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A Correlation of the Visible and Soret Spectra of Dioxygen- and Carbon Monoxide–Heme Complexes and Five-Coordinate Heme Complexes with the Spectra of Oxy-, Carboxy-, and Deoxyhemoglobins†

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ABSTRACT: A systematic investigation was carried out concerning relationships between visible and Soret spectra of heme complexes and the nature of the axial ligands. Dioxygen (O_2), carbon monoxide (CO), and five-coordinate complexes were prepared from proto-, meso-, and 2,4-dimethyldeuteroheme dimethyl esters in *N*,*N*-dimethylformamide (DMF) solution. A variety of axial ligands was employed, including imidazoles, pyridines, aliphatic amines, and very weak bases such as DMF and acetone. Variations in the ligands included differences in basicity and differences in substituents which sterically hindered coordination to heme iron. In the five-coordinate complexes, a shift to shorter wavelength in both the visible and Soret peaks accompanied a change to a more hindered axial ligand. Difference spectra obtained by taking the spectrum of a heme complex with a more hindered ligand minus that of a less hindered ligand approximated the T minus R state difference spectrum reported by Perutz et al. [Perutz, M. F., Ladner, J. E., Simon, S. R., & Ho, C. (1974) *Biochemistry 13*, 2163] for deoxyhemoglobin (deoxy-Hb) Kempsey ± inositol hexaphosphate (IHP). In the CO complexes, a decrease in the basicity of the ligand or an increase in the steric hindrance of the axial ligand also resulted in a blue-shifted λ_{max} accompanied by an increase in the ratio of intensities of the long-wavelength visible peak (α) to that of the shorter wavelength visible peak (β). The latter parameter is termed the α/β ratio. Spectra of the O_2 complexes were observed only at temperatures below −40 °C because of limited stability at higher temperatures. In O_2 complexes, shifts in λ_{max} and changes in the α/β ratio with unhindered ligands showed much the same pattern as with the CO complexes, but hindered ligands such as 1,2-dimethylimidazole and 1,2,4,5-tetramethylimidazole gave red-shifted visible λ_{max} and low α/β ratios compared to the unhindered O_2–heme–1-*n*-butylimidazole complex. This observation is interpreted as being due to the greater ease with which the Fe is distorted from the porphyrin plane in O_2 complexes than in CO complexes. Comparison of α/β ratios of model O_2– and CO–protoheme complexes with those of hemoglobins and myoglobins formed the basis for the suggestion that the proximal histidine is restrained by the protein, producing a relatively weak axial Fe–N interaction, both in liganded R-state hemoglobins and in common myoglobins. T minus R state difference spectra reported by Perutz et al. [Perutz, M. F., Kilmartin, J. V., Nagai, K., Szabo, A., & Simon, S. R. (1976) *Biochemistry 15*, 378] for O_2- and CO-Hb Kansas ± IHP were approximated by model protoheme complexes of a stronger minus a weaker axial ligand. These results are interpreted in terms of T-state steric conflict between coordinated O_2 or CO and amino acid side chains on the distal side of the heme. Transition to T state would result in the heme complex being forced toward the proximal imidazole, thereby strengthening the axial Fe–N bonding interaction and introducing strain in both the heme complex and the protein.

The visible spectra of oxyhemoglobins and oxymyoglobins are characterized by two peaks, α (~575 nm) and β (~540 nm), of which the α peak usually has slightly the greater intensity. The spectra of the CO complexes are similar, but the α peak is rarely observed to have a higher absorbance than the β peak. Deoxyhemoglobins have a single absorption band in the visible spectrum with a long-wavelength shoulder. Difference spectra have been reported recently for O_2-, CO-, and deoxy-Hb's[1] under a variety of conditions: for example,

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[1] Abbreviations used: Hb, hemoglobin; Mb, myoglobin; IHP, inositol hexaphosphate; TPP, tetraphenylporphyrin; DMF; *N*,*N*-dimethylformamide; BI, 1-*n*-butylimidazole; CMI, 5-chloro-1-methylimidazole; DMI, 1,2-dimethylimidazole; MEI, 1-methyl-2-ethylimidazole; TMI, 1,2,4,5-tetramethylimidazole; Fe–N_ϵ, bond between heme iron and $N_{\epsilon 2}$ of F8 His.

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±IHP (Perutz et al., 1974, 1976; Adams & Schuster, 1974; Knowles et al., 1975; Cassoly, 1976), high pressure minus atmospheric pressure (Gibson & Cary, 1975; Cary et al., 1977), room temperature minus low temperature (San Biagio et al., 1977), and β chains minus α chains (Sugita, 1975; Knowles et al., 1975). In all cases, differences are observed in the intensities and λ_{max} of the visible and Soret peaks which doubtlessly reflect differences in the coordination and/or environment of the heme. Differences are also apparent between the spectra of hemoglobins and myoglobins from various species. A dramatic example is Ascaris, oxy-Hb where the α peak has a much lower intensity than the β peak (Wittenberg et al., 1972).

It has long been recognized that the visible spectrum of ferroheme complexes is characteristic of the axial ligands (Williams, 1956; Drabkin, 1961), but attempts to correlate spectral parameters with structure have met with little success insofar as they can be applied to hemoproteins [see Anson et al. (1925), Corwin et al. (1963), Chien (1978), and Drabkin (1978)]. However, in recent years conditions have been found for preparing heme five-coordinate and O_2 complexes, which with the CO complexes presented an opportunity to carry out a systematic study of structure/spectra relationships. Therefore, in an effort to determine if quantitative correlations could be found between electronic spectra and structural or electronic parameters of heme complexes, we have examined a range of proto- and mesoheme diester–O_2 and –CO complexes as well as a number of five-coordinate heme complexes in which coordination of the axial ligand is varied by adding substituents which exert steric and/or electronic effects on the heme coordination.

Spectral correlations are made within each type of complex, i.e., the five-coordinate, the O_2, and the CO series. In each series, λ_{max} of both the Soret and the visible peak(s) differs as a function of the axial ligand. In the O_2 and CO series the relative intensities of the α and β peaks also appear to vary systematically with the axial ligand. Therefore, we have attempted to correlate the observed λ_{max} and the ratio of intensities at λ_{max} (α/β ratio) with structural and electronic differences between the complexes. In the model complexes studied, ligand strength is a function of both ligand basicity and steric factors which restrain coordination. However, in hemoglobin the proximal imidazole appears to be within hydrogen-bonding distance of the F4 Leu carbonyl oxygen in both liganded (Ladner et al., 1977; Heidner et al., 1976) and unliganded (Fermi, 1975) forms, and, therefore, its basicity presumably is not significantly altered as a result of the conformational change. If such is the case, the correlations between spectra and heme complexation can be applied with some confidence as a means of assessing restraints imposed on the proximal imidazole–heme interaction (Fe–N_ϵ) by the protein.

Experimental Section

Materials. Protohemin dimethyl ester was prepared as previously described by Wang et al. (1958). Mesohemin dimethyl ester was prepared by reduction of protoporphyrin dimethyl ester and insertion of iron (Falk, 1964). 2,4-Dimethyldeuterohemin dimethyl ester was prepared as previously described (Parker & Brinigar, 1976). All were purified by chromatography on alumina eluting with $CHCl_3$. *N,N*-Dimethylformamide (DMF) was purified according to method II of Thomas & Rochow (1957). For complexes with the weaker ligands, it is of the utmost importance that trace amounts of amine impurities be removed from DMF; method II is effective for this purpose. Most of the nitrogenous ligands were commercial products from Aldrich, Eastman, and Chemical Samples Co. 1,2,4,5-Tetramethylimidazole (TMI) was a most generous gift from the BASF Wyandotte Corp., Parsippany, NJ, and is gratefully acknowledged. It was recrystallized from hexane: mp 60–61 °C; lit. mp 58 °C (Lions & Ritchie, 1941). 1-Methyl-2-ethylimidazole (MEI) was prepared by treating the sodium salt of 2-ethylimidazole with CH_3I in anhydrous liquid ammonia, followed by vacuum distillation, bp 37 °C at 1 Torr. All liquid amines were fractionally distilled over KOH before use. The solids were recrystallized to a constant melting point.

Methods. Spectra were recorded on a Cary 14 spectrophotometer. Low-temperature spectra were taken in a Dewar flask containing an inner jacket through which the blowoff from liquid N_2 was passed at a rate controlled by a flowmeter. The apparatus is similar to that described by Travers et al. (1969). Temperature was measured with a copper–constantan thermocouple in conjunction with a Keithley digital multimeter, Model 160B, or an Omega Model 2160A-T digital thermometer.

The hemin diesters (~0.05 mM) were dissolved in DMF containing the nitrogenous ligand at a concentration chosen such that no significant spectral change occurred at half or double the concentration used. The concentrations ranged from 5 to 100 mM, higher for the weaker ligands. The solutions were thoroughly degassed by repeatedly freezing, evacuating, thawing, and reequilibrating with argon. Reduction was accomplished with Pt/H_2, Pd/CaH_2 (Brinigar & Chang, 1974), or by the addition of a three- to fivefold molar excess of $Na_2S_2O_4$ in 5 μL of water per 10 mL of solution. A larger excess must be avoided because it precipitates and in some cases it reduces the porphyrin ring. The presence of water and air-oxidation products of dithionite did not alter the spectra. Spectra of several of the O_2 and CO complexes were taken over a fivefold concentration range, wherein the spectral parameters remained unchanged. Heme esters prepared by reduction with dithionite combine *reversibly* with O_2, and the Fe(II)–O_2 complex remains completely reduced at low temperature. Dithionite dissolved in DMF containing a small amount of water does not react with molecular oxygen at a perceptible rate at temperatures below −30 °C, nor does it reduce O_2 coordinated to the heme. This was a particularly important discovery because complexes of heme esters in DMF, prepared by reduction with Pd/H_2 or Pd/CaH_2, invariably undergo some oxidation upon exposure to O_2, even at low temperature (Brinigar & Chang, 1974). As a result, spectra of the pure O_2 complexes could not be observed. With strong unhindered ligands, such as 1-alkylimidazoles, the oxidation product is largely the hemichrome, which has a considerably higher extinction coefficient at wavelengths around 540 nm (β peak) than around 575 nm (α peak). Therefore, α/β ratios of O_2 complexes with strong ligands, taken from spectra where reduction was carried out with Pd/H_2 or Pd/CaH_2, were frequently lower than the actual value. The α/β ratios of O_2 complexes with weak or sterically hindered ligands, such as TMI, are also subject to error because the product of oxidation is the μ-oxo-bis(hemin ester) which has a higher extinction in the region of the α peak than in the region of the β peak. Complete reduction is sometimes difficult with the weaker ligands, leaving some hemin ester in solution which has a higher extinction in the region of the β peak than in the region of the α peak. Therefore, if incomplete reduction or significant oxidation has occurred, the α/β ratio may appear either higher or lower than the actual value. Most of the O_2 complexes listed in Table V have been

Table I: Spectral Properties of Heme Complexes with Sterically Hindered Ligands in *N,N*-Dimethylformamide[a]

ligand	λ_{max} (nm) at 20 °C		λ (nm) at −55 °C				
	vis	Soret	α		β	min	Soret
protoheme dimethyl ester							
1,2-dimethylimidazole (DMI)	557	431	557		527	540	425.5
1-methyl-2-ethylimidazole (MEI)	556	431	556		526	539	425
1,2,4,5-tetramethylimidazole (TMI)	555	428		555.5			429
2-methylpyridine	555		555		524	538	
2,6-dimethylpyridine	555		555		524	538	
N,N-dimethylformamide[b]	554	424		553.5			423
human deoxyhemoglobin[c]	555	430					
mesoheme dimethyl ester							
1,2-dimethylimidazole	547	422	546		517	531	
1-methyl-2-ethylimidazole	547	421	546		517	531	
1,2,4,5-tetramethylimidazole	546	418		546			
N,N-dimethylformamide	544	412		544			

[a] Spectra were taken in DMF solutions at heme concentrations of ~0.05 and ~0.01 mM for visible and Soret, respectively. [b] DMF used for the spectrum previously reported (Brinigar & Chang, 1974) contained an amine impurity (see Materials section). [c] Spectral data for deoxyhemoglobin are included for comparison.

prepared by using both methods of reduction. In cases where the two methods did not give identical spectra, spectral parameters were taken from the complex prepared by dithionite reduction. In several instances where reduction with Pd/H_2 was complete, the addition of aqueous dithionite to solutions of O_2 or CO complexes did not alter the absorption spectrum.

Difference spectra were obtained by preparing the protoheme diester complex of the weaker ligand and recording its spectrum, followed by introduction via syringe of the stronger ligand in an amount, previously determined by titration, which was just sufficient to completely replace the first ligand. The resulting spectrum was then recorded on the same chart as the first and the difference spectrum obtained by manually substracting the absorbances of one spectrum from the other. This method avoids the necessity of having two solutions at precisely the same heme concentration.

Results

Both five- and six-coordinate heme complexes can be prepared, and by proper choice of axial ligands, spectra can be generated which bear a striking resemblance to those of deoxy-, oxy-, and carboxyhemoglobin. DMF was chosen as the solvent for this study because O_2–heme complexes are more stable toward oxidation in DMF than in less polar solvents (Brinigar et al., 1974), the freezing point of DMF is −60 °C which allows spectra to be observed at temperatures where the O_2–heme complexes oxidize at exceedingly slow rates, heme esters do not aggregate in DMF, and DMF is itself a weak unhindered ligand (Brault & Rougee, 1974b).

