the chirality evidenced by the c.d. arises only from that of the globin environment in the broader sense.

Thus apoprotein reconstituted with a symmetric porphyrin cannot produce isomers by haem rotation. Incorporation of the symmetric porphyrin TCPPFe into

Abbreviations used: Hb_m, monomeric *G. dibranchiata* haemoglobin; Hb_m(III), monomeric *G. dibranchiata* iron(III) haemoglobin; Hb_mCO, monomeric *G. dibranchiata* iron(II) carbonyl haemoglobin; Mb(II)CO, iron(II) carbonyl myoglobin; Mb(III), iron(III) myoglobin; PPIXFe(II)CO, protoporphyrin IX–iron(II) carbonyl; TCPPFe(II)CO, *meso*-tetra-(*p*-carboxyphenyl)porphinatoiron(II) carbonyl; g, Kuhn anisotropy.

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apoMb and apoHb_m and the study of their resultant c.d. properties should provide a better understanding of the origin of dichroic peaks and provide insight into the above observations.

MATERIALS AND METHODS

Live *Glycera dibranchiata* were obtained from Woods Hole Marine Biological Laboratory. Erythrocytes were washed three times at 4 °C with 0.15 M-Na₂SO₄/10 mM-EDTA and lysed with distilled water, after which the cell ghosts were centrifuged down. The heavy (oligomeric) and light (monomeric) haemoglobin components were separated by chromatography on Sephadex G-75. The buffer used throughout was pH 7 phosphate buffer (1 mM-EDTA/0.1 M-potassium phosphate buffer, pH 7 and *I* 0.2) unless otherwise indicated.

Myoglobin was Sigma Chemical Co. type III (horse), purified by passage over Sephadex G-75.

meso-Tetra-(*p*-carboxyphenyl)porphine was generously given by Dr. Frederick R. Longo. Iron incorporation was achieved by a previously published method (Dorough *et al.*, 1951). The metalloporphyrin was recrystallized from HCl solution, washed with deionized water and dried for several days in vacuum over solid KOH. It should be noted that solutions of the Fe(II)CO form of this porphyrin oxidize more readily at pH 7 than do similar solutions of PPIXFe(II)CO. This is reasonable considering the added anionic charge.

Ethyl methyl ketone (Aldrich Chemical Co.) was purified by shaking it with an acidic solution of KMnO₄, and then distilling it from an aqueous NaHCO₃ solution. NaH₃ was Aldrich Chemical Co. reagent grade purified by recrystallization from ethanol. Haemin [PPIXFe(III)Cl] was type III (horse), from Sigma Chemical Co. 8-Anilinonaphthalene-1-sulphonic acid from Sigma Chemical Co. was recrystallized from hot water, and other reagents were used as supplied from Sigma Chemical Co., Aldrich Chemical Co. and Fisher Scientific Co. Gases were from MG Industrial Gases.

The protein nitrosyl complexes were obtained by flushing the methaemoglobin solutions for 30 min with N₂ (Boil-off from liquid N₂) followed by direct reaction of NO with the iron(III) protein. The NO was scrubbed with 5 M-KOH. The protein carbonyl complexes were obtained by passing CO over the protein solution, which contained the minimal amount of Na₂S₂O₄ necessary to reduce the protein.

ApoHb_m was prepared by using a modified version of the ethyl methyl ketone procedure first described by Teale (1959). After haem extraction, the apoHb_m was dialysed against 5 mM-NaHCO₃ solution, deionized water and several changes of pH 7 phosphate buffer. The sample was then centrifuged to remove any denatured protein. Even after multiple extractions, approx. 2% of the protein appeared to retain its porphyrin, as indicated by the Soret band. This value may be slightly higher than that normally encountered with myoglobin (< 1%; Light *et al.*, 1987). A 5 mM solution of the porphyrin was made by dissolving a weighed amount of porphyrin in minimal 0.1 M-NaOH and then diluting with pH 7 phosphate buffer.

