Kinetic and spectroscopic studies of haemoglobin and myoglobin from *Urechis caupo*

Distal residue effects

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Seven components of the tetrameric haemoglobin (Hb_u) from *Urechis caupo* were separated by preparative isoelectric focusing and characterized by their absorption spectra and pI values. The helix content and Soret $\Delta\epsilon$ values are reported for several of the components. Temperature-jump O₂-binding kinetics of the major components of Hb_u show biphasic behaviour, with the majority species having $k_{on} = 1.57 \times 10^9 \text{ mol}^{-1} \cdot \text{s}^{-1}$ and $k_{off} = 3.32 \times 10^4 \text{ s}^{-1}$. The Fourier-transform i.r. spectrum of pooled Hb_u(II)–CO displays a stretching frequency of 1942 cm⁻¹. E.s.r. of Hb_u(II)–NO demonstrates evidence of proximal strain similar to that encountered in T-state human haemoglobin. CO-driven reduction of *U. caupo* methaemoglobin, Hb_u(III) and *U. caupo* metmyoglobin [Mb_u(III)] shows much higher rates relative to haemoglobins and myoglobins known to possess a distal histidine residue. Nitrosyl auto-reduction kinetics of Hb_u(III)–NO and Mb_u(III)–NO are examined. The equilibrium binding constants of several ligands are reported for both Hb_u and Mb_u, and together with the above kinetic data suggest differences in haem pocket environments between Hb_u and Mb_u. Reaction of Hb_u with 2-chloromercuri-4,6-dinitrophenol demonstrates the presence of one reactive thiol group per globin chain. λ_{max} values and the respective molar absorption coefficients for selected ligand-bound states are reported for the major component of Hb_u and for Mb_u. The majority haem orientation in *U. caupo* haemoglobin is identical with that of human haemoglobin.

INTRODUCTION

The Phylum Echiura represents a small division of marine worms with extremely sensitive probosci; these probosci probably serve as chemoreceptive centres (Meglitsch, 1972). *Urechis caupo*, an echiuroid worm, lives in U-shaped burrows in sandy mud along the western coast of the United States (Woodward *et al.*, 1928; Redfield & Florkin, 1931) and is commonly referred to as the fat-innkeeper worm on account of its feeding habits. In *U. caupo*, a relatively large worm, coelomic fluid rich in erythrocytes is moved throughout the length of the body by peristaltic waves, as there is no defined circulatory system (Pritchard & White, 1981).

U. caupo haemoglobin (Hb_u) is tetrameric and has a molecular mass of 62700 Da (Garey & Riggs, 1986). Isoelectric dispersity of Hb_u has been reported for the pooled protein, and such dispersity is apparent even after chromatography of the pooled protein on an ion-exchange medium (Hall *et al.*, 1981). Neither homotropic nor heterotropic interactions have been demonstrated for Hb_u; the latter feature is demonstrated by the observation of similar O₂ affinities for both the intact coelomic cells and stripped haemoglobin (Mangum *et al.*, 1983). Additional O₂-binding studies of the stripped haemoglobins showed that the common effectors ATP, inositol hexakisphosphate, Cl⁻, Ca²⁺, Mg²⁺ and CO₂ have no effect on Hb_u's O₂-binding properties, and thus it was concluded that no allosteric modulator is present in the erythrocyte (Mangum *et al.*, 1983; Garey & Riggs, 1984).

An interesting feature of Hb_u is the presence of a glutamine residue in the distal site (Garey & Riggs, 1986), which replaces the distal residue histidine common to most haemoglobins and myoglobins (Krishnamoorthi *et al.*, 1984).

Additionally, a 0.5 nm- (5 Å-)resolution crystallographic study

indicates a novel subunit arrangement described as an 'inside-out' quaternary structure with the G/H helices located on the outer surface of the tetramer (Kolatkhar *et al.*, 1988). Also, the sequence indicates the presence of a proline residue at F-6, only two residues away from the proximal histidine residue (Garey & Riggs, 1986). This proline residue seems to cause a shift in the F helix and could in part be responsible for the fact that the F helix is isolated and not involved in any intersubunit contacts. The isolation of the F helix is thus consistent with the non-co-operative behaviour of this protein (Garey & Riggs, 1986; Kolatkhar *et al.*, 1988).

Little is known about the myoglobin from *U. caupo* (Mb_u). Gel-permeation studies indicated an apparent molecular mass of 19000 Da; charge heterogeneity is evident, and Mb_u appears to have a larger binding affinity for O₂ than Hb_u, although no numerical data have been reported (Hall *et al.*, 1981). Binding and kinetic characterizations, then, of Mb_u and Hb_u from *U. caupo* will further develop our understanding of these interesting proteins.

MATERIALS AND METHODS

Live *Urechis caupo* (5–15 cm in length) were obtained from Sea Life Supply Co., Sand City, CA, U.S.A. Care was taken to wash the animals with sea-water before collection of the erythrocytes, since the proboscis secretes a mucus-like substance. Coelomic fluid was removed by syringe and then further by lengthwise incision of the invertebrate. Erythrocytes were washed by centrifugation 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. Yellow material, described previously as amoeboid cells (Redfield & Florkin, 1931), was separated from

Abbreviations used: PPIX; protoporphyrin-IX; Hb, haemoglobin; Mb, myoglobin; Hb_u, *Urechis caupo* haemoglobin; Mb_u, *Urechis caupo* myoglobin; Hb_m, monomeric *Glycera dibranchiata* haemoglobin; Hb_o, oligomeric *Glycera dibranchiata* haemoglobin; Mb_l, *Aplysia limacina* myoglobin; Hb_A, human haemoglobin; Hb_e, elephant haemoglobin; Mb_e, elephant myoglobin; g, Kuhn anisotropy.