Five-Coordinate Complexes. Five-coordinate heme complexes can be prepared with sterically hindered ligands such as 1,2-dimethylimidazole (DMI) or 2-methylpyridine, and also with very weak bases such as DMF. Spectral data for a number of these complexes with both proto- and mesoheme diesters are shown in Table I. At 20 °C all of these complexes are clearly five coordinate with a single banded visible peak containing a long-wavelength shoulder, similar to the spectra of deoxyhemoglobins and -myoglobins. Spectra of this type have been previously reported for similar complexes (Wagner & Kassner, 1975; Brault & Rougee, 1974a,b; Rougee & Brault, 1975).

As the temperature is lowered little change is noted in the spectra above ~0 °C, but further lowering results in a gradual change in most of these complexes to a two-banded visible spectrum characteristic of six-coordinate hemochromes. These temperature-dependent spectral changes were reversible. Similar observations have been reported previously (Wagner & Kassner, 1974). Due to the decrease in thermal motion of the hindering alkyl groups at low temperature, shorter Fe–N_{axial} bond distances and less displacement of the iron from the porphyrin plane would result. At sufficiently low temperatures, the steric restraints become so small that the ligand can coordinate on both sides of the heme. TMI and DMF are exceptions and appear to remain completely five coordinate even at −55 °C. This is probably due to severe steric hindrance in TMI and the extremely weak basicity of DMF ($pK_a \approx -2$).

The use of some ligands such as 2-isopropylpyridine and 2-*tert*-butylpyridine even at quite high concentrations gives spectra indistinguishable from that of the DMF complex; either these ligands give complexes with spectra identical with that of the DMF complex, or the nitrogen is so severely hindered that they cannot compete effectively with DMF for coordination.

The spectra of the protoheme complexes with DMI, MEI, and TMI in both toluene and acetone were also obtained. The λ_{max} values of all these complexes in both solvents are nearly identical (±1 nm) with those in DMF, and the relative order remains the same. Thus, solvent effects on these spectra appear to be small.

From the data presented in Table I, it is apparent that a shift of the visible and Soret bands toward shorter wavelength in these five-coordinate complexes is associated with an increase in the amount of steric hindrance on the axial ligand or, in the case of DMF, with a dramatic decrease in basicity. In either case these effects result in a decrease in the bonding interaction between axial ligand and heme iron. Accompanying the blue-shifted λ_{max} is invariably an increase in extinction of the Soret peak. An example is shown in Figure 1.

A shift in these bands to the blue is also observed upon the addition of IHP to the chemically modified deoxyhemoglobins NES-des-Arg and des-Arg-Tyr as well as the mutant Hb Kempsey (Perutz et al., 1974). All three of these hemoglobins exist in an R state when completely deoxygenated, but can be converted to a T state by the addition of IHP.

Five-Coordinate Difference Spectra. When the TMI–protoheme complex was treated with a less hindered ligand such as DMI at a concentration equal to that of the TMI, a shift occurred in λ_{max} of the visible and Soret bands to longer wavelength along with a decrease in intensity in the region of λ_{max} and the shoulder around 585 nm. The resulting spectrum was identical with the spectrum of the DMI complex. The

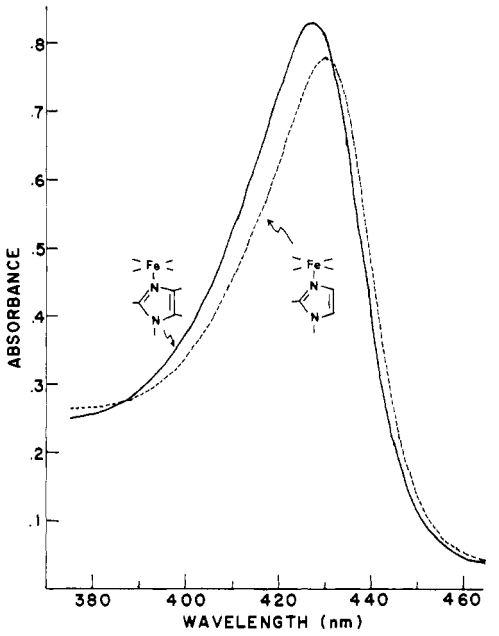


FIGURE 1: Soret spectra of five-coordinate protoheme diester complexes in DMF at 20 °C: (---) DMI complex; and (—) TMI complex.

differences between the two spectra can be seen more clearly in a difference spectrum. Figure 2 shows the visible difference spectra of the protoheme complex of TMI minus that with DMI and the protoheme complex of DMF minus that of DMI. Also shown for comparison is a reproduction of the deoxy-Hb Kempsey ± IHP difference spectrum from Perutz et al. (1974). The difference spectra of the chemically modified hemoglobins ± IHP are similar to that of Hb Kempsey (Perutz et al., 1974). Figure 3 shows the Soret difference spectra of the protoheme complex of TMI minus that of both DMI and MEI. Included for comparison is the Soret difference spectrum of NES-des-Arg-deoxy-Hb ± IHP (Perutz et al., 1974).

Note in all cases that the spectra correspond to a more hindered ligand minus a less hindered ligand or a weaker base (DMF) minus a stronger base. The difference spectra of Hb Kempsey and the chemically modified hemoglobins are the spectrum in the presence of IHP (T state) minus the spectrum in the absence of IHP (R state). The magnitudes of the difference spectra of the model complexes are comparable to that of the hemoglobin spectra. The DMF minus DMI difference is about twice that of the hemoglobins, whereas the differences for TMI minus both DMI and MEI are roughly equivalent to the hemoglobins.

Six-Coordinate O_2 and CO Complexes. The spectral parameters in both O_2 and CO complexes which appear to bear a relationship to the bonding interaction between the axial base and Fe are (a) the ratio of intensities of the visible α and β peaks, which is termed the α/β ratio, and (b) the wavelength at maximum intensity (λ_{max}) of the α, β, and Soret peaks. In general, the correlation is the stronger the Fe–N_{axial} interaction the lower the α/β ratio and the greater the shift of λ_{max} to the red. In terms of small differences in these parameters, the correlation is judged to be applicable only to complexes with axial ligands of a given structural type; that is, O_2 and CO complexes are to be considered separately, and the imidazole series, the pyridine series, etc. are to be considered separately. Temperature is also of importance, particularly with complexes of the sterically hindered ligands.

Spectra of many of these complexes have been obtained in solvents other than DMF, for example, acetone and toluene. Only small differences in the spectral parameters were observed, and the relationships between the parameters of any two complexes remained essentially unchanged with solvent. α/β ratios appear to be more insensitive to solvent than λ_{max}.

CO Complexes. Spectra of CO complexes were obtained with a variety of ligands, some of which have no steric restraints to coordination but differ in basicity, whereas others differ both in basicity and in steric effects or in steric effects alone. Examples of visible spectra are shown in Figure 4. Note that although the three complexes have very different α/β ratios, the intensities of the β bands are nearly the same. This appears to be a general property of these complexes, and, therefore, the α/β ratio can be viewed as an approximate measure of the intensity of the α peak.

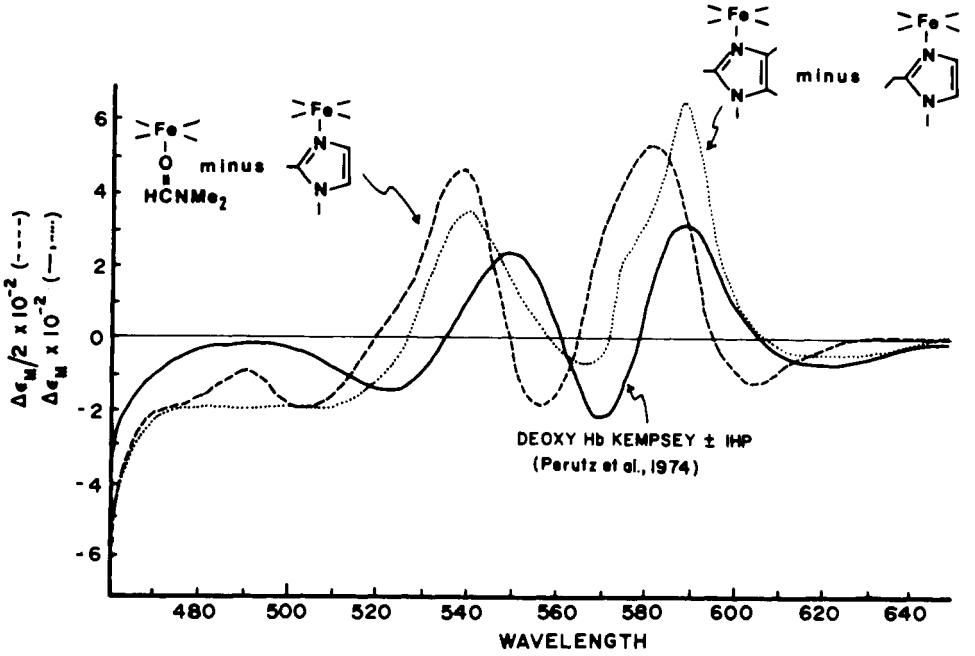


FIGURE 2: Visible difference spectra of five-coordinate protoheme diester complexes in DMF at 20 °C: (···) spectrum of the TMI complex minus the spectrum of the MEI complex; (---) the DMF complex minus the DMI complex; and (—) a reproduction of the difference spectrum of deoxy-Hb Kempsey with added IHP minus deoxy-Hb Kempsey in the absence of IHP taken from Perutz et al. (1974).

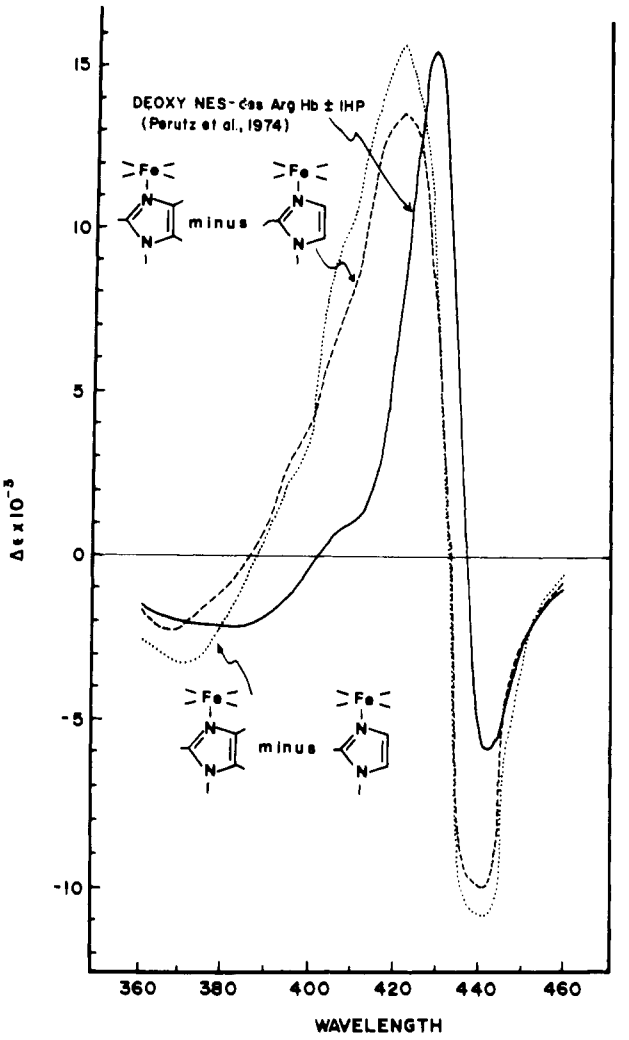


FIGURE 3: Soret difference spectra of five-coordinate protoheme diester complexes in DMF at 20 °C: (···) spectrum of the TMI complex minus that of the DMI; (- - -) the TMI complex minus the MEI complex; and (—) a reproduction of the difference spectrum of NES-des-Arg-deoxy-Hb in the presence of IHP minus the spectrum in the absence of IHP reproduced from Perutz et al. (1974).

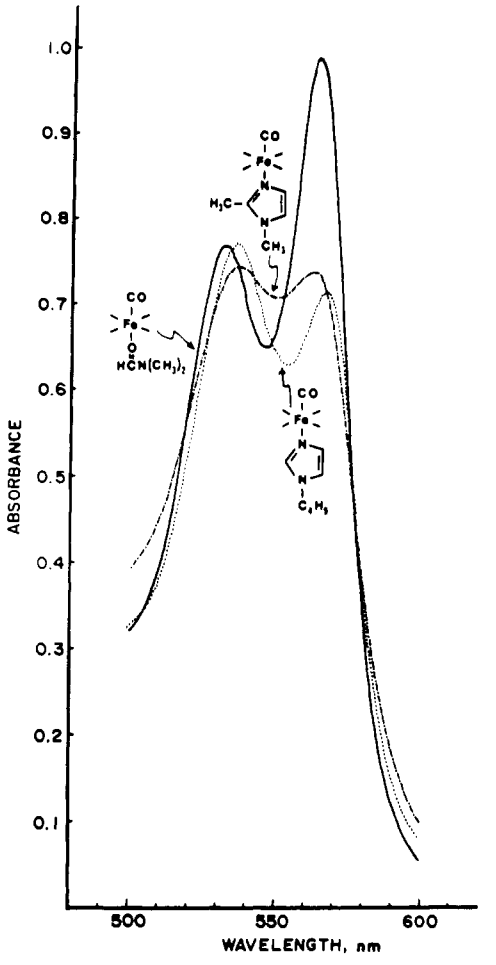


FIGURE 4: Visible spectra of CO–protoheme diester complexes in DMF at 20 °C: (···) BI; (-·-) DMI; (—) DMF.

