Influence of Globin Structures on the State of the Heme. Ferrous Low Spin Derivatives†

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ABSTRACT: Studies of high spin ferrous and ferric derivatives led us to conclude that in the quaternary R structure the state of the hemes is similar to that in the free α and β subunits, but in the T structure a tension acts on the hemes which tries to pull the iron and the proximal histidine further from the plane of the porphyrin. We have now studied the effect of inositol hexaphosphate (IHP) on the three low spin ferrous compounds of hemoglobin with O_2, CO, and NO. IHP failed to switch the quaternary structure of carbonmonoxy- and oxyhemoglobin A to the T state, but merely caused a transition to an as yet undefined modification of the R structure. IHP is known to cause a switch to the T structure in hemoglobin Kansas. We have found that this switch induces red shifts of the visible α and β absorption bands and the appearance of a shoulder on the red side of the α band; these changes are very weak in carbonmonoxy- and slightly stronger in oxyhemoglobin Kansas. As already noted by previous authors, addition of IHP to nitrosylhemoglobin A induces all the changes in uv absorption and CD spectra, sulfhydryl reactivities, and exchangeable proton resonances normally associated with the R → T transition, and is accompanied by large changes in the Soret and visible absorption bands. Experiments with nitrosyl hybrids show that these changes in absorption are caused predominantly by the hemes in the α subunits. In the accompanying paper Maxwell and Caughey (J. C. Maxwell and W. S. Caughey (1976), *Biochemistry,* following paper in this issue) report that the NO in nitrosylhemoglobin without IHP gives a single ir stretching frequency characteristic for six-coordinated nitrosyl hemes; addition of IHP causes the appearance of a second ir band, of intensity equal to that of the first, which is characteristic for five-coordinated nitrosyl hemes. Taken together, these results show that the R → T transition causes either a rupture or at least a very dramatic stretching of the bond from the iron to the heme-linked histidine, such that an equilibrium is set up between five- and six-coordinated hemes, biased toward five-coordinated hemes in the α and six-coordinated ones in the β subunits. The reason why IHP can switch nitrosyl-, but not carbonmonoxy- or oxyhemoglobin A, from the R to the T structure is to be found in the weakening of the iron–histidine bond by the unpaired NO electron and by the very short Fe–NO bond length.

Heme–heme interaction, consisting of a rise of the oxygen affinity of hemoglobin solutions with rising oxygen saturation, occurs only when the reaction with oxygen is accompanied by a transition between two alternative quaternary structures of the globin. In the oxy or R structure the ligand affinities of the hemes appear to be similar to those in free α and β subunits, but in the deoxy or T structure they are lowered by the equivalent of about 15 kJ/heme. Spectroscopic and magnetic studies of high spin or mixed spin derivatives indicate that this effect is linked to a tension exerted by the globin on the heme, a tension which tries to pull the proximal histidine and the iron further from the plane of the porphyrin and thus opposes the transition to the low spin state which is needed for combination with oxygen (Perutz et al., 1974a–c). In this paper we examine the changes in the state of the hemes accompanying the R → T transition in carbonmonoxy-, oxy-, and nitrosylhemoglobin.

Ferric high spin or mixed spin derivatives normally have the quaternary R structure, but by addition of 1 mol equiv of IHP[1] per mol of tetramer several of them can be switched to a structure showing SH reactivities, uv electronic absorption and circular dichroism (CD) spectra, and an exchangeable proton resonance line characteristic of the T structure (Perutz et al., 1974b; Fung and Ho, 1975). Of the three ferrous low-spin derivatives only nitrosylhemoglobin responds to IHP in this manner, while oxy- and carbonmonoxyhemoglobin undergo a transition to an as yet undefined intermediate structure, similar to that observed in ferric low spin compounds. Hemoglobin Kansas (Asn G4(102)β → Thr) is an abnormal hemoglobin of low oxygen affinity which has its allosteric equilibrium shifted far to the T state (Ogawa et al., 1972). By adding IHP to concentrated solutions of this hemoglobin, it was possible also to observe the effects of the R → T transition on the visible spectra of oxy- and carbonmonoxyhemoglobin. B. Giardina, F. Ascoli, and M. Brunori (personal communication) have made similar observations using component IV of trout carbonmonoxyhemoglobin which can be switched to the T state by lowering the pH.