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the haem protein by successive passes of the protein over a Sephadex G-75 column or by $(\text{NH}_4)_2\text{SO}_4$ precipitation of the protein according to a method previously described (Brown, 1961). The buffer used throughout was pH 7 phosphate buffer (2 mM-EDTA/0.1 M-potassium phosphate buffer, pH 7 and I 0.2) unless otherwise indicated.

U. caupo Mb_u was isolated from the body-wall muscle by using literature methodology (Rice *et al.*, 1979; Fosmire & Brown, 1976; Brown, 1961): the red muscle was homogenized in deionized water in a Waring blender and the Mb_u was precipitated with $(\text{NH}_4)_2\text{SO}_4$. After redissolution in buffer, the Mb_u was separated from a minor Hb component by successive passes over a Sephadex G-75 column.

Human Hb (Hb_A) was drawn from one of the authors (T. J. D.), and purified and stripped of 2,3-bisphosphoglycerate by standard literature methods (Antonini & Brunori, 1971, pp. 2–5; Imamura *et al.*, 1972).

Elephant whole blood was obtained from a superficial vein of a mature female Indian elephant at the Philadelphia Zoological Society. After clotting and separation of the serum, the pellet was broken mechanically, and the erythrocytes were washed with 0.15 M- Na_2SO_4 /10 mM-EDTA iso-osmotic solution. After the cells were lysed with distilled water, the centrifuged elephant Hb (Hb_e) solution was twice chromatographed on Sephadex G-75 in 0.1 M-NaCl to remove 2,3-bisphosphoglycerate (Antonini & Brunori, 1971, pp. 2–5; Tomita & Riggs, 1971; Imamura *et al.*, 1972), and all the haem protein was obtained as a single band.

Butan-2-one (Aldrich Chemical Co.) was purified by shaking it with an acidic solution of KMnO_4 and then distilling it from an aqueous NaHCO_3 solution. NaN_3 was Aldrich reagent grade purified by recrystallization from ethanol. 1-Methylimidazole (Sigma Chemical Co.) was distilled under an atmosphere of N_2 . 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-NO complexes were obtained by flushing the metHb solutions for 30 min with N_2 (boil-off from liquid N_2) followed by direct reaction of NO with the protein. The NO was scrubbed with 5 M-KOH. The protein-CO complexes were obtained by passing CO over the protein solution, which contained the minimal amount of $\text{Na}_2\text{S}_2\text{O}_4$ necessary to reduce the protein. Excess $\text{Na}_2\text{S}_2\text{O}_4$ was separated from the protein by using a Sephadex G-25 column.

ApoHb was prepared by using the butan-2-one procedure first described by Teale (1959). Haem analysis was performed by using the pyridine haemochrome method (de Duve, 1948; Appleby, 1969). Protein concentrations are expressed in terms of haem molarity.

Analytical isoelectric focusing was performed on a Bio-Rad Laboratories model 1405 horizontal bed cell, with 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 onto the gel, the protein samples were dialysed against 10 mM-glycine, pH 7. The gels were stained by using a modified version of the method of Merrill *et al.* (1981) that employed decreased AgNO_3 concentration and increased number of aqueous washings, resulting in decreased background and higher resolution. Preparative isoelectric focusing was performed in a 11 cm × 9 cm × 1 cm gel matrix, prepared by mixing 5.3 g of LKB Ultradex electrofocusing gel with 84 ml of water and 7.5 ml of Pharmalyte pH 6.5–9 carrier ampholyte (Pharmacia Fine Chemicals). The slurry was allowed to stand overnight, protected from CO_2 , before use. The preparative electrofocusing was performed under reduced light intensity at 5 °C at a constant power of 10–15 W. A total run time of approx. 8–10 h was required. The pH profile

of the gel was measured by using an Ingold-6124 combination surface electrode in conjunction with a Beckman pH-meter. Ampholytes were removed from the protein as previously described (LaGow & Parkhurst, 1972).

Reaction of the protein's thiol groups with 2-chloromercuri-4,6-dinitrophenol was accomplished by adding a 10-fold molar excess of the mercurial to a solution of the protein with slow stirring for 15 min. The protein was then passed over a Sephadex G-25 column twice to remove any unused mercurial. A solution of protein at the same concentration was used as the blank for the spectrophotometric determination of the organomercury derivative concentration. The absorption coefficient of the resultant protein derivative was estimated (Means & Feeney, 1971) by determining the absorption coefficient of mercurial-modified cysteine: a weighed amount of the mercurial reagent was dissolved in methanol and then diluted with buffer so that the concentration of methanol was less than 5%. Excess L-cysteine was added and the measurements were made at 373 nm, as this wavelength provided the least amount of absorbance interference from electronic transitions in the protein; ϵ_{373} was $14400 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for the mercurial-modified cysteine. Reaction of the protein with *p*-chloromercuribenzenesulphonic acid is as described elsewhere (Means & Feeney, 1971).

U.v.-visible spectra and kinetic data were recorded on Perkin-Elmer Lambda-3B and Perkin-Elmer 320 spectrophotometers thermostatically controlled to ± 1 °C. The method of calculating the equilibrium binding constants is given elsewhere (Giacometti *et al.*, 1981a). The nitrosyl kinetic data were collected periodically at 565 nm with the sample beam blocked in between readings, as these spectrophotometers caused some photo-enhancement (Addison & Stephanos, 1986; DiFeo & Addison, 1989a). CO auto-reduction kinetics were performed as described previously (Bickar *et al.*, 1984). O_2 -binding kinetics were performed on the major Hb_u component E at pH 7.4 in 0.1 M-Tris/HCl buffer at 20 °C as described previously (Yamamoto *et al.*, 1974). Fourier-transform i.r. measurements were made with a Perkin-Elmer model 1610 Fourier-transform i.r. spectrophotometer coupled with a Dell System 200 computer. The Hb concentration was approx. 0.5 mM. The spectrum of the CO adduct of Hb_u was the sum of 1000 interferograms, from which was subtracted the same number of scans of equally concentrated Hb_u(II)- O_2 . A CaF_2 cell with a path length of 0.1 mm was used, and the temperature was maintained at 25 °C. E.s.r. spectra were obtained at 77 K on a Varian E-12 X-band spectrometer, calibrated near $g = 2$ with diphenylpicrylhydrazyl. The absorption and c.d. data were digitized manually and the Kuhn anisotropy (g versus λ) spectra (Gillard, 1968; Addison & Dougherty, 1982; DiFeo & Addison, 1989a) were computer-generated. C.d. spectra above 300 nm are reported as $\Delta\epsilon$ or g (Kuhn anisotropy) versus λ and spectra below 300 nm are reported as $[\theta]_\lambda$, the mean residue ellipticity in units of $\text{deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$. Helix contents were determined in 10 mM-phosphate buffer, pH 7, by the method of Chen *et al.* (1972). The mean residue molecular mass is estimated as 110 Da for Hb_u (Harrington *et al.*, 1973).

