Binding of CO to Mutant α Chains of Hemoglobin M Iwate; Evidence for Distal Imidazole Ligation†

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ABSTRACT: The optical contribution of the β chains to the spectrum of hemoglobin M Iwate $(\alpha^{87his\rightarrow tyr})_2\beta_2{}^A$ was subtracted with the aid of a computer so that the spectrum of ferric α chains was obtained. Tyrosinate binding to the heme is suggested from spectral resemblance to ferric heme phenolate in dimethyl sulfoxide. The slow reduction of the abnormal ferric α chains in hemoglobin M Iwate by dithionite was studied spectrophotometrically both in the presence and absence of CO. The rate of reduction was found to be dependent on the state of ligation of the normal β chains. The CO-ligated form of the reduced α chains bears strong spectral resemblance to the CO-ligated form of the reduced β chains suggesting similar structures for the heme-ligand complex. A model compound with similar optical properties to the CO-ligated protein can be prepared in dimethyl sulfoxide from hemin chloride, imidazole, and CO using chromous acetate as the heme reductant. Substitution of phenolate for imidazole produces a spectral entity so different from that observed in the protein as to rule out tyrosinate ligation to ferrous heme of the α chains when CO is bound.

The hemoglobins M constitute a group of human hemoglobins in which the hemes in either the abnormal α or β chains are oxidized and thus have lost their ability to bind oxygen (Suzuki et al., 1965, 1966; Hayashi et al., 1966, 1968; Ranney et al., 1968). Of this group, there are four in which a single histidylimidazole, either proximal or distal to the heme in either the α or β chains, is replaced by a tyrosyl residue (Perutz and Lehmann, 1968; Stamatoyannopolous, 1972). Such an example is found in hemoglobin M Iwate $(\alpha^{87his\rightarrow tyr})_2\beta_2{}^A$ where the proximal imidazoles of the α chains which are normally covalently linked to heme iron have this replacement (Miyaji et al., 1963; Shibata et al., 1964; Königsberg and Lehmann, 1965; Shimizu et al., 1965). The β chains, on the other hand, having no similar amino acid substitution near the heme, maintain the ability to reversibly bind oxygen (Hayashi et al., 1966; Gersonde et al., 1973) and carbon monoxide (Hayashi et al., 1966; Sick and Gersonde, 1974).

The single amino acid substitution, as is found in Hb M Iwate, endows the α chains with a rather unusual property. That is, unlike the β chains in this tetrameric hemoglobin, heme reduction by dithionite is extremely sluggish, taking hours instead of fractions of seconds to achieve redox equilibrium (Motokawa et al., 1964). The reduced α chains are capable of reversibly binding CO, however, and the optical properties of this CO-ligated hemoprotein are quite similar to those observed for the normal β chains in the same protein and for both types of chains of hemoglobin A where the imidazole replacement by tyrosine has not taken place.

In this communication we describe the properties of model compounds for possible heme ligand structures that might be found in CO-ligated Hb M Iwate. From these studies, we conclude that the tyrosine that is substituted for the proximal imidazole in the α chains is *not* bound to the heme when CO is ligated. It is suggested, then, that CO, which binds reversibly to the distal side of the heme in the β chains of Hb M Iwate and in both chains of hemoglobin A, is bound to the proximal side of the heme in the α chains of hemoglobin M Iwate.

Materials and Methods

The hemoglobin used in this work was Hb M Oldenburg, which has been shown to be identical with Hb M Iwate by sequence analysis of the tryptic peptide αIIX (Steffens et al., 1977). The abnormal hemoglobin was separated from the blood of an individual known to be heterozygous with respect to this mutant by a procedure described elsewhere (Gersonde et al., 1973; Mayer et al., 1973). The hemoglobin was stripped of phosphate by passage through Sephadex G-50 equilibrated with 0.1 M Tris[1]-HCl buffer (pH 8.5) containing 0.1 M NaCl. Hemoglobin concentrations were determined from the pyridine hemochromogen taking as $\epsilon_{mM}{}^{557.5} = 32$ (Appleby, 1969) using ferric sperm whale myoglobin as a primary standard (Hapner et al., 1968).

Reactions were carried out either under an argon or a CO atmosphere using a special Thunberg optical cell (lightpath 10 mm) equipped with a side arm that could be made air tight with a rubber septum stopper. Protein solutions in 0.05 M phosphate buffer (pH 6.5) and containing a tenfold molar excess of inositol hexaphosphate were equilibrated with argon (less than 1 ppm of O_2) and then with CO. Gases were saturated with water vapor using wash bottles equipped with fritted glass bubblers and containing a solution of phenazine methosulfate in contact with granular zinc. Excess sodium dithionite was introduced from the side arm of the Thunberg cuvette and the optical spectrum was scanned at various time increments with a Cary 14R spectrophotometer.

Carbon monoxide was removed from hemoglobin M Iwate that had been exposed for 18 h to dithionite and CO using a 500-W high intensity lamp. Here, the protein was gently

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EPR, electron paramagnetic resonance; Hb M Iwate, hemoglobin M Iwate.