ApoMb was prepared in the same manner as apoHb_m. ApoMb concentration was determined from its absorbance at 280 nm by using an absorption coefficient of 15.9 mm⁻¹·cm⁻¹ (Harrison & Blout, 1965). The absorp-

tion coefficient for PPIXFe(II)CO in pH 7 phosphate buffer was determined experimentally by making equally concentrated solutions of PPIXFe(II)CO in pH 7 phosphate buffer and 2% sodium borate buffer, pH 9.1. The Soret maximum shifts from 408 nm to 409 nm in moving from borate to phosphate buffer at the above pH values. The absorption coefficient in phosphate buffer is then derived from a knowledge of the absorption coefficient in borate buffer (Antonini & Brunori, 1971), giving ϵ 181 ($\pm$ 5) mm⁻¹·cm⁻¹ in pH 7 phosphate buffer.

Isoelectric focusing was performed on a Bio-Rad Laboratories model 1405 horizontal-bed cell, with the use of precast gels (LKB Ampholine PagPlates) with a pH range of 3.5–9.5. These experiments were performed at 10 °C with a linear power increase from 4 W to 15 W over 60 min. Before application on the gel, the protein samples were dialysed against 10 mM-glycine, pH 7. The gels were stained by a modified version of the method of Merrill *et al.* (1981), which employed decreased AgNO₃ concentration and increased number of aqueous washings, resulting in decreased background and higher resolution.

U.v.-visible spectra and kinetic data were recorded on a Perkin-Elmer Lambda-3B spectrophotometer thermostatically controlled to $\pm$ 1 °C. The nitrosyl kinetic data were collected at 393 nm, as this spectrophotometer caused some photoenhancement (Addison & Stephanos, 1986) at longer wavelengths. Fourier-transform i.r. measurements were made with a Perkin-Elmer model 1800 Fourier-transform i.r. spectrophotometer coupled with a PE series 7000 computer. Spectra of the carbonyl adducts of Hb_m were the sums of 300–500 interferograms from which were subtracted an equivalent number of scans of equally concentrated Hb_m(III)(OH₂). A CaF₂ cell with a path-length of 0.1 mm was used and the temperature was maintained at 25 °C.

Fluorescence spectra were obtained on a Perkin-Elmer 204A fluorescence spectrophotometer, and c.d. spectra were obtained on a Jasco J41C instrument, calibrated with ammonium (+)-camphor-10-sulphonate recrystallized from ethanol (Pearson *et al.* 1979). The absorption and c.d. data were digitized manually, and the Kuhn anisotropy (*g* versus λ) spectra (Gillard, 1968; Addison & Dougherty, 1982) were computer-generated. Error limits on derived quantities are quoted as $\pm \sigma$.

RESULTS AND DISCUSSION

G. dibranchiata absorption spectra and isoelectric focusing of monomeric haemoglobin components

The isolation of Hb_m components was based on a previously published cation-exchange chromatographic method (Parkhurst *et al.*, 1980). Although the components show similar pI values (see below), the components are readily separated by cation-exchange chromatography. Our separation utilized methaemoglobin. Hb_m(III)(OH₂) tends to undergo autoreduction on standing, and therefore pooled monomer of *G. dibranchiata* was oxidized with Fe(CN)₆³⁻ immediately before the chromatography on the Sephadex CM-50 column. The entire separation was performed at 4 °C and monitored continuously at 280 nm. The major fractions were quantified at three wavelengths: 280, 393 and 505 nm. The resultant ranges are reported in Table 1. The molecular-mass homogeneity of the components was verified by passage of individual components over a

Table 1. Some properties of *G. dibranchiata* haemoglobin and other haem proteins

The percentage abundance ranges of the components of *G. dibranchiata* separated by cation-exchange chromatography are detailed, together with their isoelectric points. Logarithms of azide-binding constants were determined spectrophotometrically at 20 °C.