RESULTS AND DISCUSSION

Absorption spectra and isoelectric focusing of Hb_u components and Mb_u

Previous attempts to isolate Hb_u components by anion-exchange chromatography have been reported in the literature (Hall *et al.*, 1981; Garey & Riggs, 1984). In each instance multiple bands were seen upon gel electrophoresis and ion-exchange chromatography of the isolated components. Similar attempts here to separate the components by anion-exchange chromatography on a DEAE-cellulose column yielded equally

Table 1. Isoelectric components of Hb_u

Components A, B, C and D had not previously been isolated. F-I, F-II and F-III refer to the nomenclature previously proposed by Garey & Riggs (1984).

Component	pI (±0.2)	Abundance (%)	Soret Δε (M ⁻¹ ·cm ⁻¹)	Helix (%) (±5 %)	Fe(II)-CO <i>A</i> _{Soret} / <i>A</i> ₂₈₀
A	8.4	1-5	—	—	3.32
B	8.2	1-5	—	—	3.36
C	7.9	1-5	+37	67	3.91
D	7.7	1-5	+40	62	3.43
E (F-I)	7.4	80-90	+39	66	3.57
F (F-II)	7.0	5-10	+40	69	3.81
G (F-II)	6.7	5-10	+45	71	3.61

little success, as multiple bands were apparent upon focusing of the major band isolated from the anion-exchange experiment. We therefore decided to isolate the components via preparative isoelectric focusing. The Fe(II)-CO form of the protein was chosen as an experimentally convenient substrate, as this form of the protein proved easy to generate and quite thermodynamically stable. Care was taken to protect this photolabile system from light. Table 1 lists the components, which are labelled alphabetically with the letter A representing the most cathodic peak. Components E, F and G are identified as the components F-I, F-II and F-III previously isolated (Garey & Riggs, 1984). We identify four additional minor bands A, B, C and D not previously isolated, which lie cathodic to the major band. These four bands are not simply artifacts of the focusing, as refocusing of the major band E displays only one component. It is then apparent from the published elution profile of the previous anion-exchange experiment (Garey & Riggs, 1984) that bands A-D are co-eluted as part of the F-I component in anion-exchange chromatography.

For each component the ratio of the Soret absorbance to the 280 nm absorbance was determined to see whether any of the bands in Table 1 could be identified as apoprotein. The presence of apoprotein should result in a band or bands shifted cathodically to the major holoprotein, and bands A, B, C and D fit this criterion. If we consider each holoprotein subunit of the tetramer to have a similar absorption spectrum (Antonini & Brunori, 1971, pp. 120-122), then each unit would contribute essentially equally to the Soret absorption. Data for fraction E show a Soret absorbance of 1.068 and 280 nm absorbance of 0.299. Thus removal of one haem unit should decrease the Soret absorbance to approx. 0.801, producing a new ratio of 2.68:1. None of the components has a ratio this small. Thus we conclude that the presence of components A, B, C and D is not the result of haem loss. In addition, haem loss in Hb_A is known to produce a decrease in helix content (Yip *et al.*, 1972); however, we find similar helix content for all the components where sufficient quantity allowed for the measurement (see below).

The pI values of two major components from Hb_u isolated from anion-exchange chromatography have been previously reported (Hall *et al.*, 1981); they report a major band at pI 8.5 and a minor band at pI 7.4. The major band in our experiments focuses at pI 7.4, a value similar to that found for other Hbs (Keilin & Hartree, 1951). We are uncertain as to the origin of the difference between our data and the unusually high value of pI 8.5 reported by Hall *et al.* (1981).

Isoelectric focusing of Mb_u(II)-CO shows one band with pI 6.5.

Absorption spectra of haem proteins in the Fe(III) state

We have compared the visible spectra of several haem proteins

Fig. 1. Absorption spectra of Hb_u and Mb_u

The absorption spectra of Fe(III) (met) states of Hb_u and Mb_u were determined in pH 7 phosphate buffer (0.1 M) at 20 °C. The samples were scanned from 700 to 450 nm and are here compared quantitatively.