In order to compare spectral relationships of CO complexes with those of the corresponding O_2 complexes, we took the CO spectra both at 20 and −55 °C. Spectra of the O_2 complexes could not be obtained at temperatures higher than −30 °C due to partial oxidation at the higher temperatures. Spectral data for the CO complexes are shown in Table II.

Complexes with nonsterically hindered ligands will be considered first. In the absence of steric restraints, the bonding interaction between iron and ligand should be primarily a function of basicity of the ligand (Wang, 1961; Alben & Caughey, 1968; Walker, 1973). With CO complexes in the pyridine series, small differences in ligand basicity have a relatively minor effect on the α/β ratios and λ_{max}. For example, compare 3,5-dimethylpyridine (pK_a = 6.1), 4-methylpyridine (pK_a = 6.0), and pyridine (pK_a = 5.2); at both 20 and −55 °C they are nearly identical, with pyridine having a slightly higher α/β ratio. However, large differences in ligand basicity have a larger effect; compare 4-aminopyridine (pK_a = 9.1, α/β = 0.96), pyridine (pK_a = 5.2, α/β = 1.03), and 4-cyanopyridine (pK_a = 1.9, α/β = 1.10). In the imidazole series, spectral changes appear to be more sensitive to basicity, although the number of examples is limited; compare 1-*n*-butylimidazole (BI) (pK_a = 7.1, α/β = 0.93) and 5-chloro-1-methylimidazole (CMI) (pK_a = 5.5, α/β = 0.98). Note that the very strong ligand CN^- gives an exceptionally low α/β ratio (0.82) and exceedingly weak bases such as DMF, acetone, water, and ethanol give CO complexes with exceptionally high α/β ratios. Acetone is such a weak ligand that it does not coordinate at all in the absence of CO or O_2, and at low temperature it appears to be replaced by CO, giving the dicarbonyl complex which has a very low α/β ratio. In all other CO complexes with unhindered ligands, the α/β ratio and λ_{max} remain nearly the same at 20 and −55 °C.

Considering next CO complexes with sterically hindered ligands, it is clear from the ease with which the hindered ligands can be replaced by equal or lower concentrations of an unhindered ligand that the hindered ligands interact less strongly with the Fe than unhindered ligands of comparable basicity. Moreover, in both the pyridine and imidazole series the α/β ratios of the hindered ligand complexes are high and λ_{max} values are shifted to the blue relative to the comparably basic unhindered ligands. The same correlation is evident in three alkylamines with increasing steric restraints, *n*-octylamine (α/β = 0.90), *tert*-butylamine (α/β = 1.04), and diisopropylamine (α/β = 1.14). In each structural series of ligands, the larger the hindering group(s), the higher is the observed α/β ratio of the CO complex. Even the small steric difference between DMI and MEI gives rise to an easily observable difference in α/β ratio.

The spectral changes which occur upon lowering the temperature are much larger with the sterically hindered ligands than with the unhindered ligands. Invariably, the α/β ratio decreases and at least some regions of the spectrum shift to the red. This result is consistent with the behavior of these

Table II: Spectral Properties of CO–Heme Complexes in *N,N*-Dimethylformamide[a]

ligand	λ (nm) at 20 °C				λ (nm) at −55 °C				α/β ratio	
	α	β	min	Soret	α	β	min	Soret	20 °C	−55 °C
protoheme dimethyl ester										
N-acetylhistamine	569	539	556		568	538	555		0.92	0.91
imidazole	569	539	555	419	568	537	555	419.5	0.92	0.91
1-*n*-butylimidazole	569	539	555	419	568	537	554	419	0.93	0.92
5-chloro-1-methylimidazole	568	538	553	417.5	567	536	553	417.5	0.98	0.98
1,2-dimethylimidazole	564	538	551	417	562	537	550		0.99	0.98
1-methyl-2-ethylimidazole	564	538	551	416	562	537	550		1.02	0.99
1,2,4,5-tetramethylimidazole	566	534	548	412	563	537	549		1.22	1.00
4-aminopyridine	566.5	537.5	553	418	566	536	552	418	0.96	0.96
3,5-dimethylpyridine	566.5	536.5	552	416.5	566	536	551	416.5	1.02	1.03
4-methylpyridine	566.5	536.5	552	416.5	566	536	551	416.5	1.02	1.03
pyridine	566.5	536.5	551	416	566	535.5	550	416	1.03	1.04
4-cyanopyridine	566	535.5	550	414	565.5	534.5	549	414	1.10	1.10
2-methylpyridine	566	535	550	414	566	536	550		1.11	1.03
2,6-dimethylpyridine	566	535	549	413	566	535.5	551		1.18	1.03
N,N-dimethylformamide[e]	566	534	547.5	411.5	565	532	547	411.5	1.29	1.33
protoheme[b]										
CN^-[c]	565	538		425					0.82	
CH_3NC[d]	564	536		422.5					1.04	
EtOH[e]	562	533		410					1.29	
H_2O[e]	562	530		406.5					1.22	
human carboxyhemoglobin[g]	568.5	539	555	420					1.00	
mesoheme dimethyl ester										
N-acetylhistamine	560	530	547		559	529	546		0.85	0.83
imidazole	560	530	547		559	529	546		0.85	0.84
1-*n*-butylimidazole	560	530	547	410	559	529	546		0.86	0.85
5-chloro-1-methylimidazole	559	529	546		558	528	545		0.92	0.91
1,2-dimethylimidazole	556	528	544		555	527	543		0.95	0.93
1-methyl-2-ethylimidazole	556	528	542		555	527	542		1.02	0.96
1,2,4,5-tetramethylimidazole	557	526.5	540		555	526	541		1.21	0.97
4-aminopyridine	558	529	546		557	527.5	544.5		0.91	0.90
3,5-dimethylpyridine	558	528	544		557	527	543		0.98	0.98
4-methylpyridine	558	528	543		557	527	542		0.98	0.98
pyridine	558	528	543	407	557	527	542		0.99	0.99
4-cyanopyridine	557.5	527.5	542.5		556.5	526	542		1.05	1.05
2-methylpyridine	557	526	541		557	526	542		1.10	0.99
2,6-dimethylpyridine	557	526	541		557	527	542		1.13	0.98
n-octylamine	558	528	543		557	527	542		0.90	0.90
tert-butylamine	557	527	542		557	527	542		1.04	0.89
diisopropylamine	557	526	541		556	526	542		1.14	0.95
N,N-dimethylformamide[e,f]	557	525	539		555	524	539		1.29	1.33
acetone[e]	555	525	535		564	533	558		1.33	0.78
2,4-dimethyldeuteroheme dimethyl ester										
1-*n*-butylimidazole	560	530	547		559	529	547		0.86	0.86
1-methyl-2-ethylimidazole	556	528	541		555	527	542		1.02	0.97
N,N-dimethylformamide[e]	557	526	539		556	524	539		1.29	1.33

[a] Spectra were taken in DMF solutions, except where noted, at the temperatures indicated. Heme diester concentrations were ~0.05 mM for visible and ~0.005 mM for Soret spectra in 1.0-cm cells. α/β ratios were reproducible to ±0.01. [b] Protoheme free acid; spectral data reported by Keilin (1949). [c] In aqueous solution. [d] In ethanol solution. [e] Ligand is solvent. [f] DMF used for the spectrum previously reported (Brinigar & Chang, 1974) contained an amine impurity (see Materials section). [g] Included for comparison.

ligands in the five-coordinate complexes and is doubtlessly due to a relaxation of steric restraint at low temperature, decreasing the Fe–N_{axial} bond distance and allowing greater interaction between nitrogen and iron. This result also implies that CO opposes movement of the Fe out of the porphyrin plane such that the interaction between Fe and N is governed largely by steric factors rather than by the basicity of the ligand.

Relationship between Spectra and Stability of CO Complexes. In L–heme–CO complexes there exists a complementary trans effect between the L–heme bond (L = ligand) and the heme–CO bond such that an increase in the L–heme bond strength will increase the heme–CO bond strength and vice versa (Wang, 1961; Alben & Caughey, 1968). Therefore, if the α/β ratio of CO complexes correlates with the strength of the L–heme bonding, it should correlate with the strength of the heme–CO bonding as well. Alben & Caughey (1968) have shown that the strength of the heme–CO bond in a simple L–heme–CO complex is reflected in its CO stretching frequency (ν_{CO}). As ν_{CO} decreases, the stability of the CO complex increases. Therefore, if α/β ratio of the L–heme–CO complexes correlates with both Fe–L bonding and Fe–CO bonding, ν_{CO} of the complexes should decrease as α/β decreases. Such a relationship is found to exist, as shown in

Table III: Relationship between ν_{CO} and α/β Ratio of CO–Heme Complexes

ligand	ν_{CO} (cm^{-1}), 2,4-diacetyldeuteroheme[a]	α/β ratio	
		protoheme	mesoheme
4-aminopyridine	1971.1	0.96	0.91
4-methylpyridine	1982.3	1.02	0.98
pyridine	1983.7	1.03	0.99
4-cyanopyridine	1988.5	1.10	1.05

[a] Alben & Caughey (1968).

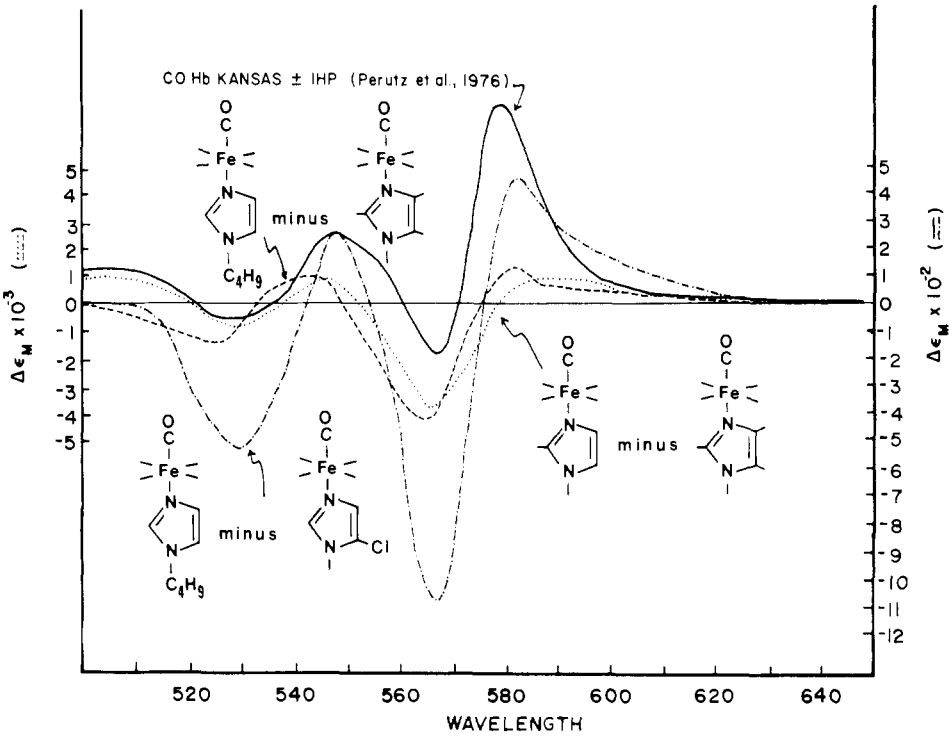


FIGURE 5: Visible difference spectra of CO–protoheme diester complexes in DMF at 20 °C: (···) spectrum of the CO complex of DMI minus that of TMI; (- - -) the CO complex of BI minus that of TMI; (–·–) the CO complex of BI minus that of CMI; and (—) a reproduction of the difference spectrum of CO-Hb Kansas in the presence of IHP minus CO-Hb Kansas in the absence of IHP, reproduced from Perutz et al. (1976).

Table III. The results suggest that the α/β ratio, like ν_{CO}, can be taken as a measure of the strength of the Fe–CO bond in simple L–Fe–CO complexes.

The same conclusion emerges from experiments in which relative stability constants are determined. If a decrease in the α/β ratio correlates with an increase in the strength of the Fe–CO bond, then an L_1–heme–CO complex having a lower α/β ratio should be more stable than an L_2–heme–CO complex having a higher α/β ratio if L_1 and L_2 are of the same structural type. Therefore, a ligand which gives a CO complex with a high α/β ratio should be replaced by a second ligand present at lower concentration than the first to give a complex with a lower α/β ratio. Many of these experiments were carried out and such was found to be the case without exception.

When the second ligand was added in increments, spectra exhibited excellent isosbesty, demonstrating the existence of only two absorbing species. Thus, the concentrations of each complex could be calculated at various relative concentrations of the two ligands. From these data relative equilibrium constants for a number of reactions of the type L_1–heme–CO + $L_2 \rightleftharpoons L_2$–heme–CO + L_1 were obtained. These are listed along with the corresponding α/β ratios of the CO complexes in Table IV.