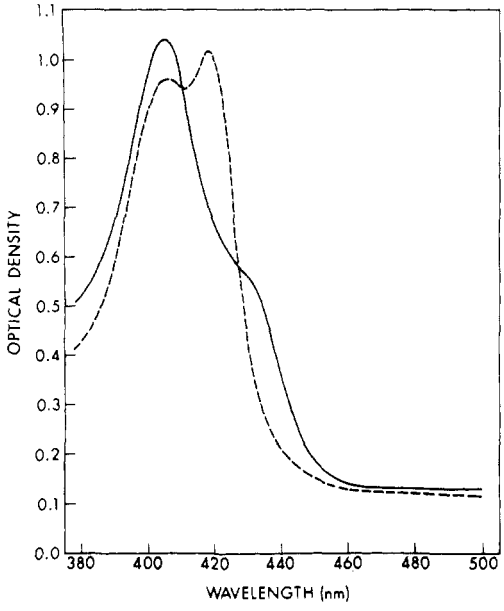

FIGURE 1: Optical absorption in the Soret region for deoxy- and carbonmonoxyhemoglobin M Iwate. For the deoxy spectrum (solid line), the protein (3.2 μM) was diluted in 10 mM K-PO_4 buffer, pH 6.5, and was equilibrated against water-saturated argon for 15 min. Clearly resolved in the spectrum is the absorption ascribed to the ferric α chain at 405 nm and the absorption ascribed to the deoxy β chains. Addition of CO (dotted lines) does not alter the position of the 405-nm peak, while a new absorption at 430 is replaced by an absorption at 418 nm ascribed to the carbonmonoxy β chains. The large absorption at 405 nm as compared with the one at 418 nm in the CO-treated protein is ascribed to the presence of oxidized β chains in the preparation (see Figure 4).

TABLE I: A Comparison of Wavelengths of Soret Maxima of Individual Chains of Tetrameric Hb M Iwate and of Heme Model Compounds[a] with Those for Hemoglobin A.

Compound	Ferric	Ferrous	Ferrous CO
Hemoglobin A[b]	405	430	419
α chains Hb M Iwate[c]	405	430	~420
β chains Hb M Iwate[c]		430	418
Hemin chloride	405	424	414
Heme + imidazole	414	426	422
Heme + sodium phenolate	402	425	415, 436
Heme + phenol	405	424	415

[a] Spectra were obtained in dimethyl sulfoxide solution. [b] From Antonini and Brunori (1971). [c] Absorption maxima for ferric α chains and ferrous β chains were determined from the spectrum of deoxygenated Hb M Iwate. For the ferrous α chains, the protein was reduced with dithionite and the spectrum was examined after 24 h. For the ferrous CO α chains, the spectrum was determined 24 h after the addition of CO and dithionite.

rocked in the Pyrex glass side arm of the Thunberg cuvette, while H_2O-equilibrated argon was passed over the surface.

In order to determine the spectral contribution of ferric α chains in hemoglobin M Iwate, only the β chains were ligated to CO while the α chains were maintained in the ferric oxidation state. This was achieved at 22 °C by reducing the hemoprotein (21.7 μM) in 50 mM phosphate buffer (pH 6.5) in the presence of a tenfold molar excess of inositol hexaphosphate with 10 mg of sodium ascorbate introduced from the side arm of a Thunberg cuvette. Before reduction, the solution was equilibrated with argon for 10 min and with CO for 5 min. After a fast initial reduction, presumably of autoxidized β chains in the preparation, the optical spectrum was stable for at least 1 h.

The optical spectrum taken between 480 and 665 nm was scanned at a rate of 15 nm/s and digitized using an Aviv Associates (Lakewood, N.J.) Cary 14 readout interface connected directly to the phototube output of the spectrophotometer. Spectra were stored as 1500 data points in a computer. Similarly, a solution of carbonmonoxyhemoglobin A of known concentration (prepared using dithionite reduction) was studied the same way and its spectrum was digitized and stored in a computer. Spectral data were subtracted by computer and plotted on a Hewlett Packard X-Y recorder connected to a time share peripheral (Danbury, Conn.) plotter-controller interface.

EPR spectra of Hb M Iwate were taken at 1.6 K on an instrument first described by Feher (1957) using cavities described by Berzofsky et al. (1971). Spectra were also recorded for the protein that was reduced with ascorbate and reacted with CO. The spectrum of hemin chloride (1 mM) in dimethyl sulfoxide to which a tenfold molar excess of sodium phenoxide was added was also taken under the same conditions.

Model compounds for the heme CO complex of α chains of hemoglobin M Iwate were prepared in dimethyl sulfoxide using chromous acetate as the heme reductant. Chromous acetate was prepared by treating an HCl-acidified solution of potassium chromium sulfate with granular zinc. After the solution turned from purple to azure blue, it was decanted into a saturated sodium acetate solution previously purged of oxygen by bubbling with purified N_2 gas. The fine red precipitate was filtered on a Buchner funnel using Whatman No. 1 filter paper, taking care not to permit air to be drawn through the precipitate. When the filtration was almost complete, two additions of 100% ethanol followed by a single addition of ether were used to wash the precipitate while on the funnel. The precipitate still adhering to the filter paper was quickly air dried and stored in small batches under oxygen-free nitrogen at −30 °C.