Haem protein	Abundance (%)	pI (± 0.1) of Hb _m (III)	log K_f of Hb _m (III) or Mb(III)
HbA	10–13	7.2	4.41 (± 0.01)
HbB	8–13	7.2	4.47 (± 0.01)
HbC	37–54	7.2	4.23 (± 0.01)
HbD	4–6	6.0	4.34 (± 0.02)
HbX	7–12	7.3	3.26 (± 0.04)
Pooled	—	—	4.27 (± 0.02)*
Mb (sperm whale)	—	—	4.51 (± 0.01)†
Mb (horse)	—	—	4.43 (± 0.01)‡

* $K_f = 3.80 (\pm 0.26)$ (Addison & Burman, 1985).

† $K_f = 4.30$ (Antonini & Brunori, 1971).

‡ $K_f = 4.40$ (Smith & McLendon, 1980).

calibrated Sephadex G-75 column; the molecular mass was 17 ± 0.5 kDa. A Sephacryl S-200 column showed apparent cation-exchange properties with resultant abnormal molecular-mass values for Hb_m and some standard proteins, and was therefore not used further.

Several groups have used Roman numerals to label the fractions; however, the same numerals used by different groups do not correspond to the same protein components (Parkhurst *et al.*, 1980; Kandler *et al.*, 1984). To alleviate this ambiguity, we report the components using a more recent convention (Cooke & Wright, 1985a,b); the components are termed HbA, HbB, HbC, HbD and HbX (see Table 1).

Fig. 1. Absorption spectra of Hb_m components

The absorption spectra of aquamet derivatives of Hb_m components were determined in pH 7 phosphate buffer at 20 °C. The samples were scanned from 700 to 450 nm and are here compared qualitatively.

Table 2. Optical absorption spectroscopy and nitrosyl autoreduction kinetic results for Hb_m components

Visible absorption-band wavelengths of the iron(III) aqua derivatives of the Hb_m components show little variation, whereas differences among the autoreduction rates are evident.

Hb _m component	$\lambda_{\max.}$ (nm) for Fe(III)H ₂ O (± 1 nm)		$10^4 \times K$ for nitrosyl autoreduction at 20 °C (s ⁻¹)
	β -Band	Soret band	
HbA	506	393	1.22 (± 0.01)
HbB	504	393	1.70 (± 0.03)
HbC	506	392	1.80 (± 0.03)
HbD	505	393	1.00 (± 0.01)
HbX	507	394	1.12 (± 0.01)
Pooled	505	393	1.27 (± 0.01)

Before their absorption spectra were obtained, the individual components were oxidized with a slight excess of $K_3Fe(CN)_6$ and then chromatographed on a Sephadex G-25 gel-filtration column equilibrated with pH 7 phosphate buffer. The spectra of the aquamet derivatives of individual components are compared qualitatively in Fig. 1 and Table 2; the maxima for the individual components are very close or the same, and the resultant spectra appear similar. The spectra of the nitrosyliron(III) derivatives of the components were obtained and the resultant α/β -bands were sharp and unsplit. For the iron(III) nitrosyl $\alpha = 564$ nm and $\beta = 530$ nm, and for the iron(II) nitrosyl $\alpha = 578$ nm and $\beta = 545$ nm. The values for the iron(III) nitrosyl were tabulated incorrectly in a previous publication (Addison & Stephanos, 1986). Splitting of the bands in Mb(III)NO has been attributed to the non-degeneracy of the $e_g(\pi^*_{x,y})$ orbitals of the porphyrin resulting from the d_{xz}, d_{yz} non-degeneracy under the influence of the distal histidine residue (Addison & Stephanos, 1986). Thus the Fe(III)NO spectra provide evidence for the absence of the distal histidine residue from all of the components.

The pI values from our isoelectric-focusing experiment reported in Table 1 compare well with previously reported results (Addison *et al.*, 1980; Cooke & Wright, 1985a,b). In our isoelectric-focusing experiments, HbA repeatedly formed a large percentage of apoprotein on the gel during focusing, as seen by the formation of a band at pI 7.7. This band was identified as apoprotein by using the criteria of a recent study (Constantinidis & Satterlee, 1987). It is interesting that HbA should form apoprotein so readily in view of its small M value relative to HbB and HbC, where $M = K_{co}/K_o$ (Parkhurst *et al.*, 1980). Model systems reveal that M decreases as a function of solvent polarity because charge separation within the Fe–O₂ complex is stabilized by a more polar environment (Suslick *et al.*, 1984). It appears likely that the more open haem crevice of HbA affords it greater solvent contact, generating a lower value of M and destabilizing the porphyrin binding.