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[1] Abbreviations used are: DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; bis-tris, *N,N*-bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PMB, *p*-mercuribenzoate; NEM, *N*-ethylmaleimide; IHP, inositol hexaphosphate; NO-TPP-Fe-Im, -tetraphenylporphinato(1-methylimidazole)iron(II); NO-TPP-Fe, -tetraphenylporphinatoiron(II); PDS, 4,4'-dithiobispyridine; Hb, hemoglobin.

In carbonmonoxyhemoglobin the R → T transition produces small red shifts of the Soret and visible absorption bands. Oxyhemoglobin shows qualitatively similar, but somewhat larger, changes. Nitrosylhemoglobin, by contrast, exhibits dramatic changes, first discovered by Cassoly (Cassoly, 1974). These include blue shifts of the Soret and visible absorption bands, a drop of 23% in the intensity of the Soret band, and the appearance of new visible absorption bands at positions normally associated with charge transfer in high spin ferric hemoglobins. The explanation for these striking changes is provided in the accompanying paper by Maxwell and Caughey (1976), who show that addition of IHP to nitrosylhemoglobin causes a splitting of the infrared NO stretching frequency. One absorption band maintains the same frequency as in the absence of IHP; this frequency is characteristic for synthetic nitrosyl heme complexes in which the sixth coordination position is taken up by a nitrogenous base. A new band equal in intensity to that of the first appears at a frequency characteristic for a synthetic nitrosyl heme complex in which the sixth coordination position is empty. These results indicate that the large changes in electronic absorption spectra observed on addition of IHP arise because the bond from the heme iron to N_ϵ of the proximal histidine is either broken, or at least dramatically stretched, in about half the hemoglobin subunits. Experiments with hybrid hemoglobins show that this effect is more marked in the α than in the β subunits. Rupture, or extreme stretching, of the bonds between the heme and the globin is the most remarkable manifestation yet found of the tension on the hemes in the quaternary T structure and confirms the conclusions about the nature of the heme-heme interaction arrived at in part III.

Methods

Preparation of Nitrosylhemoglobin. An approximately 3 mM (heme) solution of human oxyhemoglobin in 0.1 M NaCl was deoxygenated by alternate evacuation and flushing with nitrogen under shaking. 0.05 M bis-tris + 0.1 M NaCl of pH 6.5 were saturated with pure NO from a cylinder after washing the gas with concentrated NaOH. The two solutions were mixed in a nitrogen-filled glove box to give solutions 30 μM in heme; 1.5 ml of this solution was filled into each of two stoppered fluorescence cuvettes with path lengths of 10 mm in one direction and 4 mm in the other. Twenty microliters of 5 mM IHP of pH 6.5 was added to one cuvette and 20 μl of water to the other. The visible spectrum was recorded with the 10-mm and the Soret band with the 4-mm path length.

Nitrosyl Hybrids. Carbonmonoxy α and β chains were prepared according to Kilmartin et al. (1973) and converted to the deoxy form by first treating them with oxygen under a strong light to remove CO and then with nitrogen to remove oxygen (Kilmartin and Rossi-Bernardi, 1971); they were converted to the NO form by shaking in an atmosphere of 10% NO in nitrogen for 1 h. Excess NO was removed by shaking in nitrogen for a further hour. In the nitrogen-filled glove box 5 μl of 50 mM IHP of pH 6.5 was injected into one fluorescence cuvette of a matched pair and 5 μl of water into the other. The cuvettes were chilled in an ice bath. Chilled solutions of deoxy and NO subunits, 600 μM in heme, were mixed by injecting first 0.25 ml of the NO and then 0.25 ml of the deoxy solution into 10 ml of chilled 0.1 M bis-tris of pH 6.5. The mixture was immediately filled into the cuvettes which were stoppered and taken in an ice bucket to the precooled spectrophotometer, and the first spectrum was recorded just above 0°C within less than 10 min after mixing. Dilute solutions of the separate subunits were also filled into cuvettes and their spectra were checked. Difference spectra of the hybrids with and without IHP were recorded at intervals for an hour afterwards to check the rate of heme or NO exchange. At 0°C this was found to be slow enough to ensure that the amplitude of the difference spectra would have changed by less than 5% in the interval between mixing and the first recording. On leaving the solutions at room temperature overnight the spectra changed drastically, due partly to heme exchange and partly to formation of methemoglobin. Solutions of cyanomet-NO hybrids were introduced into the glove box ready mixed; for the rest, the same procedure was followed as with deoxy-NO hybrids.