Hemin chloride (6.7 μM) in dimethyl sulfoxide (Burdick and Jackson) was introduced into the cell proper of a Thunberg cuvette. The solution was equilibrated with solvent-saturated argon followed by CO. The optical spectrum was recorded. Chromous acetate (2 mg) was introduced from the side arm and once more the spectrum was taken. In order to test for reversibility of reaction, the solution was purged of CO for 10 min using argon bubbled through dimethyl sulfoxide.

In subsequent experiments, imidazole (90 mM) was introduced into the Thunberg cuvette proper together with the heme (in this case 10 μM), while, in another study, sodium phenolate (20 mg) or phenol (20 mg) was added as a solid together with the chromous acetate from the side arm of the Thunberg cuvette.

Results

Protein Reduction. The optical spectrum in the Soret region of deoxyhemoglobin M Iwate at pH 6.5 is essentially a composite of that of deoxy β chains and high-spin ferric α chains, the normal β chains absorbing at 430 nm while the abnormal α chains as a shoulder at 405 nm (Figure 1, Table I). Anaerobic addition of dithionite causes an abrupt change in the optical spectrum so that there is a sharp increase in absorption at 430 nm and, at the same time, a loss of the 405-nm absorption. This change in spectrum is ascribed to the fast reduction of ferric β chains which occur as an autoxidized impurity in the hemoglobin M Iwate preparation used for the experiment. The abrupt spectral change is then followed (Figure 2) by an

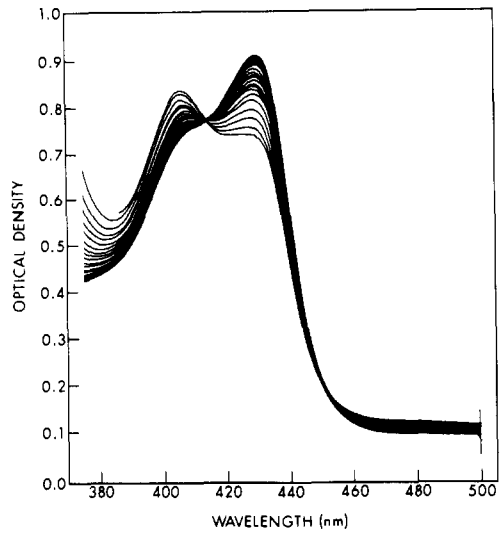

FIGURE 2: Reductive titration of hemoglobin M Iwate by dithionite. The protein (2.72 μM) contained in a Thunberg cuvette in 50 mM K-PO_4 buffer containing 25 μM inositol hexaphosphate was equilibrated with argon for 15 min. Dithionite (8 mg) was introduced from the side arm and the spectra were recorded at 4, 10, 20, 36, 40, 50, 60, 70, 80, 90, 100, 110, 130, 150, 170, 200, 230, 290, and 350 min. As can be seen, the absorption at 405 nm ascribed to the ferric α chains decreases and the absorption at 430 nm ascribed to both deoxy α and β chains increases.

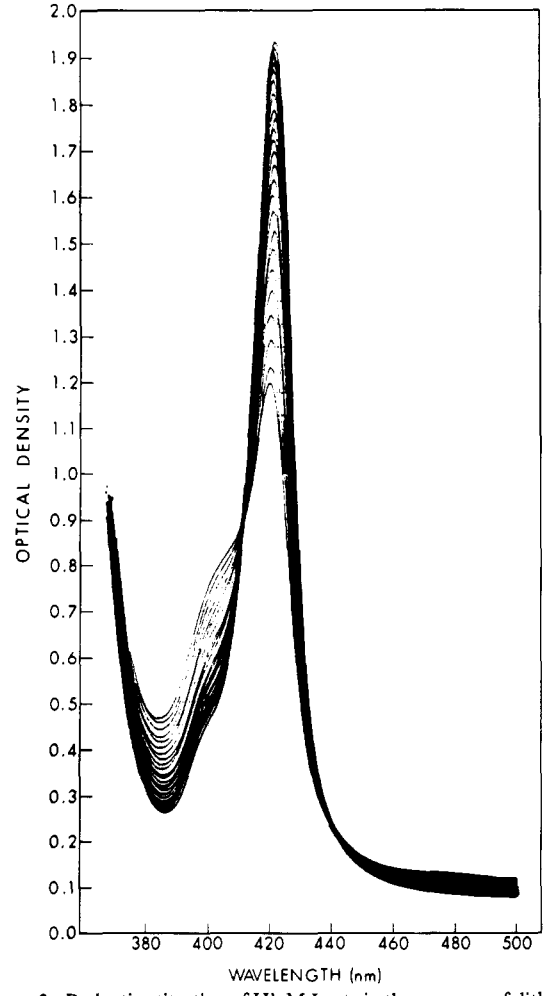

FIGURE 3: Reductive titration of Hb M Iwate in the presence of dithionite and carbon monoxide. In a Thunberg cuvette equipped with a side arm, the protein (2.72 μM) in 50 mM K-PO_4 buffer, pH 6.5, containing 25 μM inositol hexaphosphate was equilibrated for 10 min with water-saturated argon, and for 5 min with water-saturated CO. Sodium dithionite (10 mg) was introduced from the side arm and the spectra were recorded at various time intervals. During the course of reaction, the peak at 418 nm ascribed to CO-ligated heme increased and shifted to 420 nm while the peak at 405 nm ascribed to the ferric α chains decreased. Spectra were recorded at 4, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 180, 200, 220, 240, 260, 290, 320, and 380 min (time taken at spectral crossing of the 420-nm absorption). Essentially no spectral change is observed after 1000 min when the optical density of the 420 peak is 2.026.