Evidence for a more open or exposed haem site comes from analogous observations concerning the abnormal human haemoglobin Torino (CD1, Phe→Val; Dickerson

& Geis, 1983). Substitution with valine removes the support that holds the haem group tilted and pushed into the pocket. Thus the substitution favours a more out-of-the-pocket position and causes loss of haem (Fermi & Perutz, 1981). Among the components, the Phe-CD1 (where CD1 represents position 1 on the non-helical segment between helices C and D) group moves progressively away from the haem group in the order HbC, HbB, HbA, with HbA having its Phe-CD1 furthest removed from the haem group (Cooke *et al.*, 1987).

Azide binding

Studies of the binding of small molecules to haem proteins to elucidate the factors that govern haem reactivity have been the effort of many groups (Smith & Williams, 1968; Lampe *et al.*, 1972; Appleby *et al.*, 1973; Beetstone *et al.*, 1976; Addison & Stephanos, 1986; Rohlf *et al.*, 1988). Beetstone *et al.* (1976) attributed the lower azide affinity of Hb_m (compared with that for a typical mammalian methaemoglobin) to the close proximity of the iron atom to an aspartic acid residue. They suggested that, upon neutralization of the positive charge of the haem iron(III) by azide, the aspartic acid residue moves to a different position with a consequent re-adjustment of other charged groups on the surface of the molecule. Since the above hypothesis involves positional differences of groups relative to the haem group, one might also observe differences in the c.d. spectra among the components if azide-binding affinities vary.

We therefore chose to examine the binding of this ligand to each component, since binding behaviour might reflect differences in haem-pocket environment. The method of calculation of the azide-binding constant (K_f) has been given elsewhere (Giacometti *et al.*, 1981; Addison & Burman, 1985). K_f has been shown to be a function of both the type and the concentration of buffer used (Antonini & Brunori, 1971; Seamonds *et al.*, 1971; Addison & Burman, 1985), and the binding enthalpy is quite pH-dependent (Beetstone *et al.*, 1976). The binding affinities of sperm-whale and horse myoglobins were re-examined as experimental controls.

Table 1 reveals that there are only small differences in azide affinity among the components HbA, HbB and HbC. Phe-CD1 has been implicated in steric interactions in the binding of azide with Mb(III) (Rifkind, 1973). Phe-CD1 may also thus be a factor in azide binding to Hb_m. Among the three components, HbC, with its Phe-CD1 closest to the haem group, binds azide to the least degree. Component HbX, which shows greatly decreased azide binding, may have an unusual haem-pocket environment. Aside from HbX, the binding affinity of the components on the whole do not differ greatly from those of horse or sperm-whale myoglobin, which presumably indicates the decreased role of the distal histidine residue in the binding of azide. If the distal histidine residue were involved in a hydrogen bond with the iron-bound azide in the myoglobin samples, one might expect to see significantly larger binding constants for Mb versus Hb_m. The decreased role of the distal histidine residue in the binding of azide is even more evident if one considers the more polar nature of bound azide as compared with bound O₂.

The anomalous binding constant of HbX provides motivation for a c.d. study of the azide-bound components, since structural features responsible for the anomaly may be reflected in c.d. spectra (see below).