in the Fe(III) state, focusing special attention on the bands pointed out by Brill & Williams (1961) in their discussion of Fe(III) proteins. Fig. 1 shows the Fe(III) solution absorption spectra of Hb_u(III) and Mb_u(III). We have obtained the Fe(III) spectra of *Glycera dibranchiata* Hb (Hb_m) and *Aplysia limacina* Mb (Mb_L) as well. The medium-resolution crystal structure (Padlan & Love, 1974) of the major monomeric component of *G. dibranchiata* Hb reveals the alteration E7-His→Leu, and crystallographic studies of Mb_L confirm the alteration E7-His→Val (Giacometti *et al.*, 1975; Bolognesi *et al.*, 1985, 1989). The Fe(III) spectra of human Hb_A, horse Mb, sperm-whale Mb and elephant Hb_u appear to be quite similar to one another; these proteins each possess a distal histidine residue (Braunitzer *et al.*, 1982; Dickerson & Geis, 1983; Mabbutt & Wright, 1985; Stephanos & Addison, 1990). Quantitative comparison of the Fe(III) spectra of the distal histidine-containing proteins, which appear to be six-co-ordinate in their Fe(III) state (Antonini & Brunori, 1971, pp. 40-48; Perutz *et al.*, 1974; Krishnamoorthi *et al.*, 1984), with Mb_L and Hb_m, both of which are five-co-ordinate Fe(III) proteins (Giacometti *et al.*, 1981a,b; Rousseau *et al.*, 1989; D. L. Rousseau & J. J. Stephanos, unpublished work), reveals marked differences. The absorbance ratios of the 500 nm to 630 nm bands are shown in Table 2 for the Fe(III) species. It is apparent that Hbs and Mbs that co-ordinate H₂O in the Fe(III) state have a ratio of approx. 2.3-2.6:1, whereas both the five-co-ordinate Hb_m(III) and Mb_L(III) haem proteins have much larger ratios, 4.72:1 and 4.30:1 respectively. The observation that the five-co-ordinate character of Hb_m and Mb_L appears to be reflected in their Fe(III) absorption spectra is perhaps not surprising in view of the well-known effect of axial co-ordination on the absorption spectra of haem proteins (Eaton & Hochstrasser, 1967, 1968).

The results of the study of the absorbance ratios above place Mb_u in the six-co-ordinate category, though the slightly higher ratio found for Hb_u might be indicative of a system with some

Table 2. CO-driven reduction of Fe(III) proteins

Haem protein	Half-life (h)	Fe(III) A_{500}/A_{630}
Mb _u	2	2.40
Hb _m	20	4.72
Hb _u	77	3.00
Sperm-whale Mb	1200*	2.59
Hb _A	1400†	2.22
Horse Mb	No reduction	2.58
Hb _e	No reduction	2.28
Mb _L	—	4.30

* Half-life at 25 °C in 0.1 M-phosphate buffer, pH 7.5 (Bickar *et al.*, 1984).

† Half-life 1000 h at 25 °C in 0.1 M-phosphate buffer, pH 7.5 (Bickar *et al.*, 1984).

contribution from a five-co-ordinate species, which is consistent with the fluoride-binding studies below.

The spectra of the Fe(III) cytochromes *c'* show bands at 493 nm and 632 nm (Strekas & Spiro, 1974; Maltempo, 1976; Maltempo & Moss, 1976; Moore *et al.*, 1982). Cytochrome *c'* is the generic name for a class of haem proteins that have been isolated from both purple photosynthetic bacteria and a denitrifying bacterium species (Strekas & Spiro, 1974). Fe(III) cytochromes *c'* have quantum-mechanically mixed mid-spin ($S = \frac{3}{2}$) and high-spin ($S = \frac{5}{2}$) haem, which is characterized by intermediate values of paramagnetic susceptibility (Maltempo, 1976). An additional interesting feature of the cytochromes *c'* is the apparent five-co-ordinate geometry in the Fe(III) state (Ambler *et al.*, 1981; Emptage *et al.*, 1981; Weber *et al.*, 1981). It has been suggested that the formation of a six-co-ordinate species might be precluded by close packing of residues about the distal haem surface and the lack of any internal hydrogen-bonding groups capable of stabilizing a bound polar ligand (Weber *et al.*, 1981). The absorption spectrum of *Chromatium* ferricytochrome *c'* at pH 7 shows a 493 nm/632 nm absorbance ratio of 3.85:1 (Maltempo, 1976). *Chromatium* ferricytochrome *c'* lacks a distal histidine residue (Emptage *et al.*, 1981) and appears to be five-co-ordinate (Ambler *et al.*, 1981). The crystallographic structure of *Rhodospirillum molischianum* ferricytochrome *c'* at 0.25 nm (2.5 Å) resolution shows a five-co-ordinate haem iron, without the distal site occupied by any atom from the protein side chain (Weber *et al.*, 1981). The absorption spectrum of *R. molischianum* ferricytochrome *c'* shows an absorbance ratio of 3.2:1 (Moore *et al.*, 1982). Thus it appears that the ferricytochromes *c'* follow a trend in absorbance ratios similar to that of the Hbs and Mbs studied above, i.e. absorbance ratio values of greater than 3:1 are seen when five-co-ordinate species are present. The exact quantitative relationship between the absorbance ratio and the co-ordination state of the cytochromes *c'* may, however, be different from that found for Hbs

and Mbs owing to the presence of quantum mixed-spin states, which can affect the absorbance ratio (Maltempo & Moss, 1976).

Equilibrium binding studies

To characterize further the active sites of Hb_u and Mb_u we have selected a series of ligands and compared the binding affinities of Hb_u and Mb_u with those of Hb_m, Hb_A and horse Mb (Table 3). This comparison was prompted by the result of the azide-binding affinity of Hb_u(III): as is evident from Table 4, the affinity of Hb_u(III) for azide is unusually low. A value of $\log K = 2.15$ for Hb_u(III) was obtained for both the pooled protein and the isolated major fraction E. This low azide-binding constant value is independent of Hb_u concentrations and monitoring wavelength. In stark contrast, the binding affinity of Mb_u(III) for azide is quite high ($\log K = 5.1$). As pointed out previously in the literature, a $\log K_{N_3^-}$ value of 5.7 for Hb_A(III) is considered to be high for a haem protein and has been the focus of studies set out to elucidate the origin of this unusual voracity for azide (Beetlestone *et al.*, 1976). In this regard the azide-binding affinities of Hb_u(III) and Mb_u(III) are quite interesting, representing nearly the upper and lower limits of azide-binding affinity found thus far for Hbs and Mbs (see Table 4).