extremely slow spectral change, exhibiting isosbestics at 415 and 450 nm, in which the 430-nm absorption increases and the 405-nm absorption decreases. Here too, the spectrum of high-spin ferric heme, ascribed to the abnormal α chains, is converted to the spectrum of deoxyhemoglobin (λ_{max} = 430 nm). If one assumes apparent first-order kinetics, the reaction has a rate constant of 15.3 $\times$ 10^{-3} min^{-1} (Figure 4). After a period of about 350 min, no further spectral change is observed. At this point, the introduction of CO causes the loss of absorption at 430 nm and the appearance of a peak at 420 nm as measured in about 2 min after mixing. The spectrum is virtually identical with that observed when Hb M Iwate is reduced by dithionite in the presence of CO (see below) and remains substantially unchanged for at least 7 h.

The CO-Bound Form. The optical spectrum of the carbonmonoxyhemoglobin M Iwate is a composite of the spectrum of both carbonmonoxy β chains (λ_{max} = 418 nm) and ferric α chains (λ_{max} = 405 nm) (Figure 1). Dithionite addition causes an abrupt spectral change due to the reduction and subsequent CO ligation to the oxidized β chains present as an impurity in the preparation. This is followed by a slow spectral change (Figure 3), isosbestic with the abrupt change (isosbestics at 412 and 440 nm) leading to the formation of a carbonmonoxyhemoglobin with a slightly shifted Soret absorption (λ_{max} = 420 nm) from that observed for the CO-ligated β chains. The apparent first-order kinetics for the reaction (Figure 4) has a rate constant of 8.67 $\times$ 10^{-3} min^{-1}, approximately half of that observed for the reduction of heme in the absence of CO.

From extrapolation of spectral data to zero time and from a measurement of the optical density taken 4 min after the addition of dithionite, we are able to calculate millimolar extinction coefficients at 420 nm for the CO-ligated form as 220 for the normal β chains. In this calculation, it is assumed that the abrupt spectral change initially observed after the dithionite addition is solely ascribable to the β chains (Table II). After 18 h reduction, the apparent extinction coefficient of the α chains is 170. If the reduction and CO binding is performed with excess sodium ascorbate instead of dithionite, the abrupt spectral change ascribed to reduction and binding of CO to β chains which were autoxidized is once more observed (Table II) but the slow spectral change ascribed to binding of CO to α chains is not observed, even after 1 h. Now adding dithionite causes the slow change ascribed to reduction and ligation of CO to the α chains at the same rate as was observed with dithionite alone.

Reversibility of CO Ligation. Once CO is bound to both the α and β chains it is removed only with great difficulty. For example, equilibrating the fully CO-ligated hemoglobin M Iwate for 15 min with gentle rocking of the solution in the Thunberg under an argon atmosphere only depresses the 420-nm absorption by 1% (Figure 5 and Table II). Under the same conditions, CO is removed completely from hemoglobin M Iwate where only the β chains are CO-ligated, carbonmonoxyhemoglobin A or even carbonmonoxymyoglobin. The removal of CO can be facilitated, however, by exposing the argon-treated solution of fully CO-ligated Hb M Iwate to

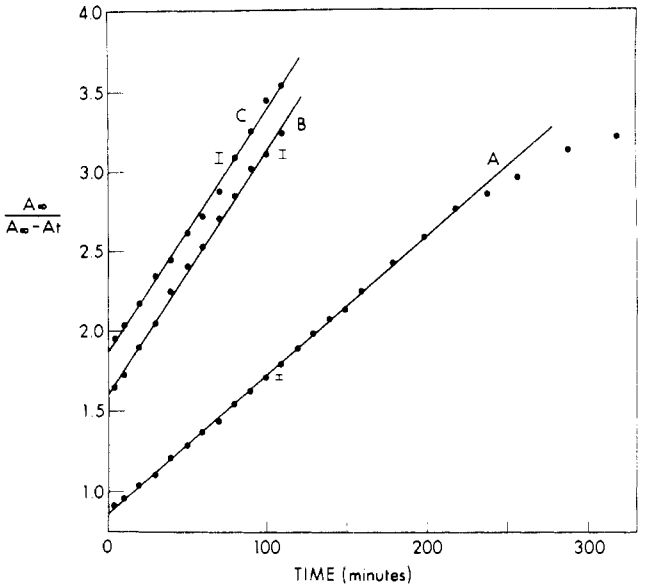

FIGURE 4: Analysis of reductive titrations of Hb M Iwate in A, the presence of CO and B and C, in the absence of CO. In this analysis, $\ln A/(A_\infty - A_T)$ is plotted against time. Here, A_∞ is defined as the optical density at the end of the reaction and A_T the optical density at time T. In curve A, the first 20 data points based on the optical density at 420 nm (Figure 2) were fit by a computer aided least-squares method. In B and C, 12 data points for the optical density at 430 and 405 nm, respectively (Figure 3), were fit the same way.