Nitrosyl autoreduction

In addition to the above experiments to probe structure variations among the components, autoreduction kinetics were monitored (Addison & Stephanos, 1986). This autoreduction corresponds to the reduction of nitrosyl-iron(III) haemoglobin to nitrosyliron(II) haemoglobin in the absence of exogenous reductants other than NO. Under pseudo-first-order conditions for the nitrosyl-iron(III) protein (0.1 MPa NO), all of the components show linear first-order plots over at least 3 half-lives. The value reported here for the pooled monomer (see Table 2) is slightly less than that originally reported, as correction has been made here for photoenhancement due to the spectrophotometer (Addison & Stephanos, 1986). A computer-generated rate curve, with quantitative contributions from each component to the rate, gives a rate constant of $1.50 \times 10^{-4} \text{ s}^{-1}$. This value is higher than that of pooled (before CM-50 separation) Hb_m and is accounted for in terms of the inherent stabilities of the components. Because HbA readily forms apoprotein under conditions where the other components are relatively stable, it is probably not quantitatively represented in the fractions collected in the cation-exchange chromatography, owing to approx. 25% denaturation on the column. Minor streaking in between the major bands on the column is observed during the separation and may well represent this partial haem loss, since the Soret band/280 nm absorbance ratio is unusually low for the minor bands.

A positive correlation for the three major components is found between the nitrosyl autoreduction rate and the O₂ off-rate (Parkhurst *et al.*, 1980) of Hb_mO₂. Off-rates for O₂ in haem proteins have been shown to be affected by doming of the haem group and the electronic nature of the proximal Fe-imidazole bond, so that small changes in haem geometry can effect large changes in reactivity (White *et al.*, 1979). For Hb_m, then, small differences among the components' haem environments may account for the differences in nitrosyl autoreduction kinetics. For example, one difference that has been shown involves the position of the E7 leucine residue, which in HbA is significantly closer to the haem normal (Cooke *et al.*, 1987), and HbA shows dramatically lower rates relative to HbB and HbC. Comparison of the three components finds that the non-polar residue is closer to the haem normal in the order HbA $\gg$ HbB $>$ HbC (Cooke *et al.*, 1987) and may be partly responsible for generating the reverse order HbC $>$ HbB $\gg$ HbA for the rate constants.

In addition, it is well known that O₂-dissociation rates in haems are sensitive to solvent polarity. The off-rate varies inversely with the solvent polarity, so that as the solvent polarity increases O₂ off-rates decrease, an observation consistent with the dipolar nature of the bound O₂ (Traylor *et al.*, 1981). Thus it is not unreasonable that nitrosyl autoreduction should also be influenced by haem-site polarity, since the iron-nitrosyl complex is polar (Addison & Stephanos, 1986).

Fourier-transform i.r. analysis

The stretching frequency for Hb_mCO has been reported to be 1970 cm^{-1} at 29 °C; it was concluded that distal effects, in particular the absence of a distal histidine residue, were responsible for the unusual stretching frequency (Satterlee, 1984). We sought to compare the value obtained for the pooled sample with that of the

Fig. 2. Fourier-transform i.r. spectra of Hb_m pooled and HbC carbonyls

The carbonyl stretching frequencies for the pooled and the major component were determined at an Hb_m concentration of 0.5 mM. Average spectrum accumulation times were 30 min.

individual components. The pooled sample gave a value of 1966 cm^{-1} . The major fraction, HbC , gave a value of 1967 cm^{-1} (Fig. 2). One would thus expect that the remaining components have stretching frequencies less than 1967 cm^{-1} , so that their contribution would bring the value into the 1966 cm^{-1} region. Unfortunately, the high protein concentrations needed for the Fourier-transform i.r. experiment for the remaining components could not be achieved and their $\nu_{\text{C-O}}$ values could not readily be determined.

C.d. studies

(1) Hb_mN_3 and Hb_mCO . Fig. 3 shows the c.d. spectra of several of the components in their azidomet forms. All of the components show broad similarities and minor differences. With regard to the low binding affinity of HbX , the variation in the c.d. spectrum is no greater for HbX than for any of the components. Apparently, the residue or residues involved in decreasing the binding affinity of HbX are not uniquely coupled to the Soret c.d. signal.