The presence of an H₂O molecule in the co-ordination site for Hb_A(III) and horse Mb(III) is well documented (Antonini & Brunori, 1971, pp. 40–48; Perutz *et al.*, 1974). A comparison of the fluoride-binding affinities (Table 3) of Hb_A(III) and horse Mb(III) with that of Hb_m(III) suggests that the inability of Hb_m(III) to co-ordinate an H₂O molecule is also reflected in its low binding affinity for fluoride, i.e. the distal residue leucine is unable to stabilize the bound fluoride via a hydrogen bond. On the basis of the binding affinity of Hb_u(III) for fluoride and the absorbance ratios above, one can infer that Hb_u(III) has its equilibrium slightly shifted towards a five-co-ordinate species, although not to the degree of Hb_m. The ability of the distal glutamine residue in Hb_u to hydrogen-bond bound H₂O is consistent with n.m.r. results from Mb_e(III) (E7-His→Gln), which demonstrate that a distal glutamine residue can act as a hydrogen-bond acceptor (Krishnamoorthi *et al.*, 1984). The

Table 4. Fe(III)-azide binding affinity comparison at 20 °C

Haem protein	$\log K$ (± 0.03)
Hb _A	5.71
Mb _u	5.10
Horse Mb	4.43
Sperm-whale Mb	4.27
Hb _m	4.23
Hb _h	3.80
Mb _L	3.40*
Hb _u	2.15

* Giacometti *et al.* (1975).

Table 3. Equilibrium binding constants for selected haem proteins

Abbreviations: MEIC, 2-(*N*-morpholino)ethyl isocyanide; N.B., no binding apparent.

Ligand and iron oxidation state	Equilibrium binding constant				
	Hb _m	Hb _u	Hb _A	Horse Mb	Mb _u
Dicyanamide, Fe(III)	176 (± 20)	16 (± 2)	5 (± 2)	N.B.	324 (± 7)
Fluoride, Fe(III)	4.4 (± 2)	10 (± 2)	60 (± 1)	210 (± 7)	28 (± 4)
MEIC, Fe(II)	550 (± 10)	15 (± 10)	N.B.	4 (± 0.5)	711 (± 36)

fluoride-binding result for Mb_u(III) together with the absorbance-ratio studies (see below) are consistent with a co-ordination equilibrium dominated by six-co-ordinate species for Mb_u(III).

It is apparent concerning 2-(*N*-morpholino)ethyl isocyanide (MEIC) and dicyanamide anion that one feature of the co-ordinated ligands might be steric repulsion interactions between the ligands and distal residues due to the large size of the ligands (Antonini & Brunori, 1971, pp. 94–95; Mims *et al.*, 1983; Johnson *et al.*, 1989). The binding affinities of Hb_u(II), Hb_A(II) and horse Mb(II) for these ligands contrasts with the much larger affinities of Hb_m(II) and Mb_u(II). A scenario consistent with the above observations concerning the relative binding affinities (Table 3) is that the haem pockets of both Hb_m and Mb_u are more open, i.e. they are less susceptible to steric repulsion by ligands because of the particular special features of their haem environments. This openness is indeed evident for Hb_m in binding studies of various bulky isocyanides (Mims *et al.*, 1983), and thus our reasoning is similar to that of those authors, who used alkyl isocyanides to demonstrate unfavourable steric interactions on the distal side of the haem group in Hbs and Mbs. As we show below, these haem pocket features of Hb_m and Mb_u might be indicated in the unusually fast carbonyl auto-reduction kinetics observed for these proteins (see below).

CO-driven reduction of metHbs and metMbs

CO-driven reduction of Fe(III) haems has been found to be a general phenomenon for O₂-binding haem proteins with reduction rates dependent on temperature, pH, protein concentration and CO concentrations (Bickar *et al.*, 1984). The striking feature of the phenomenon is that the reported half-lives for reduction of Hb_A(III) and sperm-whale Mb(III) are quite large, i.e. about 1000 h, and we find that horse Mb(III) and Hb_e(III) appear to be kinetically inert. The kinetic studies reported previously were performed in 0.1 M-phosphate buffer, pH 7.5, containing 0.1 mM-EDTA at 25 °C (Bickar *et al.*, 1984). We attempted to perform similar kinetic studies under these conditions, but protein denaturation was evident. To alleviate this problem the pH was lowered to 7.0 and the temperature was lowered to 20 °C. A first-order dependence on HO⁻ ion concentration has been reported, and thus, considering our slightly lower pH and temperature, we expected that our observed half-times would be somewhat larger. Indeed, Hb_A(III) (stripped of 2,3-bisphosphoglycerate) was observed to have a half-life for reduction of 1400 h compared with a value of 1000 h reported by Bickar *et al.* (1984). We observed no significant reduction of horse Mb after 100 h under 0.1 MPa (1 atm) of CO. Hb_e showed a similar kinetic inertness, although denaturation prevented quantitative determination (see Table 2).

The rates of the reduction of Hb_u(III), Mb_u(III) and Hb_m(III) are an interesting contrast with the rather sluggish kinetics of Hb_A(III) and the other proteins mentioned above. Reduction of Hb_u(III) is approx. 20 times faster than Hb_A(III) and sperm-whale Mb(III); Mb_u(III) is reduced at a rate 500 times greater than that reported for any other Mb. We also observed the autoreduction of Hb_m(III) from *G. dibranchiata* to be much faster than previously reported rates for Hbs. One apparent difference between the kinetically sluggish globins and the kinetically rapid haem proteins Hb_m and Hb_u is the absence of a distal histidine residue in these latter globins. The rates observed for the proteins are consistent with the trend in MEIC binding. Thus these auto-reduction rates may in part reflect some distal steric phenomenon.