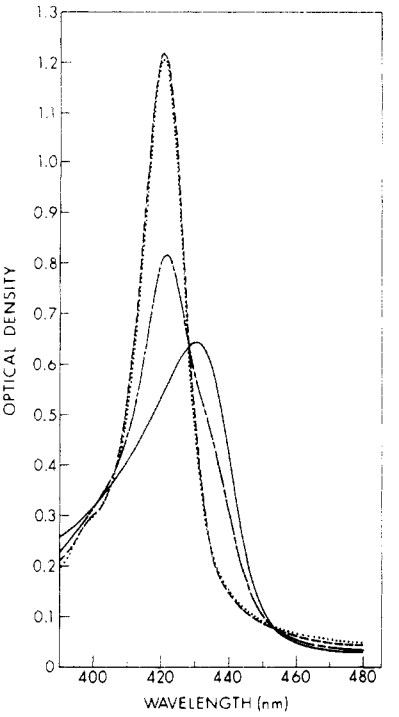

FIGURE 5: Effect of ultraviolet irradiation on the dissociation of CO from carbonmonoxyhemoglobin M Iwate. The CO-bound protein (1.66 μM) in 50 mM K-PO_4, pH 6.5, in the presence of 16.6 μM inositol hexaphosphate (- - - -) was treated with argon for 15 min (· · · ·). Only a small spectral change was effected. Irradiating with light for 5 min (- · -) and 15 min (—) under an argon atmosphere removed most of the CO.

TABLE II: Relative Absorption of the 420-nm Chromophore of Carbonmonoxyhemoglobin M Iwate.[a]

Condition	Relative absorption near 420 nm
Deoxy protein + CO	0.49
Deoxy protein + CO + ascorbate, 1 h	0.59
Deoxy protein + CO + dithionite extrapolated to zero time	0.59
Deoxy protein + CO + dithionite, 18 h	1.00
Carbonmonoxy protein + argon, 15 min	0.99
Carbonmonoxy protein + argon + light, 1 min	0.90
Irradiation, 15 min under argon + CO studied 4 min after CO addition	0.78
As above, 18 h later	0.84

[a] The reference considered to be fully ligated to CO is the protein solution reduced with dithionite under a CO atmosphere and kept at room temperature for 24 h.

strong light. Here, the irradiation was achieved by tipping the contents of the Thunberg cuvette into the glass side arm and gently rocking the solution in the light beam while argon was passed over the surface. In this way, the 420-nm absorption is depressed 10% by treatment with light for 1 min. This was accompanied by an increase of absorption at 430 nm. After 5 min irradiation, the 420-nm absorption is depressed to 60% of its initial value while after a total of 15 min, the 420-nm peak is removed from the spectrum and a single Soret band at 430 nm, characteristic of deoxyhemoglobin, is observed (Figure 5). In the visible region, one also observes a typical spectrum for the deoxy hemoprotein with a major peak at 555 nm (OD = 0.077) and a slight shoulder near 590 nm. Nowhere in the spectrum can one see indication of high-spin ferric heme or of carbonmonoxy ferrous heme.

If now CO is restored to the solution, the spectrum of the fully CO-ligated species is once more observed (λ_{max} = 420 nm) within 4 min after CO treatment with a total absorption 78% of that of the fully CO-ligated protein before irradiation (Table II). Permitting this solution to stand for 16 h under a CO atmosphere increases the 420-nm absorption to 84% of the value obtained for the fully CO-ligated protein.

EPR Spectrum of the Ferric α Chains. The EPR of hemoglobin M Iwate (Figure 6) is ascribed solely to the α chains (Watari et al., 1968) as the β chains are EPR silent either when in the deoxy state or when ligated to O_2 or CO. The spectrum is that of an $S = \frac{5}{2}$ species with nearly tetragonal symmetry and extends from the region of $g = 6$ to $g = 2$ (Peisach et al., 1971). The spectrum remains unchanged whether the β chains are ligated to CO or to O_2. Unlike ferric hemoglobin A and isolated individual ferric chains, cyanide (100-fold molar excess) has no effect on the spectrum.

The splitting of the feature near $g = 6$ is well-resolved (Figure 6A,B) and represents a departure of about 5% from axial symmetry (Peisach et al., 1971). Such splittings of the $g = 6$ feature, not observed in ferric hemoglobin A, are typical of hemoglobins M where a tyrosine phenolate has been replaced for a histidine imidazole in the vicinity of the heme. The addition of sodium phenolate to ferric heme (Figure 6C and D) elicits similar spectra, but, in this instance, the departure from axial symmetry is considerably larger.