Determination of the c.d. spectra of the carbonyl-iron(II) derivatives (Fig. 4) of all the components was important for our studies of the pooled monomer with

Fig. 3. C.d. spectra of azidomet forms of Hb_m components

Azidomet forms were generated by adding excess NaN_3 solution (pH 7 phosphate buffer) to each Hb_m component. The Soret c.d. band maxima are at 428 nm. $\Delta\epsilon$ values were estimated by using a millimolar absorption coefficient of $131\text{ mM}^{-1}\text{ cm}^{-1}$ (Seamonds *et al.*, 1971).

the symmetric porphyrin (see below), since the sign of the band is at issue. Fig. 4 clearly shows that all of the components generate a negative Cotton effect at 426 nm. The g values range from -1.3×10^{-4} to -1.6×10^{-4} .

(2) $\text{Hb}_m\text{TCPPFe(II)CO}$. Iron was incorporated into the symmetric porphyrin and the resultant metallo-porphyrin was oxidized and titrated with NaOH . The spectrum of the hydroxoiron(III) porphyrin was compared with that of the related tetraphenylporphyrin

Fig. 4. C.d. spectra of the carbonyliron(II) derivatives of Hb_m components

The carbonyl derivatives each show a negative Soret c.d. maximum at approx. 426 nm. $\Delta\epsilon$ values were estimated by using a millimolar absorption coefficient of $232 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Seamonds *et al.*, 1971).

compound (Dorough *et al.*, 1951) and found to be similar.

A 30% excess of TCPPFe(II)CO was added to the apoHb_m, which was then passed over a short Sephadex G-25 column to remove any non-bound haem. The Soret c.d. and g value (-1.3×10^{-4}) showed little change over several days. The striking result here is the production of a negative dichroic band seen at 428 nm (Fig. 5). The incorporated symmetric porphyrin produces a negative Cotton effect similar to that of native PPIXFe(II)CO. The component rotational strengths, which contribute to

Fig. 5. C.d. spectrum of Hb_mTCPPFe(II)CO

A negative Soret c.d. band at 428 nm is found for Hb_m reconstituted with a symmetric porphyrin; a similarly negative band at 425 nm is found in the native protein.

the overall sign and magnitude, are a function of relative orientation and distance between the haem group and aromatic side chains (Hsu & Woody, 1971). The similar g values of native Hb_mCO and Hb_mTCPPFe(II)CO clearly demonstrate that the chirality of the globin environment is an important factor in producing the overall negative dichroic band.

N.m.r. data for the components (Cooke & Wright, 1985a,b, 1987; Cooke *et al.*, 1987) were compared with the experimentally determined values of g from our c.d. experiments (Table 3). Previous studies of various myoglobins have suggested a relationship between the magnitude of the c.d. signal and the percentage haem disorder (Bellelli *et al.*, 1987; Light *et al.*, 1987). Here we compare g values, since minor differences in absorption coefficients among the components may exist. However, from Table 3 it is not at all clear, because of the large ranges in the n.m.r. data and temperature differences among the experiments, that a correlation between the magnitudes of the g values and the percentage haem reversals among the components actually exists. The difference between correlations drawn for a species of myoglobin (Light *et al.*, 1987) and such attempts for monomeric haemoglobin components is that the components have minor structural differences that could complicate the result. This very fact may provide the impetus for a future parallel quantitative c.d. and n.m.r. study of the components.

Table 3. C.d. and n.m.r. data for Hb_m components

Kuhn anisotropy values of the carbonyl adducts of the components (present work) are compared with calculated values for percentage reversed haem from n.m.r. studies of the components (Cooke & Wright, 1985a,b, 1987; Cooke *et al.*, 1987).