Clearly, a relationship exists between the α/β ratio and ligand affinity, although it is obviously not strictly a quantitative one. However, it should be useful because, as mentioned above, the strength of the L–heme bond is related to the strength of the heme–CO bond. Therefore, the relative association constants in Table IV are not only the relative affinities of heme–CO for L but should also at least approximate the relative affinities of L–heme for CO. Rougee & Brault (1975) have measured these two affinity constants separately for the deuteroheme complexes of imidazole and 2-methylimidazole and found the equilibrium constant of L–heme + CO to be approximately 10 times larger than that of heme–CO + L. These results were confirmed by White et al. (1979) and extended to mesoheme, where the two equilibrium constants were found to differ by a factor of 10^4 with both 1-methylimidazole and 2-methylimidazole as ligands. The CO affinity with imidazole as ligand was found to be approximately 200 times that with the sterically hindered 2-methylimidazole as ligand. In the proto- and mesoheme diester complexes employed here, we found a difference of 100–200 in relative affinities of heme–CO for L, where L is BI and DMI.

Difference Spectra of CO Complexes. For purposes of comparing the entire spectrum of the model complexes with hemoglobin spectra, difference spectra were generated between pairs of protoheme diester–CO complexes. Three examples in the visible region are shown in Figure 5: the spectrum of the BI complex minus the CMI complex, the spectrum of the DMI complex minus the TMI complex, and the spectrum of the BI complex minus the TMI complex. Also shown for comparison is a reproduction of the CO-Hb Kansas ± IHP difference spectrum taken from Perutz et al. (1976). Note that the model complex spectra are a *stronger* ligand minus a *weaker* ligand, the *reverse* of the five-coordinate difference spectra, and the CO-Hb Kansas spectrum is the spectrum in the presence of IHP (T state) minus the spectrum in the absence of IHP (R state). It should also be noted that the three difference spectra include (1) a pair in which the axial ligands differ only in basicity, (2) a pair in which one ligand is sterically hindered and the other not, and (3) a pair where both are sterically hindered to differing extents. The magnitude of the difference spectrum appears to be larger where at least one of the ligands is sterically hindered than when the two ligands differ in basicity by ~2 pH units. This result suggests that a substantial change in the restraint imposed on the axial ligand should lead to easily observable changes in the visible spectrum of CO–heme complexes. Although magnitudes of the model spectra are considerably greater than

Table IV: Relative Affinities of Ligands for the Heme-CO Complex in *N,N*-Dimethylformamide at 20 °C[a]

ligand	α/β ratio	K_{rel}[b]
protoheme dimethyl ester		
1-*n*-butylimidazole	0.93	3×10^5
1,2-dimethylimidazole	0.99	3×10^3
N,N-dimethylformamide	1.29	1
3,5-dimethylpyridine	1.03	1×10^3
2,6-dimethylpyridine	1.18	1
mesoheme dimethyl ester		
1-*n*-butylimidazole	0.86	2×10^2
1,2-dimethylimidazole	0.95	1
3,5-dimethylpyridine	0.98	2×10^3
2,6-dimethylpyridine	1.13	1
4-methylpyridine	0.98	1×10^2
2-methylpyridine	1.10	1
4-aminopyridine	0.91	1×10^5
pyridine	0.99	2×10^4
N,N-dimethylformamide	1.29	1

[a] DMF solutions containing protohemin diester and a known concentration of the weakest ligand (L_1) to be used were reduced with dithionite as described under Methods. CO was added to 1 atm and the spectrum recorded. A second ligand (L_2) was added in known increments, allowing equilibrium to be achieved before recording the spectrum. In cases where DMF itself was the first ligand, this was followed by the stepwise addition of a third ligand, stronger than the second. Concentrations of each complex were calculated by using previously established extinction coefficients and Beer's law. The relative equilibrium constants were then calculated, $K_{rel} = ([L_2\text{-heme-CO}][L_1])/([L_1\text{-heme-CO}][L_2])$, using the known values of $[L_1]/[L_2]$. [b] Only those complexes enclosed within each brace are to be compared with one another.

the CO-Hb Kansas spectrum, the positive region centered at ~580 nm is of greater relative magnitude in the hemoglobin spectrum than in the model spectra. This positive region has its origin in the red shift of the spectrum as CO-Hb is converted from R state to T state. Clearly this red shift is considerably smaller in the model complexes than in hemoglobin, and the question naturally arises as to whether this difference is due to interactions which distort the CO in CO-Hb (Huber et al., 1970; Heidner et al., 1976).

Figure 6 shows the Soret spectra of the protoheme diester–CO complexes of BI and MEI along with their difference spectrum (BI complex minus MEI complex). This difference spectrum is exceedingly similar to that reported for trout IV CO-Hb at low pH minus high pH (Giardina et al., 1975) and to the *inverse* of those reported for human CO-Hb (Soni & Kiesow, 1977) and carp CO-Hb (Knowles et al., 1975) at high pH minus low pH. Low pH stabilizes the T conformational state relative to the R state, and this is particularly dramatic with Root-effect fish Hb's such as carp (Pennelly et al., 1975) and trout IV (Brunori, 1975).

O_2 Complexes. The O_2 complexes oxidize at a significant rate in DMF at temperatures above −30 °C, and therefore −55 °C was taken as the standard temperature for collection of the spectral data. Representative spectra are shown in Figure 7, and the data obtained are in Table V along with the pK_a values of the ligands used. In the O_2 complexes, like the CO complexes, the β peak intensity remains relatively constant within a series of complexes with structurally similar ligands, and here again the α/β ratio may be taken as an approximate and experimentally convenient way of expressing changes in the intensity of the α peak.

In O_2 complexes of the unhindered ligands, the α/β ratio and λ_{max} correlate with ligand basicity. In the pyridine series, where the number of complexes is largest, the relationship between the α/β ratio and pK_a is essentially linear. As with the CO complexes, low temperature tends to reduce the apparent contribution of steric hindrance and only small differences are observed in the spectra of the O_2 complexes of pyridine ligands with pK_a values between 5 and 7.

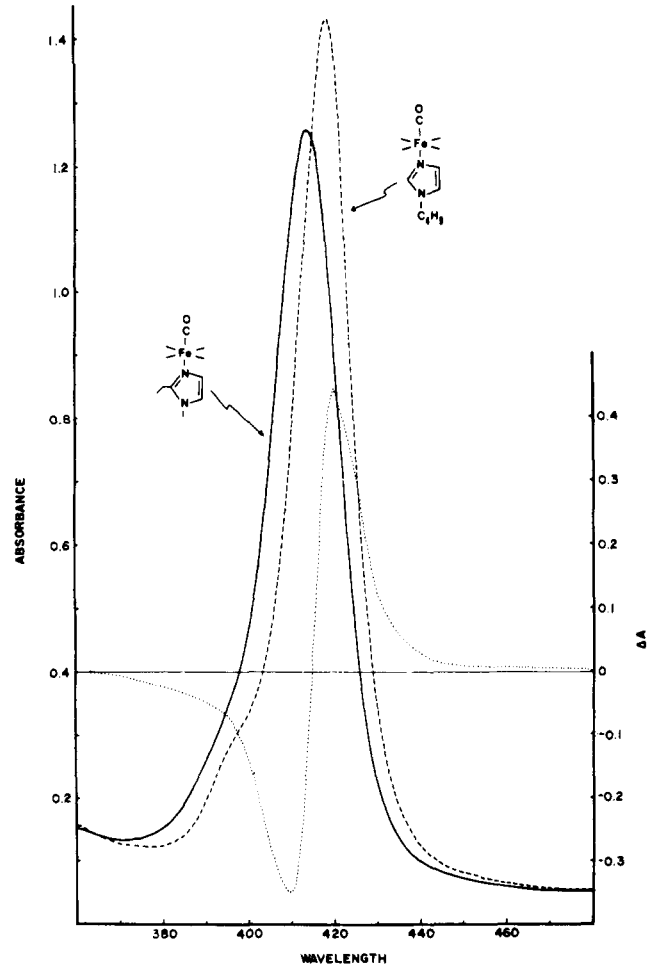


FIGURE 6: Soret spectra and difference spectrum of CO–protoheme diester complexes in DMF at 20 °C: (—) MEI; (- - -) BI; and (···) difference spectrum.

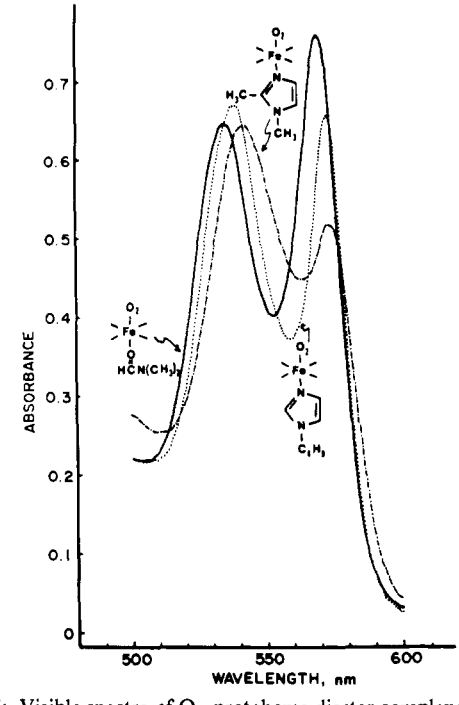


FIGURE 7: Visible spectra of O_2–protoheme diester complexes in DMF at −55 °C: (—) DMF; (-·-) DMI; and (···) BI.

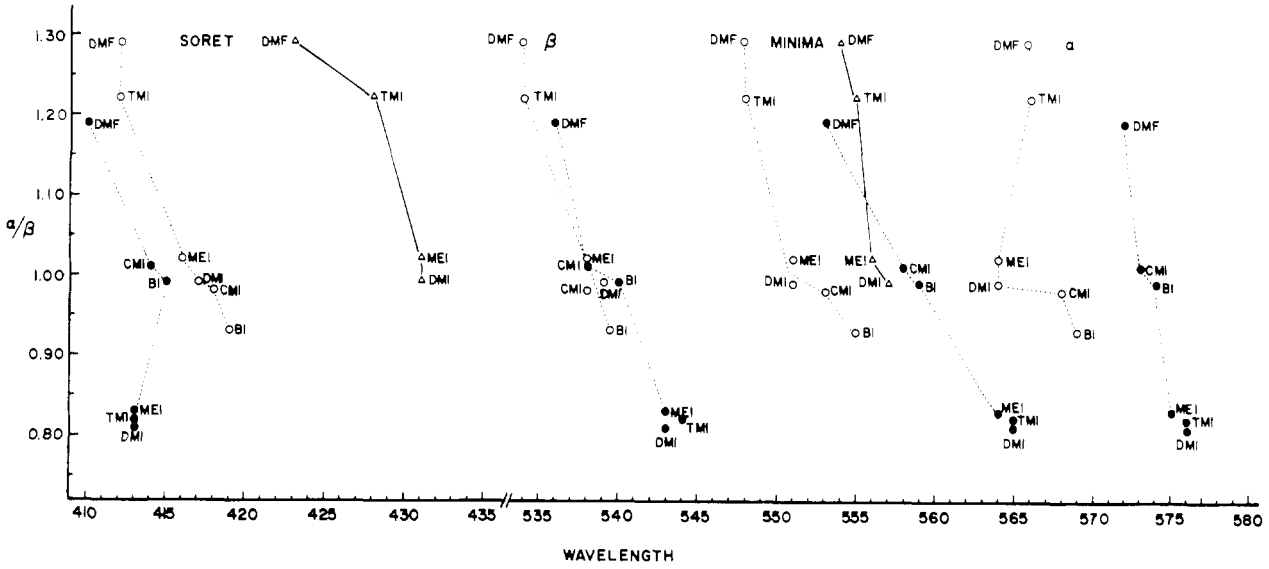


FIGURE 8: Relationship between α/β ratio and wavelength of absorption maxima and minimum for protoheme diester–O_2 and –CO complexes and five-coordinate complexes with imidazole ligands. All spectra are taken with complexes in DMF solution. (○) CO complexes at 20 °C; (●) O_2 complexes at −55 °C; and (△) five-coordinate complexes at 20 °C. α/β ratios of the CO complexes are used for plotting the respective five-coordinate complexes. Connecting lines are merely for visual assistance.

In the imidazole series, the two unhindered ligands BI and CMI give O_2 complexes with relative α/β ratios consistent with their pK_a values. However, complexes of the hindered imidazoles (DMI, MEI, and TMI) give exceptionally low α/β ratios relative to that of the BI complex. This is just the inverse of the relationship for CO complexes, where hindered imidazoles give complexes with higher α/β ratios than unhindered imidazoles at both 20 and −55 °C. Obviously, steric hindrance in the axial ligand affects the spectra of O_2 complexes and CO complexes differently, but basicity has the same effect. A reasonable explanation for this anomaly emerges when one considers that O_2 is a much weaker ligand to Fe than is CO. As a result, sterically hindered ligands can more easily pull the Fe out of the porphyrin plane when O_2 is the trans ligand than when CO is the trans ligand. Since all of these sterically hindered ligands are stronger bases than the unhindered ligands, the actual Fe–N_{axial} interaction could be stronger with the hindered compared to the unhindered ligands of lower basicity. The effect of steric hindrance alone is still apparent in the spectral differences between the DMI and MEI complexes. The basicity of these two ligands is essentially the same, but the slightly greater steric bulk of an ethyl group compared to a methyl makes MEI the slightly weaker ligand, and the α/β ratio of the MEI complex is observably higher than that of the DMI complex. TMI is more hindered than MEI and DMI but is also a stronger base. The two effects appear to cancel, resulting in only small differences between the spectra of these three O_2 complexes.