Optical Contribution of the Ferric α Chains. The optical spectral characteristics of the abnormal ferric α chains in hemoglobin M Iwate are different from those of ferric hemoglobin A. With the use of a computer (Figure 7) we were able to strip away the optical spectral contribution of CO-ligated β chains from the protein treated with CO in the presence of ascorbate using the optical spectrum of carbonmonoxyhemo-

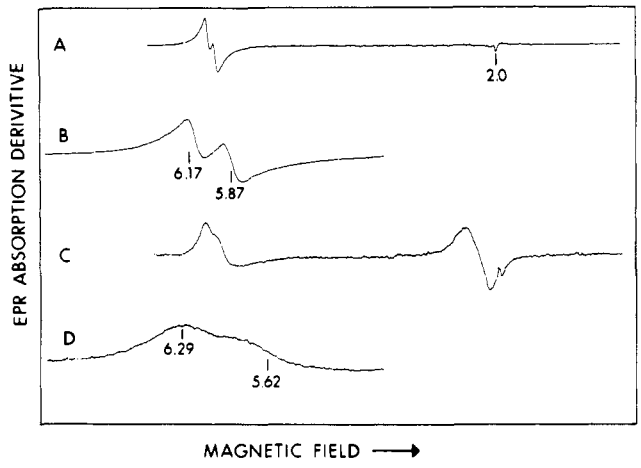

FIGURE 6: EPR spectra of the ferric α chains in hemoglobin M Iwate (A and B) and of phenolate ferric heme complex (C and D). In A and B, hemoglobin M Iwate (140 μM) in 0.01 M $K-PO_4$, pH 6.5, and 1 mM inositol hexaphosphate, was reacted with 3 mM sodium ascorbate in order to reduce partially oxidized β chains in the preparation. Spectrum B is recorded over a magnetic field of 5000 G in 2.5 min, while spectrum A was taken at one-fifth the sweep rate and is thus a fivefold expansion of the low-field features of the spectrum. In C and D, 100 μM hemin chloride was reacted with 1 mM sodium phenoxide in a 1:1 chloroform–dimethyl sulfoxide solution. Spectrum D is a fivefold expansion of the spectrum in the region near $g = 6$. In spectrum C, the large feature to lower field of the $g = 2$ end of the spectrum is ascribed to adventitious iron which is an impurity of the reagents and is not part of the heme spectrum.

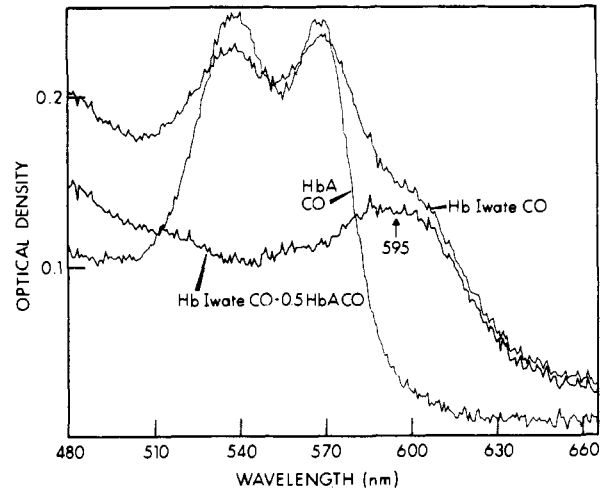

FIGURE 7: Computer-generated curves in which the optical spectrum of CO-ligated hemoglobin A (HbA-CO) is subtracted from the spectrum of CO-ligated hemoglobin M Iwate ($\alpha_2^{Mmet}\beta_2^{CO}$) (Hb-Iwate-CO). By subtraction of half the spectral contribution of HbA-CO from the spectrum of the CO-ligated Hb M Iwate, one obtains the spectrum ascribed to the ferric α chains.

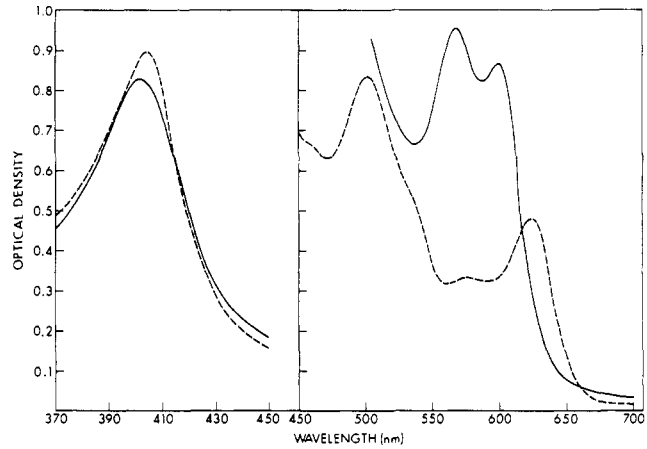

FIGURE 8: Optical spectra of ferric heme in the presence of sodium phenolate. The dashed curve is that of hemin chloride (100 μM) in dimethyl sulfoxide. The solid curve is that of the complex formed by the addition of a tenfold molar excess of sodium phenoxide. Raising the phenoxide concentration to 2 mM does not significantly alter the spectrum. The Soret absorptions shown to the left of the visible region were studied with a 1-mm optical cell.

globin A as a standard. Using a trial and error method, it was found that one-half of the optical spectral contribution of an equimolar solution of carbonmonoxyhemoglobin A was required to subtract the features at 537 and 568 nm from the spectrum. The resultant spectrum of the ferric α chains has a well-defined peak at 595 nm, quite different from the 635-nm peak of ferric hemoglobin A. The peak corresponding to the 500-nm peak in ferric hemoglobin A is not well-resolved by the procedure we employed, although an absorption near 560 nm is suggested.