Hb _m component	$10^4 \times g$ at 25 °C (from c.d.)	Percentage reversed haem at 40 °C (from n.m.r.)
HbA	-1.6	70-80
HbB	-1.3	70-80
HbC	-1.4	90-95
HbD	-1.5	90-95
HbX	-1.3	-

(2a) ApoHbC + PPIXFe(II)CO. Incorporation of haem into apoprotein results initially in the formation of equal amounts of haem rotational isomers (LaMar *et al.*, 1984; Levy *et al.*, 1985). Upon incorporation of PPIXFe(II)CO into apoHbC, a positive Soret c.d. signal of $g = +1.1 \times 10^{-4}$ is produced by the 50/50 mixture of rotational isomers. The native protein produces a Soret g value of -1.4×10^{-4} , with approx. 95% of the haem in the reversed form. The data of Lecomte & LaMar (1985) indicate that native horse myoglobin contains approx. 5% reversed haem and we observe a Soret value of $g = +3.8 \times 10^{-4}$, whereas a freshly reconstituted 50/50 mixture of rotational isomers produces a g value of $+1.9 \times 10^{-4}$. During the prosecution of this work, Aojula *et al.* (1988) showed that a linear relationship exists between $\Delta\epsilon$ and isomer composition. Adopting a viewpoint similar to that used by Hawn *et al.* (1979), we might consider the chiroptical properties of the haem to arise from two sources: (i) that asymmetry associated with the haem orientation (coupling strongly with vicinal globin chromophores) and (ii) that arising from the more distant effect of globin environment. Thus for native HbC we may write $-1.4 \times 10^{-4} = 0.95a + b$, where 0.95 is the fraction of the haem groups in the reversed orientation, a represents the globin-reversed haem orientation interaction, b is a constant contribution from globin environment and -1.4×10^{-4} is the g value for the native protein. Using the data for the native proteins and the 50/50 rotational isomer mixtures, we can determine these coefficients for HbC and Mb.

For HbC $a = -5.5 \times 10^{-4}$ and $b = +3.8 \times 10^{-4}$, and for Mb $a = -4.4 \times 10^{-4}$ and $b = +4.1 \times 10^{-4}$. The interesting result here is the larger value of a for HbC relative to Mb. This difference in values between Mb and HbC represents the difference in globin-reversed haem orientation interaction in the two proteins, and is also evident in the differing appearances of the c.d. spectra of the two proteins reconstituted with the symmetric porphyrin.

Our data are consistent with those of Aojula *et al.* (1988), who predicted a small negative Soret c.d. signal for the reversed haem in Mb; the much larger value seen for HbC is now understandable in the light of the very different globin environments.

The conclusion regarding the relationship between reversed haem orientation and a negative Soret c.d. pointed out by Santucci *et al.* (1988) seems justified for Hb_m. However, the apparent discrepancy pointed out by Santucci *et al.* (1988) between their prediction of a negative signal for Hb_m and the work of Light *et al.* (1987), who predicted a small or near zero value for Mb, is now resolved: globin environment effects are significant and must be considered in the discussion of Soret c.d. signals.

(3) MbFe(II)PPIXCO and MbTCPPFe(II)CO. A 30% excess of PPIXFe(II)CO was added to the apoMb, which was then treated as described above for Hb_m. The c.d. and absorption spectra were monitored for 1 month.

The symmetric porphyrin TCPPFe(II)CO incorporated into apoMb produces a c.d. spectrum quite different from those of either native MbCO or apoHb_m plus TCPPFe(II)CO (trace a in Fig. 6). It consists of two bands: a positive one at 436 nm and a negative one at 423 nm. The intensities of the bands are approximately equal. Hb_m TCPPFe(II)CO shows only one band.

Fig. 6. C.d. spectra of apoMb reconstituted with the symmetric porphyrin TCPPFe(II)CO and titrated with PPIXFe(II)CO

Upon addition of excess TCPPFe(II)CO, spectrum *a* is generated, displaying two Soret c.d. bands of opposite sign. Spectra *b*, *c*, *d* and *f* represent quantitative additions of PPIXFe(II)CO equalling 0.15, 0.45, 0.90 and 1.5 times the amount of the protein present. Spectrum *e* represents a 1.5 fractional excess of PPIXFe(II)CO incorporated into apoMb for comparison with spectrum *f*.