Relationship between α/β Ratio and Wavelength Maxima and Minima. With the exception of the sterically hindered imidazoles, λ_{max} and λ_{min} of the O_2 complexes, as with the CO complexes, shift to longer wavelength as the α/β ratio decreases. This is also true with the hindered imidazole complexes in the visible, but the Soret undergoes the opposite shift. The reasons for this exception are not clear, but it is tempting to relate the red-shifted visible and blue-shifted Soret with out-of-plane distortion of the Fe by the sterically hindered ligands.

The relationship between α/β ratio, λ_{max}, and λ_{min} for the O_2, CO, and five-coordinate complexes is plotted in Figure 8. α/β ratios of the CO complexes are used for plotting the five-coordinate complexes.

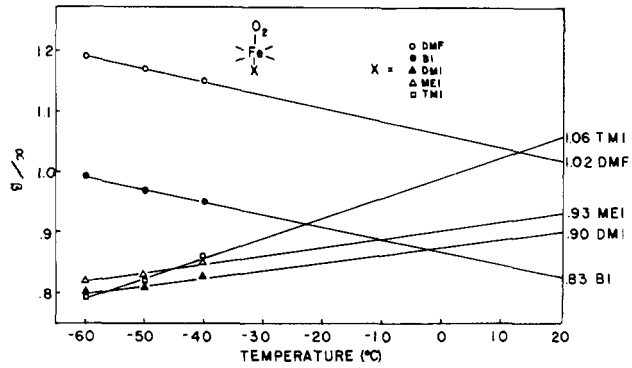


FIGURE 9: Relationship between α/β ratio and temperature for O_2–protoheme diester complexes in DMF: (○) DMF; (●) BI; (▲) DMI; (△) MEI; and (□) TMI. Extrapolated values of the α/β ratio are indicated at the right.

Effect of Temperature on α/β Ratio. α/β ratios of the O_2 complexes are more sensitive to change in temperature than are those of the CO complexes. With unhindered ligands the α/β ratio of O_2 complexes decreases progressively and λ_{max} values of the visible and Soret bands shift ~0.5 nm to longer wavelength as the temperature is increased from −60 to −40 °C. This behavior corresponds to an increasing Fe–N_{axial} interaction as the temperature increases. Spectral properties of O_2 complexes with hindered ligands behave like those of the analogous CO complexes with α/β ratios increasing with increasing temperature. Clearly, this reflects increased thermal motion of the hindering groups, resulting in a weaker Fe–N_{axial} interaction. A plot of the α/β ratio against temperature for a number of O_2 complexes is shown in Figure 9. Although the curves may not remain linear to +20 °C, this is the only means presently available for estimating spectral parameters of O_2–protoheme complexes at room temperature. The extrapolated α/β ratios are of interest for comparison with α/β ratios of hemoglobins and myoglobins as a means of assessing restraint exerted by the protein on the proximal histidine.

Relationship between Spectra and Stability of O_2 Complexes. For CO complexes the α/β ratio is indicative of relative ligand affinities within a structural series: the lower the α/β ratio the more stable the complex. Such is not invariably the case with O_2 complexes. The exceptions are the

Table V: Spectral Properties of O_2-Heme Complexes in *N,N*-Dimethylformamide[a]

ligand	pK_a[b]	λ (nm) at −55 °C: α	β	min	Soret	α/β ratio at −55 °C
protoheme dimethyl ester						
1-*n*-butylimidazole (BI)	7.10	574	539.5	559.5	416	0.99
5-chloro-1-methylimidazole (CMI)	5.45	573	538	558	414	1.01
1,2-dimethylimidazole (DMI)	7.85	575.5	544	565	413	0.81
1-methyl-2-ethylimidazole (MEI)	7.85	575	543	565	413	0.83
1,2,4,5-tetramethylimidazole (TMI)	8.92	575.5	544	565	413	0.82
4-aminopyridine	9.11	574	540	562	413	0.93
4-methylpyridine	6.02	573	539	561	412	0.98
pyridine	5.25	573	539	561	411	1.00
2-methylpyridine	5.97	573	539	561		1.00
2,6-dimethylpyridine	6.77	573	538	560		1.02
4-cyanopyridine	1.90	571.5	536.5	558	410	1.11
N,N-dimethylformamide[c]	−2.0	571	536	553.5	410	1.19
human oxyhemoglobin[d]		577	542	560	415	1.06
mesoheme dimethyl ester						
1-*n*-butylimidazole	7.10	565	532	552		0.94
5-chloro-1-methylimidazole	5.45	564	530	550		0.98
1,2-dimethylimidazole	7.85	566	535	558		0.77
1-methyl-2-ethylimidazole	7.85	566	535	557		0.80
1,2,4,5-tetramethylimidazole	8.92	566	535	558		0.77
4-aminopyridine	9.11	564	531	552		0.83
pyridine	5.25	563	530	549.5		0.96
4-cyanopyridine	1.90	562.5	528.5	548		1.00
N,N-dimethylformamide	−2.0	561.5	528	545.5		1.09
acetone		561	528	545		1.16
2,4-dimethyldeuteroheme dimethyl ester						
1-*n*-butylimidazole	7.10	565	532	551		0.94
1-methyl-2-ethylimidazole	7.85	566	536	557		0.80
N,N-dimethylformamide	−2.0	562	528	546		1.09

[a] Spectra were taken in DMF solutions at −55 °C, ~0.05 mM in heme for visible spectra and ~0.005 mM for Soret. Ligand concentrations range from 5 to 100 mM, depending on the strength of the ligand. Numerical values of the α/β ratio are reproducible to ±0.01 and are unaffected by heme diester concentration. [b] From Sober (1968) or determined by titration with standard sulfuric acid by Michael Vitali. [c] DMF used for the spectrum previously reported (Brinigar & Chang, 1974) contained an amine impurity (see Materials section). [d] Included for comparison.

complexes with sterically hindered ligands. For example, when the TMI–heme–O_2 complex was treated with increasing amounts of BI, the spectrum became identical with that of BI–heme–O_2 when the [BI]/[TMI] ratio was less than 0.5. Under the same conditions, BI completely replaced DMI and MEI. These results are consistent with the Fe being severely distorted away from the porphyrin plane in O_2 complexes of the hindered ligands. Severe distortion would weaken interactions between Fe and the pyrrole nitrogens as well as those with O_2. Thus, despite a stronger Fe–N_{axial} interaction, the stability of the entire complex would be lower than that of a complex with a less basic but unhindered ligand, which permitted the Fe to bond optimally with the other ligands.

Relative Affinities of O_2–Heme and CO–Heme for Hindered and Unhindered Ligands. If, as suggested above, O_2 as a sixth ligand permits greater distortion of the Fe than CO as a sixth ligand, then given a choice between a strongly basic hindered ligand and a weakly basic unhindered ligand, O_2–heme should exhibit a preference for the trans-hindered ligand whereas CO–heme should prefer the unhindered ligand. Results of such a competition experiment with DMF and TMI as unhindered and hindered ligands, respectively, are shown in Figure 10. Clearly, the ratio [TMI–Fe–CO]/[DMF–Fe–CO] is lower by at least a factor of 5 than the ratio [TMI–Fe–O_2]/[DMF–Fe–O_2] at all observed TMI concentrations. Although subtle differences between the bonding of O_2 and CO to Fe may contribute to the competition, the most obvious explanation for this result is that Fe is more easily distorted from the porphyrin plane by steric restraints in an axial ligand when O_2 is the trans ligand compared to the case where CO is the trans ligand.

Difference Spectra of O_2 Complexes. Here again spectral differences between the model complexes can be plotted as difference spectra for comparison with hemoglobin difference spectra. Figure 11 shows spectra obtained with unhindered ligands (BI minus CMI), both hindered and unhindered ligands (TMI minus BI), and two hindered ligands (TMI minus DMI) compared with the O_2-Hb Kansas ± IHP difference spectrum reproduced from Perutz et al. (1976). Note that the model spectra are plotted with the complex having the lower α/β ratio minus the complex with the higher α/β ratio. In terms of base strength alone, this corresponds to a strong ligand minus a weaker ligand. In terms of steric hindrance alone, two of the spectra correspond to a more hindered ligand minus a less hindered ligand. Note that the hemoglobin spectrum is O_2-Hb Kansas in the presence of IHP (T state) minus O_2-Hb Kansas in the absence of IHP (R state). As in the case of the CO complexes, the oxyhemoglobin T minus R state difference spectrum is approximated by a complex with a stronger Fe–N_{axial} interaction minus a complex with a weaker Fe–N_{axial} interaction, although this assignment is not compelling in complexes of the sterically hindered ligands.

The magnitude of the TMI minus DMI spectrum is somewhat smaller than the Hb Kansas spectrum, whereas the BI minus CMI spectrum is roughly comparable, and the TMI minus BI spectrum is of considerably greater magnitude than the Hb Kansas spectrum. Other oxyhemoglobin difference spectra have been reported, and although they probably do not involve an actual T minus R state conformational difference, they are similar to the Hb Kansas ± IHP spectrum. Examples are shown in Figure 12 along with the model

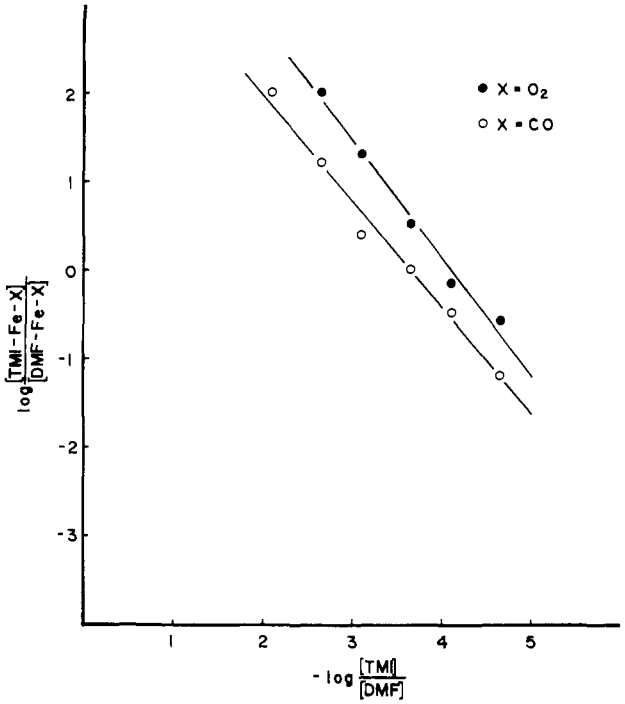


FIGURE 10: Competition between DMF and TMI for coordination to protoheme diester complexes of O_2 (●) and CO (○) at −55 °C. DMF solutions of protohemin diester containing TMI at the concentrations indicated were reduced by dithionite as described under Methods. After the mixture was cooled to −55 °C, O_2 was admitted to 1 atm of pressure and the spectrum recorded. O_2 was then displaced with CO, and after equilibrium was attained the spectrum was recorded again. The ordinate values were calculated from the absorbance of the mixture and the extinction coefficients of the TMI and DMF complexes, or more simply from the α/β ratios of the two complexes and the α/β ratio of the mixture. The lines were calculated by the least-squares method.

difference spectrum of TMI minus DMI.

Difference spectra can also be generated by using a single O_2 complex at two different temperatures. The BI complex is shown as an example in Figure 13. The difference spectrum of higher temperature minus lower temperature is remarkably similar to the one recently reported for hemoglobin by San Biagio et al. (1977). This result suggests for both oxyhemoglobin and O_2–heme complexes with unhindered ligands that the Fe–N_{axial} interaction becomes stronger as the temperature increases within a limited temperature range.

Discussion

The data reported in Tables I, II, and V clearly show that the electronic spectra of simple heme complexes can be correlated with electronic and structural properties of the axial ligand. Certain restrictions apply to the correlations: (1) spectral parameters of five-coordinate complexes, O_2 complexes, and CO complexes must be considered separately; (2) relatively large differences in the α/β ratio or λ_{max} relate to large differences in the strength of the Fe–L (L = axial ligand) bonding interaction regardless of the nature of the axial ligand; and (3) small differences in these spectral parameters can only be compared within a series of complexes possessing structurally similar axial ligands, e.g., the imidazole series or pyridine series. Of course, each porphyrin must also be considered separately; i.e., protoheme complexes cannot be compared directly with mesoheme complexes.

Reliable spectral data on these complexes can only be obtained when complete reduction of the iron from +3 to +2 oxidation states is achieved and maintained. This is particularly difficult for the O_2 complexes because partial oxidation of Fe(II) to Fe(III) occurs even at low temperature. This problem was overcome by using a three- to fivefold excess of $Na_2S_2O_4$ in a small amount of water to reduce the Fe(III) porphyrin diester in DMF solution. At low temperature the excess dithionite reduces free O_2 and O_2 coordinated to heme exceedingly slowly but rapidly reduces any Fe(III) porphyrin to Fe(II). In many cases where other methods of reduction were employed, the addition of dithionite, its oxidation products, and water caused no observable change in the absorption spectra.

Solvents other than DMF, such as toluene and acetone, were also employed, and the spectral parameters were found to be only slightly different than in DMF. Thus, large changes in the solvent environment of the heme complexes appear to have a much smaller effect on electronic spectra than even minor changes in the axial ligand.