Additionally, it has been effectively argued that one of the key functions of the distal histidine residue is the stabilization of the Fe³⁺-O₂⁻ moiety via hydrogen-bonding (Phillips & Schoenborn, 1981; Olson *et al.*, 1988; Springer *et al.*, 1989). This function is

exemplified in the 40–350-fold increase in autoxidation of ten mutant oxyMbs where the distal histidine residue was replaced by other amino acid residues (Springer *et al.*, 1989). The distal histidine residue most probably increases the stability of the Fe(III)OH₂ species as well, since the distal histidine residue is known to hydrogen-bond with co-ordinated H₂O in Hbs and Mbs (Ladner *et al.*, 1977; Johnson *et al.*, 1989). This added stability would then be reflected in the kinetically sluggish CO-driven reduction rates of distal histidine-containing globins.

The CO-driven reduction results appear to divide these haem proteins into two basic categories: kinetically sluggish distal-histidine-containing proteins and the kinetically fast non-distal-histidine-containing proteins; this would consequently place Mb_u in the non-distal-histidine-containing category.

O₂-binding kinetics

A range of O₂ association and dissociation constants have been reported for Hbs and Mbs: $k_{on} \sim 0.01 \times 10^8 - 3 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_{off} \sim 10 - 10^3 \text{ s}^{-1}$ (Yamamoto *et al.*, 1974; Mims *et al.*, 1983). The larger rate constants of $k_{on} = 1.57 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_{off} = 3.32 \times 10^4 \text{ s}^{-1}$ found for Hb_u are perhaps consistent with the observed enhanced CO-driven reduction of this Hb, i.e. Hb_m shows a similarly enhanced CO-driven reduction rate and has O₂ on and off rates at the upper limit of the typical ranges listed above. Kinetic studies detailing the quenching of protein fluorescence by O₂ (Lakowicz & Weber, 1973; Jameson *et al.*, 1984) suggest that a diffusion-controlled rate of O₂ binding most probably approaches $k \sim 10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$. Thus the observed rate constant for Hb_u is probably not diffusion-controlled, although the rate appears to be the highest recorded for an Hb thus far. Fig. 2 shows a plot of the concentration-dependence of $1/\tau$ for the reaction of O₂ with the major component of Hb_u fraction E.

The appearance of a second minor component (approx. 20%) in the kinetics might be explained by the presence of an additional component with nearly the same isoelectric point as the major component of fraction E. The rate constants of the minor component could not be determined reliably owing to its relatively small abundance.

Nitrosyl auto-reduction kinetics

Several Fe(III)-NO Hbs have been shown to undergo auto-reduction (Addison & Stephanos, 1986; DiFeo & Addison, 1989a). This auto-reduction corresponds to the reduction of Fe(III)-NO Hb to Fe(II)-NO Hb in the absence of exogenous

Fig. 2. O₂-binding kinetic plot for Hb_u.

The Figure shows a plot of the reciprocal relaxation times for O₂ kinetics of Hb_u as a function of Hb + O₂ concentration at pH 7.4 in 0.1 M-Tris/HCl buffer at 20 °C. The intercept and slope yield k_{-1} and k_{+1} respectively.

Fig. 3. Kinetic logarithmic plot of the nitrosyl auto-reduction of Mb_u(III)-NO

The Figure shows a kinetic logarithmic plot $\ln(A - A_{\infty})$ versus time] from absorbance data taken at 403 nm during the auto-reduction of 8.9 μ M-Mb_u(III)-NO with a large excess of NO (0.1 MPa $\equiv$ 1.72 mm) in pH 7 phosphate buffer (0.1 M) at 20 °C. The process is pseudo-first-order with respect to protein.

reductants other than NO. The reaction has been shown to be photo-enhanced (DiFeo & Addison, 1989a), and therefore samples were thermostatically controlled in the dark between readings. The nitrosyl auto-reduction of Hb_u and of Mb_u each exhibits behaviour pseudo-first-order with respect to protein, as demonstrated for Mb_u by Fig. 3.

The optical spectra of the Fe(III)-NO derivatives of Hb_u and Mb_u show unsplit α -/ β -bands with $\epsilon_{\alpha} > \epsilon_{\beta}$. Splitting of the bands in horse Mb(III)-NO has been attributed to the non-degeneracy of the $e_g(\pi^*_{x,y})$ orbitals of the porphyrin resulting from the $d_{x,y}$, $d_{y,z}$ non-degeneracy under the influence of the distal histidine residue (Addison & Stephanos, 1986). The presence of unsplit bands of Fe(III)-NO cytochrome *c* peroxidase (Yonetani *et al.*, 1972) is consistent with the crystal structure (Edwards *et al.*, 1987), which suggests that the distal histidine residue is further from the haem plane than in horse Mb. Additionally, we observe unsplit α -/ β -bands of Fe(III)-NO of cytochrome *c* from *Saccharomyces cerevisiae*, the distal residue of cytochrome *c* being methionine (Creighton, 1984). The appearance of the spectra of the Fe(III)-NO derivatives of Hb_u and Mb_u is consistent with the results of CO auto-reduction kinetics, which suggest the absence of a distal histidine residue.

Fourier-transform i.r. analysis

Fig. 4 presents the Fourier-transform i.r. spectrum of pooled Hb_u. The observed C-O stretching frequency of the carbonyl ligand is 1942 cm⁻¹ with $\Delta\nu_{\frac{1}{2}} = 10$ cm⁻¹. Studies of the factors that govern the stretching frequency of bound CO have suggested various sources for the frequency-dependence (Satterlee *et al.*, 1978; Ormos *et al.*, 1988). Free protohaem-CO shows a band centred at 1964 cm⁻¹ (Ormos *et al.*, 1988), and Satterlee *et al.* (1978) proposed three protein influences that lower the frequency of the carbonyl stretch in haem proteins: (1) nucleophilic interaction, (2) bending in the Fe(II)CO unit and (3) the *trans* effect. We shall concern ourselves with the first two effects, since the *trans* effect appears to be similar in most haem proteins (Satterlee *et al.*, 1978). The nucleophilic interaction was first proposed by Caughey (1970), and entails electron-density donation from the distal histidine tertiary nitrogen lone pair to the CO π^* -orbital of the bound CO, weakening the carbon-oxygen bond and thereby lowering its stretching frequency. In Hb_u, the distal glutamine residue may similarly engage in nucleophilic interaction via its amide carbonyl oxygen donating electron density into the CO π^* -orbital. This interpretation is consistent with observations regarding another haem protein, Mb_e, which

Fig. 4. Fourier-transform i.r. spectrum of Hb_u(II)-CO

The Fourier-transform i.r. spectrum of pooled Hb_u(II)-CO shows a stretching frequency of 1942 cm⁻¹. The concentration of Hb_u(II)-CO was 0.5 mM and the average spectrum accumulation times were 45 min.