Incorporation of the symmetric porphyrin into apoMb gave a less stable product than did incorporation into apoHb_m. Upon treatment of apoMb with the symmetric porphyrin TCPPFe(II)CO, in the same fashion as described for PPIXFe(II)CO, only a very weak c.d. signal is generated. This signal can be intensified by the addition of excess of TCPPFe(II)CO, forcing the equilibrium towards active-site binding. Below we present evidence of active-site binding of the symmetric porphyrin TCPPFe(II)CO to apoMb.

(a) Free TCPPFe(II)CO has a Soret absorbance maxi-

mum at 416 nm. HoloMb(II)CO has a maximum at 423 nm. ApoMb plus TCPPFe(II)CO yields a value of 426 nm. Upon addition of TCPPFe(II)CO to holoMb(II)CO, the maximum shifts from 423 to 418 nm. If the equilibrium described above entailed non-haem-pocket-site binding of TCPPFe(II)CO, then one would have expected to see a Soret maximum value around 416–418 nm. We observe a value of 426 nm, which would hardly be expected for non-haem-pocket-site binding, since the holoMb Soret band is blue-shifted from 423 to 418 nm by non-haem-pocket-site binding of TCPPFe(II)CO.

(b) Upon addition of TCPPFe(II)CO to apoMb, the c.d. signal produced is distinguishable from that of the native Mb(II)CO as described above. The MbTCPPFe(II)CO signal can be gradually diminished with the concomitant formation of the native protein's c.d. signal by titration with PPIXFe(II)CO. The native porphyrin binds more stably to the haem pocket and displaces the TCPPFe(II)CO from the haem pocket (Fig. 6). The intensity and transition energy of the c.d. band thus produced is nearly identical with that of apoMb plus PPIXFe(II)CO. If TCPPFe(II)CO bound at a non-haem site were responsible for producing a c.d. signal, this would be evidenced by the production of a final spectrum in the PPIXFe(II)CO titration different from that of simply apoMb plus PPIXFe(II)CO.

(c) PPIXFe(II)CO has a β -band at 530 nm (Antonini & Brunori, 1971). Upon its incorporation into apoMb, this band shifts from 530 nm to 540 nm. Similarly, TCPPFe(II)CO has a β -band at 536 nm, which upon incorporation into apoMb shifts to 546 nm.

Fluorescence experiments

8-Anilinonaphthalene-1-sulphonic acid has been shown to bind at the haem active site in sperm-whale apomyoglobin, whereupon its fluorescence intensity increases 200-fold, and the emission maximum shifts from 515 to 454 nm (Stryer, 1965). Thus 8-anilinonaphthalene-1-sulphonic acid is an excellent probe of active-site binding.

TCPPFe(II)CO was added in excess to apoMb, generating the c.d. signal discussed above. Upon addition of portions of 8-anilinonaphthalene-1-sulphonic acid, the fluorescence emission intensity maximum at 460 nm increased (365 nm excitation). The 8-anilinonaphthalene-1-sulphonic acid was added until the emission intensity reached a plateau, at which point the Soret c.d. signal intensity was 20% its original value. Thus equilibrium displacement of the symmetric haem by 8-anilinonaphthalene-1-sulphonic acid provides additional evidence for the active-site binding of TCPPFe(II)CO.

CONCLUSIONS

The variations in azide-binding affinities and nitrosyl autoreduction rates are reflections of structural variations among the Hb_m components. These results are significant, since they present chemical and spectroscopic means for determining structure–function relationships for amino acid substitutions.

The factors that govern the sign and magnitude of the c.d. signal are intimately bound to the structural details of the haem pocket and coupled chromophores. Thus the negative Soret c.d. band of native Hb_m results from reversed haem orientation and from contributing negative rotational strengths resulting from the relative posi-

tions of the coupled haem and protein chromophores. The sign and number of observed bands in the Soret c.d. spectrum depends on both the energy difference and the relative magnitude of the haem transition moments. These in turn are affected by, among other things, haem-pocket environment, as seen in the differing appearance of the Soret c.d. spectra of Hb_mTCPPFe(II)CO and MbTCPPFe(II)CO.

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