Oxy- and Carbonmonoxyhemoglobin A. Difference spectra of these derivatives were examined between 0 and 5°C in order to avoid dissociation of ligand by IHP. They were examined in unbuffered solutions or in 0.1 M Hepes of pH 7.0 or in 0.05 M bis-tris of pH 7.0, at various concentrations of NaCl.

Oxy- and Carbonmonoxyhemoglobin Kansas. This abnormal hemoglobin was kindly provided by Dr. S. Ogawa. It has a very high dissociation constant from tetramers to dimers (400 μM), so that its concentration has to be as high as 3 mM heme in order to keep it tetrameric, even in the presence of IHP. To unbuffered solutions in 0.1 M NaCl, IHP was added in the form of a 0.2 M solution of pH 5.3, the low pH compensating for the absorption of protons which occurs on dilution of IHP and as a result of binding to hemoglobin and converting it to the T state. All solutions were kept in an ice bath throughout and the absorption spectra observed just above 0°C in matched 0.2-mm pathlength cuvettes.

All absorption spectroscopy was done with a Cary 17 spectrophotometer. Circular dichroism was measured as described in part II, and SH reactivities were determined as described by Kilmartin et al. (1975).

Results

Evidence Relating to Changes in Quaternary Structure. The R and T quaternary structures are defined by the different distances between the iron atoms and the different dovetailing of the $\alpha_1\beta_2$ contacts in crystal structures of the two forms, as determined by x-ray analysis (Perutz and Ten Eyck, 1971). In solution, the transition from the R to the T state can be correlated with a difference absorption spectrum in the uv, containing positive peaks at 279, 287, 294, and 302 nm; the appearance of a negative peak in the CD spectrum at 287 nm; a large drop in the first-order rate constant of the reaction with *p*-mercuribenzoate (PMB) or the second-order rate constant of the reaction with either 2,2′- or 4,4′-dithiobispyridine (PDS), the magnitude of the drop depending on the tertiary structure of the β subunits (Perutz et al., 1974a,b); and the appearance of an exchangeable proton resonance at about 9.4 ppm from HDO or 14 ppm from DSS (Patel et al., 1970). All these effects have been observed on addition of IHP to deoxyhemoglobin derivatives so modified that without IHP they have the R structure, or to stripped high spin or mixed spin ferric derivatives, but not to low spin derivatives of either valency. These appeared to undergo a transition to an as yet undefined intermediate form (Perutz et al., 1974a,b).

Addition of IHP to solutions of oxy- and carbonmonoxyhemoglobin Kansas or of nitrosylhemoglobin A produces