Table 5. Carbonyl stretching frequencies for Fe(II)-CO haem proteins

Haem protein	Frequency (cm ⁻¹)
Hb _m	1966*
Hb _A	1951†
Horse Mb	1944†
Hb _u	1942‡
Mb _e	1937§

* DiFeo & Addison (1989a).

† Volpe *et al.* (1975).

‡ Present study.

§ Kerr *et al.* (1985).

also contains the E7-His→Gln substitution (Kerr *et al.*, 1985). Raman studies of Mb_e show a main C≡O stretching frequency of 1937 cm⁻¹ (Table 5) and a larger CO binding constant than in sperm-whale Mb due to a lower CO off rate. The authors concluded that there exists a stabilizing interaction between the glutamine residue and bound CO that is responsible for the observed stretching frequency of CO and the enhanced stability of the carbonylated protein.

More recent studies of Mb-CO indicate a reciprocal relationship between the haem-CO normal angle and the stretching frequency of the CO (Ormos *et al.*, 1988). This effect corresponds closely to the second parameter considered by Satterlee *et al.* (1978). It is apparent, however, that a tilting, rather than an actual bending, of the linear Fe-C-O unit occurs (Kerr *et al.*, 1985; Ormos *et al.*, 1988). Tilting of the Fe-C-O unit would most probably occur if the distal residue is as sterically demanding as histidine (Springer *et al.*, 1989).

In the absence of CO kinetic data, however, we cannot exclude the steric effect as a primary source of the decreased frequency, although the glutamine residue is expected to provide less steric hindrance to the CO than histidine (Kerr *et al.*, 1985).

One might then expect to see for Hb_u(II)-CO a carbonyl frequency higher than the 1944 cm⁻¹ observed in sperm-whale Mb(II)-CO, owing to less steric interaction between glutamine and CO than between histidine and CO. The observation of 1942 cm⁻¹ in Hb_u(II)-CO is perhaps indicative of a larger-than-

Fig. 5. Cryogenic e.s.r. spectrum of $\text{Hb}_u(\text{II})\text{-NO}$

The Figure shows the first-derivative e.s.r. spectrum of $\text{Hb}_u(\text{II})\text{NO}$ at 77 K. The protein concentration was approx. 1 mM, in pH 7 phosphate buffer. The vertical intensity scale is arbitrary, and the horizontal axis (calibrated as g -factor value) is linear in magnetic field.

expected steric role of glutamine or possibly reveals a nucleophilic interaction between the carbonyl oxygen of glutamine and the carbon of the bound CO.

E.s.r. spectroscopy of $\text{Hb}_u(\text{II})\text{-NO}$

The e.s.r. spectrum of $\text{Hb}_u(\text{II})\text{NO}$ (Fig. 5) is similar to that of other $\text{Hb}(\text{II})\text{-NO}$ and $\text{Mb}(\text{II})\text{-NO}$ systems and falls into the 'type 2' classification of Yonetani *et al.* (1972), with $g = 1.968(\pm 0.003)$, $2.009(\pm 0.010)$ and $2.068(\pm 0.003)$. The interesting feature of this e.s.r. spectrum is the presence of a hyperfine coupling pattern similar to that encountered in T-state Hb (Trittelvitz *et al.*, 1975; Szabo & Perutz, 1976; Scholler *et al.*, 1979; Nagai *et al.*, 1980; Addison & Stephanos, 1986). This pattern is consistent with the preliminary report (no numerical data reported) of CO and O_2 association-dissociation kinetics in the nanosecond time regime that show very little geminate recombination, a characteristic of T-state Hb (Garey & Riggs, 1986). We conclude that in $\text{Hb}_u(\text{II})\text{-NO}$ this superhyperfine coupling intensity pattern is characteristic of strain or tensioning of the proximal Fe-N (proximal histidine) bond.

C.d. studies and determination of the molar absorption coefficient

The protoporphyrin-IX-Fe moiety may assume two orientations within the active site of a haem protein, thus producing conformers that differ by 180° rotation about the α - γ (methine carbon) axis of the haem plane. Several haem proteins, including native Mb, have been shown to exist in two such conformations, which are distinguishable by n.m.r. (LaMar *et al.*, 1978).

C.d. studies of haem proteins reconstituted with a synthetic symmetric porphyrin have demonstrated that the c.d. signal is a function not only of haem orientation but also of the surrounding globin environment (DiFeo & Addison, 1989a), and it has recently been proposed that c.d. spectroscopy can be used to deduce haem orientation in Hbs and Mbs (DiFeo & Addison, 1989b). A regression equation was developed relating the orientation of the haem (determined from n.m.r. data) in several proteins as a function of both the Soret c.d. signal of the native $\text{Fe}(\text{II})\text{-CO}$ and the non-equilibrium 50/50 mixture of rotational isomers. This regression equation enabled quantitative estimates of haem orientation to be made for proteins otherwise as yet structurally uncharacterized.