In contrast, the optical spectrum of ferric heme in the presence of phenolate (Figure 8) also exhibits absorptions (λ_{max} = 402, 567, 598 nm) distinctly different from ferric heme in the absence of this potential heme ligand (λ_{max} = 404.5, 501, 624 nm). The spectrum of heme phenolate is strongly reminiscent of that of the bis(hydroxyferric)-heme complex first described by Keilin (1949) with absorptions in the visible near 560 and 590 nm.

Optical Spectrum of Phenolate-Heme-CO Model Compound. The binding of phenolate to ferrous heme shifts the optical spectrum of the CO complex. Even in the absence of protein, reduced heme can react with CO. Adding chromous acetate to ferric heme in dimethyl sulfoxide under anaerobic conditions shifts the Soret absorption from 405 to 424 nm, while the visible absorption is now at 557 nm with a minor peak at 525 nm. Introduction of oxygen with shaking restores the 405-nm absorption ascribed to ferric heme signifying that chromous acetate can reversibly reduce heme under the conditions of our experiments.

Adding CO to heme that was reduced by chromous acetate yields a species with a Soret absorption at 414 nm and with peaks in the visible at 537 and 568 nm. This is the spectrum ascribed to a heme CO complex, as purging the solution with argon restores the Soret at 424 nm.

When these same experiments are performed in the presence of imidazole, the Soret maximum of ferric heme is now near 414 nm.[2] The Soret for the heme-CO complex prepared in the presence of imidazole (λ_{max} = 422 nm) is nearly at the same wavelength as is found for CO-ligated derivatives of hemoglobin A and the individual chains of Hb M Iwate. Much in the same way as for hemoglobins, the binding of CO to the model compound is reversible since purging the solution with argon restores the spectrum of ferrous heme (λ_{max} = 424 nm).

These results are to be compared with those obtained when sodium phenolate is added to heme. Reduction of the heme by chromous acetate leads to the formation of a spectral species absorbing in the Soret at a position not very different from other ferrous heme complexes (Figure 9 and Table I), while

[2] The large difference observed in the Soret for the imidazole ferric heme complex compared with that for ferric heme proteins is ascribed to the difference in spin states. The bisimidazole complex is low spin while, under neutral conditions, ferric heme proteins are high spin.

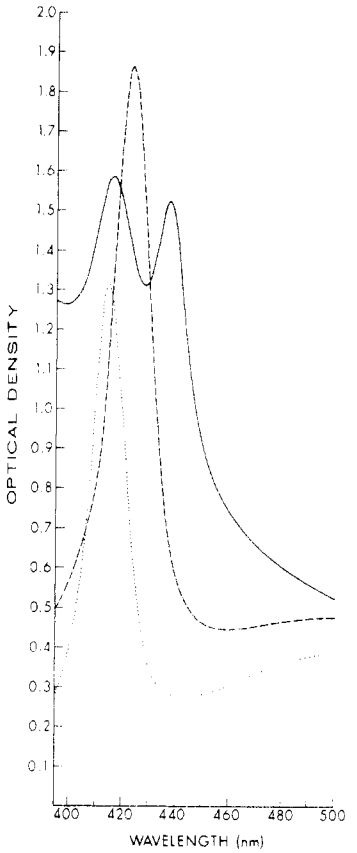

FIGURE 9: Optical spectra of ferrous heme in the presence of saturated sodium phenolate. Hemin chloride (5.6 μM) in dimethyl sulfoxide was reduced anaerobically with excess chromous acetate (- - - - -). CO was added (—). Removal of CO restored the original spectrum. For a control (· · · · ·) saturated phenol was used instead of sodium phenoxide.

the visible shows a sharp absorption at 590 nm and a shoulder at 540 nm. Adding CO now produces two spectral species, one absorbing in the Soret region at 415 nm, close to the same wavelength observed in the absence of sodium phenolate, and the other at 436 nm.

Control experiments performed with phenol instead of sodium phenolate show that the Soret and visible absorptions for ferric heme (λ_{max} = 405, 500, and 620 nm) are essentially unshifted by this reagent. The addition of phenol does not alter the spectrum of the species prepared by the addition of CO to ferrous heme. It is the deprotonated form of phenol, phenoxide anion, that shifts the Soret to 436 nm.

By computer-aided subtraction of the optical spectrum of the heme-CO complex prepared in the absence of added ligand from that of the complexes obtained in the presence of phenolate so that the peak at 415 nm was subtracted from the spectrum, it was determined that the conversion to the 436-nm-absorbing species was about 65% under the conditions of our experiment. Reducing the concentration of sodium phenolate used in these studies yields less 436-nm-absorbing material. In the presence of phenolate, purging with argon restores the spectrum of ferrous heme (λ_{max} = 424 nm).

Discussion

Experiments with heme model compounds are often performed under aqueous conditions. With ethoxide or phenoxide as potential heme ligands, this is not possible as these materials are unstable and generate hydroxide anion. One is forced then to resort to nonaqueous systems. Whereas dithionite can be used as a heme reductant in aqueous solutions, this compound has virtually no solubility in organic solvents and one must seek other reductants that have both the proper redox potential and reasonable solubility in organic media. One such reductant is chromous acetate.