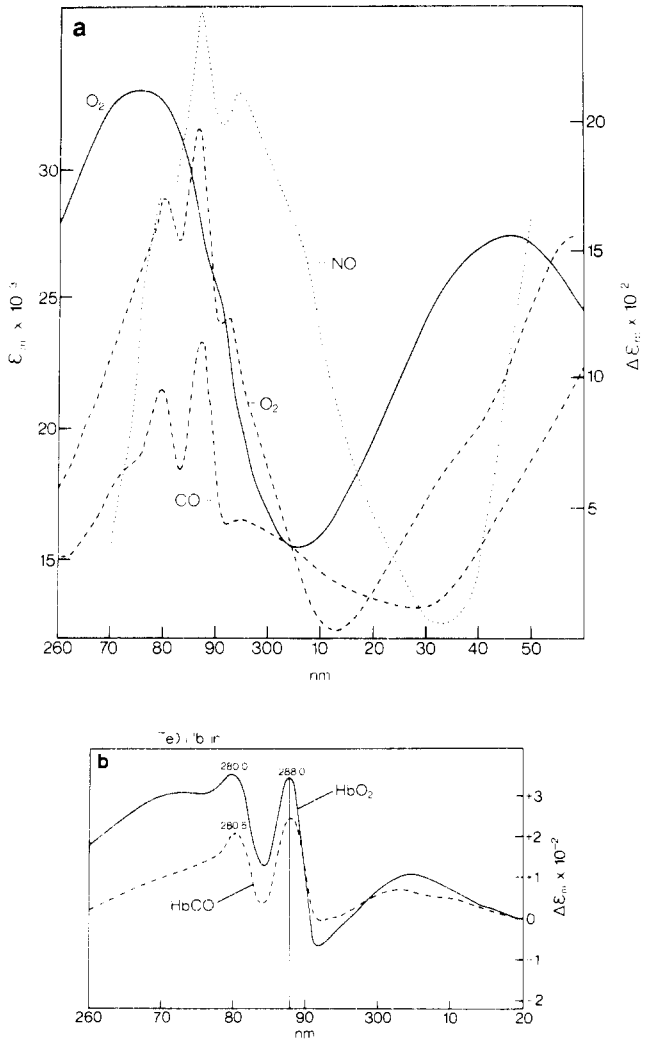

FIGURE 1: (a) Uv difference spectra. The full line gives the spectrum of a 60 μM (Fe) solution of oxyhemoglobin Kansas. The dotted and broken lines show the difference spectra of nitrosylhemoglobin A and oxy- and carbonmonoxyhemoglobin Kansas ± IHP. Note the peak at 294 nm which is due to tryptophan-37β and is an essential diagnostic for the R → T transition. (b) Uv difference spectra of oxy- and carbonmonoxyhemoglobin A ± IHP. Note that the diagnostic peak at 294 nm is absent, that the peak positions are slightly different, and the molar amplitudes are smaller than in Figure 1a.

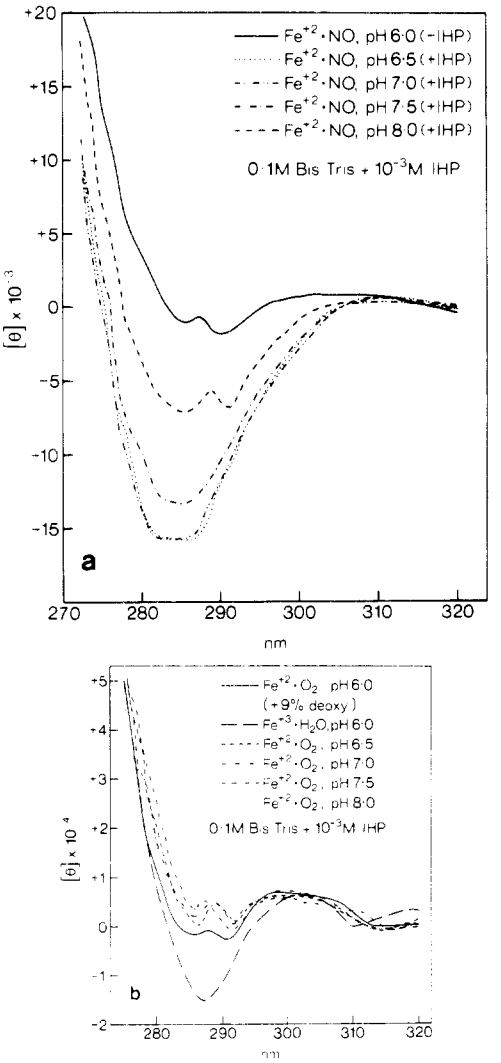

FIGURE 2: (a) CD spectra of 370 μM (Fe) solutions of nitrosylhemoglobin A in 0.1 *M* bis-tris + 1 mM IHP. (b) CD spectra of solutions of oxy- and methemoglobin. Conditions as in (a).