The immediate interest in the spectral correlations presented here is to determine if they can be used to provide new insight into structural differences and changes among the O_2-transporting hemoproteins. Attempts to use these correlations in this way should be prefaced by a word of caution. In any study involving the use of simplified models to simulate a more complex process, the danger always exists that the model is not a faithful mimic. We have found one way to approximate hemoglobin spectra with simple complexes, but of course there may be others. This is of real concern because both five- and six-coordinate hemoglobin difference spectra result largely from small wavelength shifts in the absorption spectra. Many perturbations in the structure or electron distribution in the heme could give rise to small differences in the ground state or excited state energy levels. Knowles et al. (1975) have shown that the observed liganded hemoglobin difference spectra can be approximated rather closely by simply differentiating the absorption spectrum. Therefore, they suggested that the difference spectra may not have a unique chemical origin. In one sense we see that similar spectral changes can have different chemical origins. The hemoglobin difference spectra can be approximated with pairs of model complexes where the ligands differ in terms of steric restraint or in terms of basicity. However, both steric restraint and basicity influence the strength of interaction between ligand and iron or the electron density on the iron, and in this sense the spectral changes do have a common chemical origin. Although a large amount of spectral data is presented in support of the correlations, some ambiguity remains and, even if the ambiguities were resolved, limitations in the application of the spectral correlations to hemoproteins would not be clear without more detailed structural data on the model complexes. Therefore, for the present, deductions derived from imposing model spectral correlations upon hemoproteins can be considered compelling only when they are supported by other types of experimental evidence.

Five-Coordinate Complexes. In the five-coordinate complexes it is apparent that as the axial ligand is altered by increasing the steric restraints to coordination; the λ_{max} of both the visible and Soret peaks shifts progressively to shorter wavelength. A more dramatic shift to the blue is observed when any of the nitrogenous ligands is replaced by the exceedingly weak base DMF. That steric hindrance, and not basicity, is the principal factor governing the strength of the Fe–N_{axial} interaction was shown by the ability of a less hindered ligand such as DMI to replace a more highly hindered ligand such as TMI, even though TMI is the stronger base. In addition, the spectrum of the slightly more hindered MEI

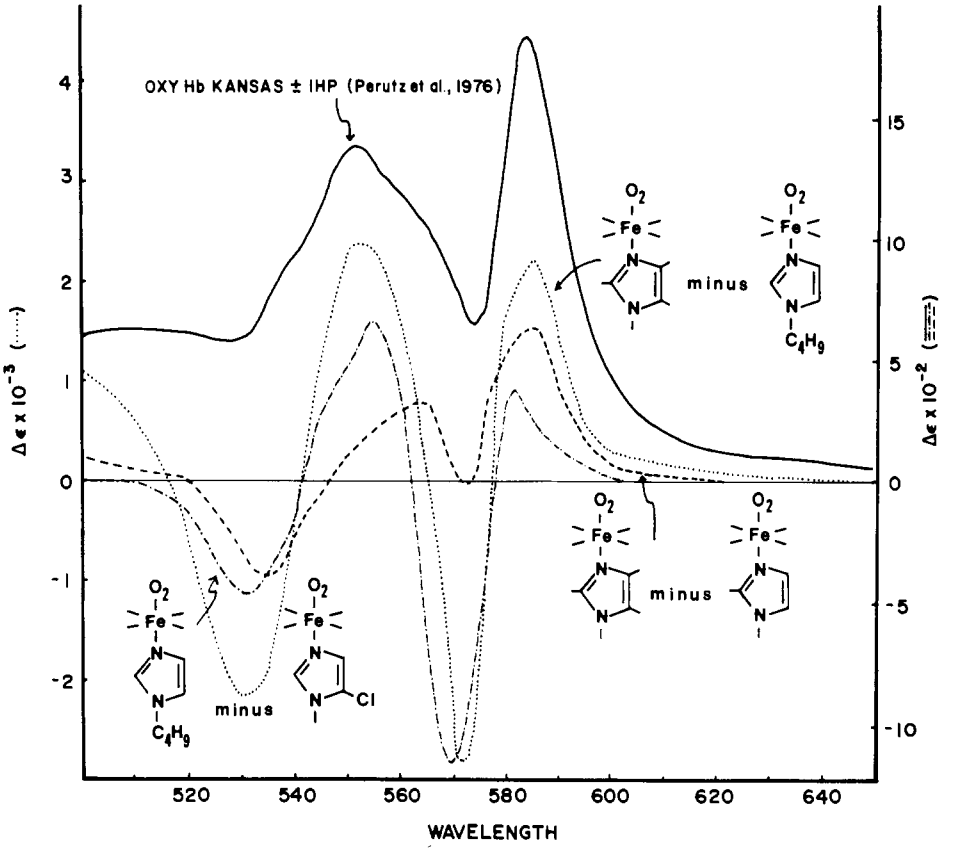


FIGURE 11: Visible difference spectra of protoheme diester–O_2 complexes in DMF at −55 °C: (---) spectrum of the O_2 complex of TMI minus that of DMI; (···) the O_2 complex of TMI minus that of BI; (−·−) the O_2 complex of BI minus that of CMI; and (—) oxy-Hb Kansas with IHP minus oxy-Hb Kansas with no IHP, reproduced from Perutz et al. (1976). Note the differences in ordinate scales.

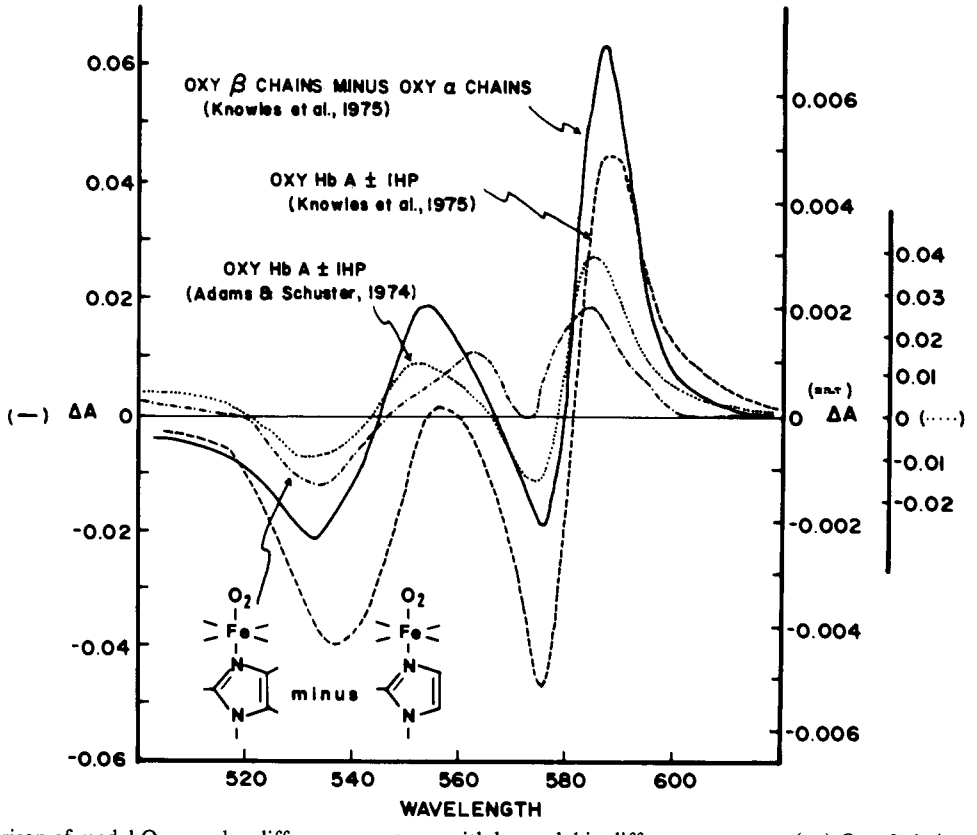


FIGURE 12: Comparison of model O_2 complex difference spectrum with hemoglobin difference spectra. (—) Oxy β chains minus oxy α chains in 0.05 M phosphate, pH 7.0 and 20 °C (left ordinate scale), reproduced from Knowles et al. (1975); (---) oxy-HbA plus IHP minus oxy-HbA in 0.05 M Tris and 0.1 M NaCl, pH 7.0 and 20 °C (inner ordinate scale on right), also reproduced from Knowles et al. (1975); (···) oxy-HbA plus IHP minus oxy-HbA in 0.1 M Hepes, pH 7.0 and 5.6 °C (outer ordinate scale on right), reproduced from Adams & Schuster (1974); and (−·−) the O_2–protoheme diester complex of TMI minus the O_2 complex of DMI in DMF at −55 °C (ordinate scale on left).

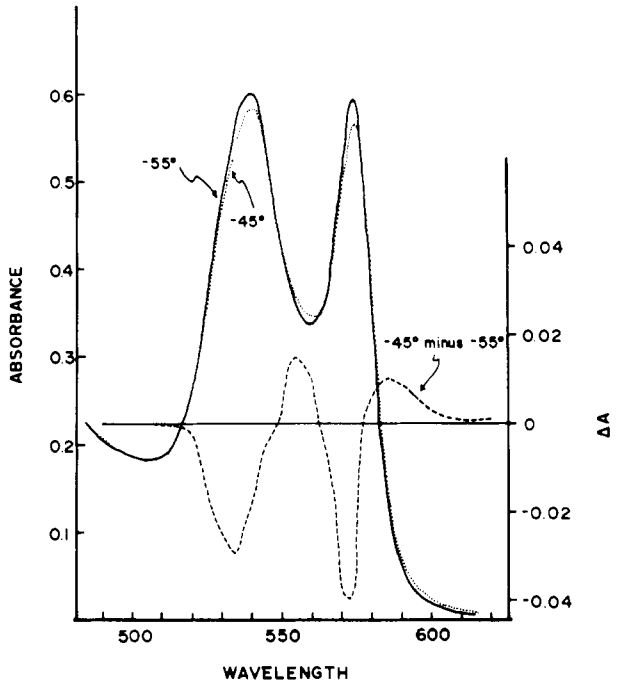


FIGURE 13: Visible spectra of the O_2–protoheme diester complex of 1-*n*-butylimidazole in DMF at −45 (···) and −55 °C (—) along with the difference spectrum (- - -), −45 minus −55 °C.

complex is blue shifted compared to the DMI complex. All the nitrogenous ligands replace DMF at much lower concentrations than the DMF, and the λ_{max} values of their complexes are all red shifted compared to the DMF complex. So the stability of these complexes can be related to the λ_{max} of both the visible and Soret peaks, a blue shift corresponding to a decreased axial interaction. Associated with the blue shift in all cases was an increase in extinction of the Soret peak (Figure 1). This characteristic appears to be the most sensitive indication of change in the axial interaction. In this connection, Olson (1976) observed that the Soret peak of the α-deoxyhemoglobin chains increases approximately 30% upon aggregation with β-deoxy chains, whereas the spectrum of the β chains changes little. This suggests a decrease in the Fe–N_ϵ interaction in the α chains and no change in the β chains upon assembly of the deoxy $\alpha_2\beta_2$ tetramer.

The deoxy T minus R state difference spectra observed by Perutz et al. (1974) with Hb Kempsey, NES-des-Arg-deoxy-Hb, and des-Arg-Tyr-deoxy-Hb in the presence of IHP minus in the absence of IHP were closely approximated by taking the spectrum of a complex with a more highly hindered ligand minus that of a less hindered ligand (Figures 2 and 3). Thus, by analogy with the model spectra, the Fe–N_ϵ interaction in hemoglobin would appear to be weaker in the T state than in the R state. This result was anticipated from considerations developed by Perutz (1976), whereby conformational restraints in the T state exert tension on the proximal histidine, pulling it away from the heme iron and increasing the Fe–N_ϵ bond length. However, the recent extended X-ray absorption fine structure (EXAFS) results of Eisenberger et al. (1976, 1978) and the resonance Raman results of Kincaid et al. (1979) suggest that the Fe–N bond distances of deoxy-Hb Kempsey do not change by more than a few hundreths of an angstrom as a result of the transition from R to T states. The results here do not speak directly to how much the bond distances change, but only indicate the direction of the change. Insofar as a decrease in the axial interaction can be associated with an increase in the axial bond length, the spectral changes accompanying the R → T transition indicate that the axial bond length increases, but obviously this increase could be small. Of course, the structural changes could involve changes in orientation and tilting of the proximal imidazole relative to the heme (Gelin & Karplus, 1977) rather than a simple movement of the proximal imidazole perpendicular to the heme. The magnitude of the Hb Kempsey ± IHP difference spectrum is approximately equivalent to that obtained from the TMI complex minus the DMI or MEI complexes. Both DMI and MEI can tilt in the plane of the imidazole ring in order to reduce unfavorable van der Waals contact between the 2-alkyl group and the porphyrin ring, whereas TMI cannot. So the differences in Fe–N_{axial} interactions between these ligands could be due to differences in geometry as well as different Fe–N_{axial} bond lengths. A difference in Fe–N_{axial} bond length of a few hundreths of an angstrom between R and T states would not appear to be inconsistent with the visible and Soret difference spectra.

It is not clear to what extent the blue-shifted spectrum can be associated with out-of-plane distortion of the Fe. No doubt some distortion occurs with the hindered ligands because most of them form a six-coordinate complex at low temperature where decreased thermal motion of the alkyl group lessens the steric restraint; however, the most hindered ligand, TMI, does not, and neither does the unhindered but exceedingly weak ligand DMF. It seems probable that a blue-shifted spectrum results from a weaker Fe–N_{axial} interaction, whether it results from a decrease in basicity of the axial ligand with no structural distortion of the complex or from the introduction of steric restraints which pull the Fe away from the porphyrin plane in order to achieve maximum bonding with all five ligands.