Fig. 6. C.d. spectra of $\text{Hb}_u(\text{II})\text{-CO}$ and $\text{Hb}_A(\text{II})\text{-CO}$

—, C.d. spectrum of $\text{Hb}_u(\text{II})\text{-CO}$; ----, c.d. spectrum of $\text{Hb}_A(\text{II})\text{-CO}$.

C.d. results in Fig. 6 show that the general appearance and magnitude of the Soret c.d. signal of the native $\text{Fe}(\text{II})\text{-CO}$ forms of Hb_u and Hb_A are similar. Unfortunately, attempts to produce apo Hb_u by conventional methods (Teale, 1959) proved unsuccessful. Similarly, attempts at producing the 50/50 haem rotational mixture by the pH-jump method (Aojula *et al.*, 1988) produced copious protein precipitation when the solution was returned to pH 7. We note, however, that in the case of Hb_A the g (Kuhn anisotropy) value from the c.d. pH-jump experiment measured at pH 2.3 differs by only 10% from the value produced by the protein solution returned to pH 7. Thus by measuring the Soret c.d. value at pH 2.3 for Hb_u we can estimate the g value for the 50/50 haem orientation mixture and calculate a percentage reversed haem orientation in native Hb_u of 20%. This value of 20% is again similar to that obtained for Hb_A by c.d. spectroscopy, and thus the majority haem orientation in Hb_u is identical with that of Hb_A .

Helix contents for several of the Hb_u components are listed in Table 1. These values are similar to that found for Hb_A under similar experimental conditions. A helix content of 77% has been reported for horse Mb (Chen *et al.*, 1972). C.d. studies of Mb_u reveal a Soret $\Delta\epsilon$ value of $36 \text{ M}^{-1}\cdot\text{cm}^{-1}$ and indicate a helix content of $76 \pm 5\%$, and the 0.5 nm (5 Å) structure of Hb_u suggests substantial helix content (Kolatkhar *et al.*, 1988).

Table 6 displays the calculated molar absorption coefficients for the major component of Hb_u and for Mb_u . The wavelengths of the maxima and their associated absorption coefficients are typical of Hbs and Mbs for the various ligand-bound states (Seamonds *et al.*, 1971).

Thiol-group reactivity in Hb_u

Mercurials are commonly used to probe reactive thiol groups since they react rapidly and specifically with thiol groups of proteins and the dissociation constants of the resultant mercaptides are quite small ($< 10^{-20}$) (Means & Feeney, 1971). The amino acid sequence of the major E component of Hb_u indicates the presence of one cysteine residue at position 21 in the sequence (Garey & Riggs, 1986). Initial studies of the reaction of Hb_u with *p*-chloromercuribenzenesulphonic acid, however, produced no dissociation of the tetramer. We then chose a chromophoric arylmercurial, 2-chloromercuri-4,6-dinitrophenol, to investigate the reactivity of this cysteine residue. An advantage of this reagent is that it has virtually no optical absorption at

Table 6. Optical spectroscopic properties of Hb_u major component and Mb_u

Ligand	α -Band		β -Band		Soret band	
	Wavelength (nm)	$10^{-3} \times \epsilon$ (M ⁻¹ ·cm ⁻¹)	Wavelength (nm)	$10^{-3} \times \epsilon$ (M ⁻¹ ·cm ⁻¹)	Wavelength (nm)	$10^{-3} \times \epsilon$ (M ⁻¹ ·cm ⁻¹)
Haemoglobin						
Fe(III) N ₃ ⁻	575	8.70	538	11.0	418	140
Fe(III) NO	565	16.6	531	14.5	419	141
Fe(II) CO	570	14.8	539	14.5	421	182
Fe(II) O ₂	578	15.2	542	14.3	416	118
Fe(II) NO	574	15.2	543	16.0	418	122
Myoglobin						
Fe(III) N ₃ ⁻	575	11.0	540	12.9	417	120
Fe(III) H ₂ O	—	—	505	13.6	403	128
Fe(II) CO	570	16.8	540	16.1	420	183
Fe(II) Deoxy	—	—	555	15.4	431	115

570 nm and thus the concentration of the Hb can be determined concurrently in the presence of the mercurial reagent.

Hb_u that has reacted with the arylmercurial produces $A_{373} = 0.67$ as compared with $A_{373} = 0.41$ for an equal concentration of unmodified protein ([protein] 21.2 μ M). Thus an absorbance of 0.26 arises solely from the mercurial-modified cysteine, indicating the presence of 0.86 reactive thiol group per globin chain.

Gel-permeation chromatography of Hb_u that has reacted with the arylmercurial indicates that the tetramer does not dissociate. The difference in tetramer stability of Hb_u relative to Hb_A is consistent with sequence data (Garay & Riggs, 1986), which indicate that of the 17 residues that form the $\alpha_1\beta_1$ contacts in Hb_A only two residues are identical in Hb_u; a similar comparison for the $\alpha_1\beta_2$ interface demonstrates that only two of the 13 residues are the same in Hb_u relative to Hb_A, and these sequence differences are presumably reflected in the relative stability of the Hb_u tetramer.

Conclusions

Preparative isoelectric focusing separates seven components of Hb_u in the Fe(II)-CO form. The pI value of the major component-CO and the helix content and Soret c.d. spectra of the components are typical of Hbs, but the kinetic rates for the CO-driven reduction of the Fe(III) protein and O₂ binding are unusual. The results for the CO-driven reduction of Fe(III) proteins divide the haem proteins studied into two broad categories: the kinetically sluggish distal-histidine-containing proteins and the rather kinetically rapid non-distal-histidine-containing proteins. O₂-binding studies show fast binding kinetics for Hb_u.

CO-driven reduction kinetic studies as well as the appearance of the Fe(II)-NO absorption spectra of Mb_u indicate the likely presence of a non-histidine distal amino acid, and ligand-binding studies reflect a relatively open haem pocket reminiscent of that in *G. dibranchiata* Hb.

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