Although chromous acetate is a colored material, its extinction coefficient is much smaller than that of the heme Soret. Therefore, its optical contribution is negligible. One is able, then, to compare reduced heme model compounds in nonaqueous environments with reduced heme proteins.

From x-ray studies (Kendrew, 1962; Perutz et al., 1968) it is well established that heme iron in hemoglobin and in myoglobin is covalently linked to the iron via a proximal imidazole from a histidine residue. Carbon monoxide and oxygen bind to the ferrous heme on the distal side of the heme. In the case of α chains of Hb M Iwate, this particular imidazole-heme structure is not present because, in this mutant hemoglobin, a tyrosine has been substituted. The possibility exists, then, that the tyrosyl residue replaces the imidazole as the covalent link to the protein structure.

Comparing the optical properties of high-spin heme in ferric hemoglobin A with the ferric heme in α chains of Hb M Iwate (Figure 7), one notes that the characteristic absorption at 635 nm has been shifted to 595 nm. The addition of sodium phenolate[3] to ferric heme in dimethyl sulfoxide also shifts this absorption to the blue. From these findings and from the similarity of the EPR of ferric heme phenolate with that of hemoglobin M Iwate (Figure 6), it is suggested that the tyrosyl residue is indeed bound to the heme in the abnormal α chain of hemoglobin M Iwate. The lack of effect of phenol on the optical spectrum of ferric heme in dimethyl sulfoxide leads one to conclude that the tyrosyl residue in the protein, when bound, is deprotonated.

The large differences observed in the Soret for heme-CO complexes with a variety of trans ligands including imidazole, phenolate and even mercaptide anion (Stern and Peisach, 1974) lead us to believe that the wavelength of the Soret can be used as a marker of heme ligand structure (Table I). The absorption at 436 nm which is dependent on phenolate concentration is taken as a spectral signature for a phenolate-heme-CO complex (Figure 9). The fact that the CO complex of the abnormal α chains of hemoglobin M Iwate bears spectral resemblance to that of the CO-complexed β chains and also to carbonmonoxyhemoglobin A (where the imidazole is known to be bound to heme) is taken as strong evidence that tyrosinate is not bound to the heme when the α chains are bound to CO. Of course, the possibility exists that another nitrogenous ligand on the proximal side of the heme replaces the tyrosinate which is bound to the heme when in the ferric state or, less likely, that tyrosinate oxygen is bound to the heme in an uncharged form, that is, bound to a proton and to heme iron at the same time. This latter suggestion is ruled out since phenol does not appear to perturb the optical spectrum of ferric or carbonmonoxy heme model compounds, and one would expect a Soret absorption in this case near 415 nm.

An attractive alternative explanation is that imidazole is the trans axial ligand when the α chains of Hb M Iwate are reduced and bound to CO. Since the closest imidazole to the heme is the distal (E7) one, this suggests that either the heme or the E helix has been perturbed from its original position so that this imidazole can now bind. This is not an unreasonable

[3] Structurally, phenolate anion resembles tyrosinate anion, the only difference being an aliphatic substitution para to negatively charged oxygen.

suggestion since optical and EPR evidence has lead us to conclude that, under various conditions, this distal imidazole can bind to heme in ferric hemoglobin A and to its isolated ferric α and β chains as well (Rachmilewitz et al., 1971; Peisach et al., 1973). One is then lead to the conclusion that the CO binds proximally.

Of course, CO cannot bind to the heme unless the protein is reduced. The redox potential for heme reduction must be extremely low since excess dithionite does not drive the reaction to near completion at pH 6.5, while ascorbate has essentially no effect at all. Yet, the rate and extent of reduction is itself dependent upon the presence of CO (Figure 4). In contrast to studies of heme thiol interaction (Stern and Peisach, 1974), the presence of CO does not enhance reduction since its presence slows down electron transfer from dithionite. This suggests that the redox potential of the α chains is a cooperative property, dependent in part on the state of ligation of the β chains. The difficulty in reducing the α chains as compared with the β chains may be an expression of the difficulty of adding a negative charge to ferric heme bound to the negatively charged ligand, tyrosinate.

Once the CO binding has taken place, the tyrosine is no longer available to occupy the original position that it once held in the ferric protein since removal of CO with strong light leads to the formation of a deoxy heme which is now capable of readily binding CO (Table II). This new, readily formed CO complex has the same spectral properties as the one formed after slow dithionite reduction of the ferric protein. The loss of absorption at 420 nm (Table II) for this newly formed heme-CO complex as compared with the one first formed after dithionite addition may be due to heme breakdown caused by the photoillumination of the sample or less likely may be due to a change of the extinction coefficient of the CO-ligated α chains.

In summary, we present evidence that the tyrosyl residue which is substituted for the proximal imidazole is bound to the heme of the abnormal chain of hemoglobin M Iwate. We also present evidence that this residue is not bound when CO is ligated to the heme. It is believed that the distal imidazole is bound as the ligand trans to the CO. The possibility exists that ligands other than CO can also bind in like manner to the otherwise unreactive α chains of Hb M Iwate after the distal imidazole is bound to the heme. This possibility is presently being investigated.

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