CO Complexes. As the axial ligand is changed in CO complexes, the shift in λ_{max} is analogous to the shifts observed in the five-coordinate complexes (Table II). With unhindered ligands differing in basicity, a blue shift occurs in λ_{max} as a more strongly basic axial ligand is replaced by a weaker base. With the hindered ligands, as the hindrance to coordination increases λ_{max} shifts to the blue despite increases in basicity. Accompanying the blue shift is an increase in the relative intensity of the α peak (~565 nm) compared to the β peak (~535 nm). This spectral parameter is termed the α/β ratio. The relationship between the α/β ratio and λ_{max} is shown in Figure 8.

With complexes of the unhindered ligands, lowering the temperature from 20 to −55 °C has little effect on the spectral parameters, generally slightly shifting the visible λ_{max} to the blue. However, with the hindered ligands, lowering the temperature results in a dramatic decrease in the α/β ratio and small changes in λ_{max}. This property of the CO complexes parallels that of the five-coordinate complexes; as thermal motion in the hindering alkyl groups subsides, the Fe–N_{axial} interaction increases.

The relative stabilities of the CO complexes are clearly related to their α/β ratios. Within a structurally related series of ligands, the lower the α/β ratio the greater the equilibrium constant of the reaction L + heme–CO ⇄ L–heme–CO is. Alben & Caughey (1968) have shown that the stability of L–heme–CO complexes is inversely related to their CO stretching frequencies (ν_{CO}). Obviously, the α/β ratio should also bear a direct relationship to ν_{CO}, and such is found to be the case (Table III). These results suggest that the α/β ratio also can be taken as a measure of the strength of the Fe–CO bond as well as the L–Fe bond, and therefore the α/β ratio should be inversely related to the affinity of L–heme complexes for CO. Although at present we have only an indirect measure

of a few relative CO affinities (Table IV), the conclusion appears to be valid.

Wang (1961) first demonstrated that an increase in CO affinity resulted from increasing the strength of the trans ligand and proposed that back π bonding from Fe to CO is facilitated by strong ligands trans to CO. Since the α/β ratio appears to be most directly related to the strength of the L–Fe bonding interaction, it would be expected to be a measure of CO affinity as well. As will be discussed below, the same spectral characteristics appear to be related to O_2 affinities. Obviously, this correlation might prove applicable to O_2-transporting hemoproteins as a means of comparing intrinsic affinities, i.e., affinities of the hemoproteins for CO and O_2 due exclusively to the heme–proximal imidazole complex, devoid of steric or other factors operative in the heme pocket.

O_2 Complexes. When only the complexes with nonhindered ligands are considered, the same correlations apply to the O_2 complexes as to the CO complexes; as ligand basicity decreases, λ_{max} shifts to the blue and the α/β ratio increases (Table V). However, spectral properties of the O_2 complexes with hindered imidazole ligands are quite different compared to those of the corresponding CO complexes. All three of the hindered imidazoles give O_2 complexes with exceptionally low α/β ratios, and their visible λ_{max} values are red shifted and their Soret λ_{max} values blue shifted compared to the O_2 complex of the unhindered BI.

The spectra of the O_2 complexes could be observed only at low temperature, where steric factors clearly have a smaller inhibitory effect on the ligand coordination than they do at room temperature. Nevertheless, when O_2 complexes are compared to the corresponding CO complexes at −55 °C, the anomaly is still apparent. These results suggest that a difference of fundamental importance exists between O_2– and CO–heme complexes. A reasonable explanation for the difference emerges when one considers the consequences of CO being a stronger ligand than O_2. If we compare the three diatomic gases which coordinate to heme complexes, NO, CO, and O_2, NO is by far the strongest ligand followed by CO and O_2. In six-coordinate Fe(II) porphyrin–NO complexes, the Fe was found ~0.1 Å out of the porphyrin plane in the direction of NO (Scheidt et al., 1977). CO forms a weaker bond, and the Fe is essentially in the plane of the porphyrin nitrogens (Peng & Ibers, 1976). O_2 is such a weak ligand that a strong ligand trans to O_2 might pull the Fe out of the plane away from the O_2. One would expect the distortion to become particularly large with sterically hindered or otherwise restrained ligands. If this is the case, steric hindrance in the ligand trans to O_2 will have less effect on the Fe–N_{axial} interaction than it would in the corresponding CO complex where distortion is more strongly opposed by CO. As a result, the strength of the Fe–N_{axial} interaction in the O_2 complexes with hindered ligands is largely a function of ligand basicity, particularly at low temperature where the steric restraints are small. If this explanation is correct, then one would expect a highly hindered but strongly basic ligand to more easily replace a weak unhindered ligand when O_2 is the trans ligand compared to CO as the trans ligand. This expectation was realized as shown in Figure 10.

On the basis of the foregoing discussion, an anomaly between the relative stability constants and α/β ratios can also be anticipated for O_2 complexes with hindered ligands. If the Fe is displaced from the porphyrin plane or the heme ring system is otherwise distorted in the O_2 complexes with hindered ligands, then the Fe–$N_{porphyrin}$ and Fe–O bonds will be weakened relative to an equivalent undistorted complex. As a result, the complexes with hindered ligands might be thermodynamically less stable than the unhindered complexes, despite their low α/β ratios which suggest a stronger Fe–N_{axial} interaction and greater stability. Such was found to be the case. BI will replace any of the hindered imidazole ligands essentially completely at concentrations less than half the hindered imidazole concentration.

It is unfortunate that spectra of the O_2 complexes cannot be observed at higher temperatures because at sufficiently high temperature steric effects in the hindered ligands should predominate over basicity and relative values of α/β ratios in the O_2 complexes should parallel those in the CO complexes. However, it is possible to gain an approximate idea of what the spectral parameters would be at room temperature. Spectra of the O_2 complexes are much more sensitive to temperature change than are the CO spectra, so one can observe the spectral changes over a fairly narrow temperature range and then extrapolate to higher temperature. Of course there is no assurance that the spectral changes remain linear to +20 °C; however, the trends are unmistakable and the results, shown in Figure 9, are those reasonably anticipated. All the complexes with hindered ligands increase in α/β ratio as the temperature is increased, the slope being greater for the more highly hindered. The complexes with unhindered ligands decrease in α/β ratio with approximately the same slope for two vastly different types of ligand (BI and DMF). Thus, what appeared to be an anomaly in the correlation between α/β ratio and strength of the axial interaction in the O_2 complexes can be attributed to the ease in which the O_2 complex is distorted by sterically hindered ligands and to the low temperature, which decreased the effect of steric hindrance on ligand strength relative to the effect of basicity. Distortion of the complex no doubt affects the electronic spectrum, and this is likely the basis for the Soret peak of O_2 complexes with hindered ligands being blue shifted compared to the Soret peak of the complexes with unhindered ligands (Figure 8).

The spectral changes of oxyhemoglobin as a function of temperature parallel those of the model complexes with unhindered ligands; the temperature difference spectrum with the BI complex shown in Figure 13 is exceedingly similar to the one reported by San Biagio et al. (1977) for oxyhemoglobin.

The most striking aspect of the α/β ratio vs. temperature plot (Figure 9) is that the extrapolated α/β ratio of the BI complex is so low (0.83) compared to that of common vertebrate oxyhemoglobins (1.06), whereas the extrapolated α/β ratios of the DMF and TMI complexes are roughly its equivalent. The basicity of BI is nearly the same as a histidine side-chain imidazole, and therefore if the proximal imidazole–heme iron interaction were at the minimum of the potential energy well, one would expect the spectral characteristics of oxyhemoglobin and the BI–heme–O_2 complex to be comparable. In fact, the extrapolated α/β ratio of the BI complex is very close to that reported for Ascaris Hb (0.85), the highest affinity hemoglobin known (Wittenberg et al., 1972). The α/β ratio of the BI–heme–CO complex at 20 °C (0.93) is also considerably lower than the α/β ratio of common carboxyhemoglobins (1.0). This appears to be a clear implication that the proximal imidazole of common hemoglobins is restrained in some manner by the globin in its interaction with Fe in the *high-affinity R state*. It might be argued that the basicity of the proximal imidazole is somehow lowered by its unique environment, but the only apparent interaction of the F8 proximal imidazole is a hydrogen bond from N_{δ_1} to the F4 carbonyl oxygen, and this interaction would increase, rather

than decrease, the basicity of the imidazole.

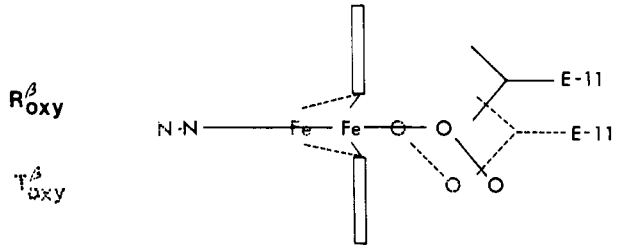
Further support for the suggestion that the Fe–N_ϵ interaction is weak in R-state hemoglobins comes from the existence of a number of hemoglobins and myoglobins with α/β ratios much lower than those of common vertebrate hemoglobins. Examples, in addition to Ascaris, O_2-Hb are Aplysia O_2-Mb (α/β = 0.92) (Rossi Fanelli & Antonini, 1959), Paramecium O_2-Hb (α/β = 0.88) (Smith et al., 1962), and Strongylus O_2-Hb (α/β = 0.85) (Davenport, 1949). With the exception of Mb Aplysia, all of these hemoglobins have exceedingly high O_2 affinities, commensurate with a strong Fe–N_ϵ interaction (see below). Moreover, X-ray structures of sperm whale O_2-Mb (Phillips, 1978) (α/β = 1.07) and Chironomus O_2-Hb (Weber et al., 1978) (α/β = 0.97) have been reported recently where the Fe is found to be out of the porphyrin plane on the proximal side. In both structures the Fe appears to be at least as far out of the plane as it is in the deoxy structures. In addition, the X-ray structures of a number of carboxyhemoglobins and -myoglobins have been reported where the heme ring appears to have moved closer to the F helix compared with the deoxy structure, or the Fe is slightly out of the plane on the proximal side (Norvell et al., 1975; Padlan & Love, 1974; Heidner et al., 1976; Huber et al., 1970). Taken together, these observations constitute strong evidence that the proximal histidine is restrained in some manner in both myoglobins and in R state hemoglobins whose oxy spectra have an α/β ratio greater than $\sim$0.9.

Relationship between α/β Ratio and Affinity. It has been established for a number of transition metal–O_2 complexes that a low electron density on the metal weakens the metal–oxygen bond and vice versa (McGinnety et al., 1969; Stynes et al., 1973). The same generalization holds for CO complexes (Wang, 1961; Alben & Caughey, 1968). Indirect experimental results indicating the magnitude of this effect on the affinity of heme complexes for CO are shown in Table IV. Rougee & Brault (1975) measured a number of affinity constants directly and found that the affinity of imidazoledeuteroheme for CO is 200 times that of 2-methylimidazoledeuteroheme. So it seems clear that if the α/β ratio is a measure of the strength of the Fe–N_{axial} interaction, then α/β ratios of the O_2 and CO complexes should correlate with the relative affinities of the complexes for O_2 and CO, respectively. Obviously, exceptions are to be expected in cases of O_2 complexes where the ligand is both a strong base and is severely hindered. This complication presumably would not arise in hemoglobins and myoglobins where the basicity of the proximal imidazole probably remains essentially the same and the strength of the Fe–N_ϵ interaction is a function only of the restraints imposed upon it by the protein. However, whether or not the basicity of the proximal imidazole varies in hemoglobins and myoglobins, their α/β ratio should be a measure of the intrinsic affinity (i.e., affinity due exclusively to the Fe–N_ϵ interaction) for O_2 and CO. Of course, intrinsic affinities are difficult to establish, but estimates of relative values can be obtained by comparing hemoglobins with different hemes and the same globin. The clearest example is mesoheme and 2,4-dimethyldeuteroheme where electronic effects of ring substituents are essentially the same for both (Seybert et al., 1977; Parker & Brinigar, 1976), and, as shown in Tables II and V, these two heme esters give both O_2 and CO complexes with indistinguishable spectra. However, the spectrum of oxymesohemoglobin is significantly different from that of 2,4-dimethyldeuterohemoglobin, as are their p_{50} values. Oxymesohemoglobin has an α/β ratio of 0.90 and a p_{50} of 1.8 Torr (Sugita & Yoneyama, 1971), whereas oxy-2,4-dimethyldeuterohemoglobin has an α/β ratio of 0.93 and a p_{50} of 3.0 Torr (Seybert et al., 1977). One can also compare isolated α- and β-hemoglobin chains. Oxy and CO α chains have α/β ratios of 1.06 (Sugita, 1975) and 1.02 (Banerjee et al., 1969), respectively, with O_2 and CO p_{50} values of 0.5 Torr (Antonini et al., 1965) and 2.5×10^{-3} Torr (Brunori et al., 1966), whereas O_2 and CO β chains have α/β ratios of 1.04 (Sugita, 1975) and 1.01 (Banerjee et al., 1969) with p_{50} values of 0.43 Torr (Antonini et al., 1965) and 1.6×10^{-3} Torr (Brunori et al., 1966), respectively. These p_{50} values do not represent actual intrinsic affinities, but other factors which influence affinity should be roughly equivalent; therefore, in these two cases, differences in p_{50} values should largely reflect differences in intrinsic affinities. Although the evidence is limited, the indication is that the relationship between α/β ratio and affinity applies to hemoproteins as well as model complexes.

Application of the Spectral Correlations to Hemoglobin Cooperativity. If α/β ratios of O_2- and CO-Hb are as sensitive a measure of the Fe–N_ϵ interaction as they appear from the model complexes, then a comparison of the spectra of both O_2- and CO-Hb in R and T states (Figures 5, 6, 11, and 12) indicates that the Fe–N_ϵ interaction changes little as a result of the conformational transition. This follows from the very small change in α/β ratios and shifts in λ_{max} of both O_2- and CO-Hb Kansas upon the addition of IHP (Perutz et al., 1976). Hemoglobin Kansas is a low-affinity mutant hemoglobin in which the allosteric equilibrium is shifted far to the T state. Oxy- and carboxy-Hb Kansas exist in an R state, but addition of IHP causes the conformation to shift to a T state without significant dissociation of O_2 or CO (Ogawa et al., 1972; Salhany et al., 1975; Perutz et al., 1976).

Although changes in the heme coordination sphere appear to be small as a result of the R $\rightarrow$ T conformation change, the changes in the spectra which do occur can be related to changes in the Fe–N_ϵ interaction. In both O_2- and CO-Hb Kansas the addition of IHP results in a slight decrease in the α/β ratio and a red shift of λ_{max}. Clearly these changes are associated with an increase in the Fe–N_ϵ interaction. This conclusion is supported by the complexes used to simulate the T minus R state difference spectra (Figures 5, 6, and 11). For both O_2 and CO complexes the T minus R state Hb Kansas spectra were closely approximated by several pairs of model complexes: L_1–heme–X minus L_2–heme–X, where L_1 was a stronger ligand than L_2. Thus, it appears that in liganded hemoglobins the transition from R to T states causes the Fe–N_ϵ interaction to become stronger, whereas in deoxyhemoglobins the Fe–N_ϵ interaction becomes weaker. A reasonable explanation consistent with these results and the X-ray structures of liganded and deoxyhemoglobins emerges when one considers structural changes which occur on the distal side of the heme. When the transition from R to T state occurs, the side chains of E7 His and E11 Val come into steric conflict with the bound O_2 or CO, forcing the Fe–O_2 or Fe–CO entities, and/or the entire heme complex, toward the proximal imidazole. Thus, the Fe–N_ϵ bond distance could be shorter in T state than in R state, as is shown schematically in Scheme I. The representation is not meant to imply that relative movements of the proximal imidazole and heme are necessarily along an axis perpendicular to the heme nor that the heme ring remains rigid or fixed. The scheme represents only the basis of the structural change which is proposed to produce a stronger Fe–N_ϵ interaction in the T state. Regardless of the structural details, strain would be introduced not only in the heme complex but also in the E helix region of the protein as well. Thus, the

Scheme I



lowered affinity of T-state hemoglobins would be due to steric conflict between bound ligand and the distal side chains and to distortion of the Fe out of the porphyrin plane caused by restraint on the proximal imidazole.

It should be added that steric obstructions to O_2 binding are clearly more severe in the β subunits than in the α subunits (Heidner et al., 1976; Fermi, 1975; Baldwin & Chothia, 1979). It is conceivable that in the α subunits the Fe–N_ϵ bond actually lengthens somewhat during the oxy R → T transition, and in the β subunits distal steric strain causes the Fe–N_ϵ bond distance to shorten, the latter change being larger than the former in compliance with the difference spectra. If such were the case, EXAFS determination (Eisenberger et al., 1976, 1978) of average Fe–N bond distances would be much smaller than the individual differences in each of the two subunits. This could have a significant effect on estimates of the strain energy accompanying distortion of the heme complex (Eisenberger et al., 1976; Kincaid et al., 1979). However, all presently available evidence indicates that the excess free energy of the T state which is localized in the heme complex is small. It seems likely that the major portion of the energy of cooperativity is due to distortion of the protein and not to distortion of the heme. By analogy to two springs in steric conflict, the O_2–heme spring has the larger force constant and therefore is distorted less than the protein spring. Most of the energy of the system is thereby located in the protein spring.

Application of "Distal Strain" to Other Properties of Hemoglobins and Myoglobins. The term "distal strain" is used in order to distinguish strain arising from steric conflict on the distal side of the heme from strain resulting from steric interference on the proximal side and doming of the heme ring system (Gelin & Karplus, 1977; Warshel, 1977) or strain originating in movements of the proximal histidine (Perutz, 1976).

In 1974, Adams & Schuster reported the difference spectrum reproduced in Figure 12 when IHP was added to oxy-HbA. Similar spectra of lower magnitude were also observed when DPG or ATP was added to stripped oxy-HbA. Although these spectra probably do not represent an actual transition from R to T states (Perutz et al., 1976; Knowles et al., 1975), nevertheless the Adams–Schuster spectra are similar to the oxy T minus R state difference spectra reported by Perutz et al. (1976) and the difference spectra obtained by Knowles et al. (1975) under a variety of conditions (Figures 11 and 12). Adams & Schuster presented evidence that IHP and DPG bind to liganded Hb in the region of the central cavity where they are known to bind in deoxy-Hb (Arnone, 1972; Arnone & Perutz, 1974). More compelling evidence that such is the case comes from the observation that carbamylation of the β-subunit N termini is inhibited by DPG and IHP in oxyhemoglobin as well as deoxyhemoglobin (Jensen et al., 1973). Therefore, it seems clear that organic phosphates bind to liganded Hb in the region of the central cavity and induce structural changes which are of lesser magnitude but qualitatively the same as the changes which occur upon transition to an unliganded T state. The suggestion naturally emerges that this may be a general property of allosteric effectors.

The considerations developed here also can be employed to analyze the anomalous behavior of nitrosylhemoglobin (Perutz et al., 1976; Szabo & Perutz, 1976). Nitric oxide binds avidly to heme iron with a preferred geometry (Fe–N–O bond angle of ~140°) close to that expected for O_2 (Scheidt, 1977). Thus, the steric effect encountered by O_2 on the distal side of the heme should be better approximated by NO than CO. Maxwell & Caughey (1976) found that NO-HbA gave a single NO-stretching absorption, but upon the addition of IHP two NO-stretching bands were observed, one corresponding to a six-coordinate complex and the other to a five-coordinate complex. The weakened or lost Fe–N_ϵ bond is apparently in the α subunits (Szabo & Perutz, 1976; Maxwell & Caughey, 1976). In six-coordinate complexes of NO–Fe(II)–TPP, the Fe is found to be displaced from the plane of the porphyrin nitrogens by 0.07–0.10 Å, whereas in the five-coordinate complex the Fe is displaced by 0.21 Å, both displacements in the direction of NO (Scheidt, 1977). Therefore, NO bound to Hb pulls the Fe away from the proximal imidazole in both subunits. However, NO-HbA is in an R state (Salhany et al., 1975) where there is little steric interference to bound NO and a weak Fe–N_ϵ interaction is maintained. When IHP is added, the conformation changes to a T state (Salhany et al., 1975) where the β-subunit distal side chains exert a force on the heme–NO complex, pushing it toward the proximal imidazole. However, in the α subunits, distal strain is small or absent and the Fe–N_ϵ bond is lost. This seems to be a clear indication that the α-subunit proximal imidazole is retracted from the heme in the R → T transition of liganded hemoglobin and emphasizes differences in the factors contributing to triggering the conformational change in the α compared to the β subunits.

The tension theory (Perutz, 1976) has been criticized (Little & Ibers, 1974; Basolo et al., 1975) for its failure to account satisfactorily for the substantial cooperativity (Imai et al., 1977) exhibited by coboglobin (cobaltohemoglobin). It seems likely that the reluctance of Co to be distorted from the porphyrin plane would make the O_2 affinity of the T-state β subunits much lower than that of the α subunits because of the steric obstruction of the β-subunit distal valine. So the α subunits would oxygenate first, but little additional strain would be introduced in the protein because Co changes position only slightly. However, when a β subunit oxygenates, the distal strain would be severe because of the intense crowding. Thus, triggering in coboglobin may be attributable almost exclusively to distal strain in the β subunits. The triggering mechanism operative in the β subunits would appear to be relatively independent of the nature of the metal ion so long as it binds O_2 with a geometry similar to Fe–O_2.

Although the data available are limited, little correlation is apparent between the α/β ratio and O_2 and CO affinities of hemoglobins and myoglobins from various species. Most of the points are clustered around an α/β ratio a little greater than 1, but with considerably different p_{50} values. The one obvious correlation is that hemoglobins or myoglobins having exceedingly high O_2 affinities also have low α/β ratios, but having a low α/β ratio does not ensure a high O_2 affinity: for example, O_2-Mb Aplysia, α/β = 0.92 and p_{50} = 2.5 Torr (Rossi Fanelli & Antonini, 1959; Wittenberg et al., 1974). This raises the question of how the hemoglobin molecule has evolved in order to meet the diverse requirements of various organisms. Clearly a strong Fe–N_ϵ interaction is necessary for extremely high O_2 affinity, but for the vast majority of

animals, hemoglobins with an extremely high O_2 affinity would not maintain a sufficient O_2 concentration at the tissue level. It is reasonable then for the proximal imidazole in the hemoglobins of most animals to be restrained by the protein such that the O_2 affinity falls within an acceptable range. The fine adjustments would appear to be made largely by steric intervention on the distal side of the heme. A particularly dramatic case in point may be the teleost fish hemoglobins, which have O_2 and CO α/β ratios similar to those of mammalian hemoglobins, but their O_2 affinities become vanishingly low when the transition to T state occurs (Brunori, 1975; Pennelly et al., 1975). Morover, a pronounced *red shift* occurs in the Soret of trout IV and carp carboxyhemoglobins as a result of lowering the pH from 8 to 6 (Giardina et al., 1975; Knowles et al., 1975), which we now recognize to be associated with an increase in the Fe–N_ϵ interaction. The considerations developed here indicate that the low affinity of these T-state fish hemoglobins cannot be due to a weakened Fe–N_ϵ interaction, but is most likely due to intense steric obstruction of the O_2-binding site. The poor correlation between α/β ratios and affinities in general seems to suggest that nature utilizes variations on the distal side of the heme not only to fine tune O_2 affinity but also to regulate the relative affinities for O_2 and CO. Also, restraint on the proximal imidazole in R-state conformations should reduce the affinity for CO to a greater extent than that for O_2 because of the greater ease with which the Fe can distort from the porphyrin plane in O_2 complexes.

We have selected the strength of the Fe–N_{axial} interaction as an appropriate property to correlate with spectral parameters. In this form the correlation appears reasonable and hopefully will prove useful in its application to hemoprotein chemistry. However, a more fundamental relationship surely exists which should develop as additional structural analyses of O_2– and CO–heme complexes are carried out and a more detailed analysis of the transitions responsible for the visible and Soret spectra becomes available.

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Binding of *all-trans*-Retinal to the Purple Membrane. Evidence for Cooperativity and Determination of the Extinction Coefficient†

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ABSTRACT: At low bacteriorhodopsin concentration the binding of *all-trans*-retinal to the apomembrane of *Halobacterium halobium*, as monitoried by the absorbance change at 568 nm, occurs in a cooperative manner. The simplest way of analyzing the binding data is based on an all-or-none model and results in a Hill coefficient of 3.0 ± 0.2 and an apparent association constant of 2.8×10^6 M^{-1}. The same sigmoidal binding curve was obtained by using the change in circular dichroism at 365 nm (displacement of retinal oxime) or at 263 nm (retinal-induced change in bacterioopsin). Moreover, the trivial explanation of our results, namely, that the sigmoidal shape is caused by a suitably varying extinction coefficient, could be excluded, since the extinction coefficient was shown to be independent of the degree of binding. The Hill coefficient of close to 3 suggests that protein–protein interactions within bacteriorhodopsin trimers are responsible for the observed cooperative effect. Such an interpretation is consistent with the structure of the reconstituted apomembrane which consists of a hexagonal lattice of bacteriorhodopsin in which the bacteriorhodopsin molecules are arranged in clusters of three. The surprisingly small value of the association constant shows that retinal binding to the apomembrane is not irreversible. This was confirmed by exchange experiments between $retinal_1$ and $retinal_2$ which show that bound $retinal_2$ can be displaced by $retinal_1$ and vice versa. At bacteriorhodopsin concentrations much higher than the reciprocal of the association constant, all the retinal added is bound until all the binding sites are occupied. It is therefore possible to determine the extinction coefficient of the chromophore from the slope of the binding curve. The extinction coefficient obtained in this way is based on a knowledge of the retinal concentration and does not depend on a determination of the protein concentration. The resulting value of 62 700 ± 700 M^{-1} cm^{-1}, at 568 nm, refers to the light-adapted state of the purple membrane at 25 °C in 0.02 M phosphate buffer, pH 6.9, and is corrected for light scattering.

Purple membrane patches consist of a hexagonal array of identical protein units. At each vertex of the unit cell, three bacteriorhodopsin molecules are arranged in a cluster, in such a way that intratrimer distances are smaller than intertrimer distances (Henderson & Unwin, 1975; Unwin & Henderson, 1975). In harmony with this structural evidence, chemical cross-linking with bifunctional reagents produces bacteriorhodopsin trimers in a very high yield (Dellweg & Sumper, 1978). At present, the functional significance of the lattice structure is not yet clear. In view of the structural evidence, it is conceivable that bacteriorhodopsin trimers are the true functional units. Cooperative binding of ligands is a well-known phenomenon for water-soluble oligomeric proteins made up of identical subunits. Little is known about cooperative effects in the binding of ligands to intrinsic membrane proteins which are embedded in the two-dimensional matrix of a membrane. Whereas cooperative binding to linear arrays of

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