the spectral effects characteristic for an R → T transition (nitrosylhemoglobin Kansas has the T structure even in the absence of organic phosphates; Salhany et al., 1974, 1975). The ultraviolet difference spectra for the three derivatives are shown in Figure 1a and the CD spectra, for nitrosylhemoglobin only, in Figure 2a. Note that as in methemoglobin, the effect of IHP weakens above pH 7.0 and disappears at pH 8.0. The negative CD peak at 287 nm in nitrosylhemoglobin A had already been found by Salhany (Salhany, 1974). The exchangeable proton resonance at 14 ppm from DSS in carbonmonoxyhemoglobin Kansas + IHP has been demonstrated by Ogawa et al. (1972), and in stripped nitrosylhemoglobin Kansas and nitrosylhemoglobin A + IHP by Salhany et al. (1974, 1975). The effects of IHP on the reactivities of cysteine-93β in deoxy-, CO-, and NO-hemoglobin A are compared in Table I. IHP reduces the half-time of the reaction of 4,4′-dithiobispyridine with the sulfhydryl groups of deoxy- or CO-hemoglobin approximately twofold, but that with NO-hemoglobin 17-fold, indicative of a change in quaternary structure. Similar results have been obtained with nitrosylhemoglobin A by Taketa et al. (1975).

Adams and Schuster (1974) found that addition of IHP to human oxyhemoglobin in 0.1 M Hepes of pH 7.0 at 5.6°C produced changes in the visible absorption spectrum which they attributed to a change of quaternary structure to an "oxy T-form". We have reinvestigated this reaction in order to discover whether IHP really does cause a transition to the quaternary T structure or merely to some other, intermediate, structure as in ferric low spin compounds. We have confirmed the appearance of the visible difference spectrum on addition of IHP to fully saturated solutions of oxyhemoglobin under the conditions used by Adams and Schuster and found that it also appears in unbuffered salt-free solutions (Figure 3). However, the accompanying uv difference spectrum lacks the essential peak between 294 and 296 nm which is due to the changed environment of Trp-37β in the T state, and the amplitude of the difference spectrum is 2.5 times smaller than in the R → T transition (Figure 1b). Addition of IHP does not produce the negative CD peak at 287 nm characteristic for the T state (Figure 2b). IHP increases the half-time of the reaction of the cysteines-93β with 4,4′-dithiodipyridyl only two- to threefold and the reaction with oxygen remains cooperative (Table I).

Table I: Reactivity of SH Group with 4,4′-Dithiobispyridine.[a]

Sample	Stripped $t_{1/2}$ (min)	With IHP $t_{1/2}$ (min)	Ratio $t_{1/2}$
CO-Hb	2.0	4.5	2.2
NO-Hb	1.3	22	17
Deoxy-Hb	103	215	
O_2-Hb in Hepes	5.7	11.6	
O_2-Hb in bis-tris	5.7	16.8	

	Oxygen Equilibrium Parameters			
	Stripped		With IHP	
Sample	Log p_{50}	Hill's Constant n	Log p_{50}	Hill's Constant n
Hb in Hepes			1.29	2.2
Hb in bis-tris	0.50	3.0	1.40	2.0

[a] SH reactivity was carried out as described by Kilmartin et al. (1975). The conditions for CO-Hb, NO-Hb, and deoxy-Hb were hemoglobin concentration 0.8 mg/ml, 0.1 M bis-tris, 0.1 M KCl (pH 6.6 at 25°C), and 0.5 mM PDS with or without 0.4 mM IHP. The deoxy-Hb solutions contained 1.5 mM ferrous citrate. The buffers for O_2-Hb (0.8 mg/ml) were either 0.1 M Hepes (pH 7.0) or 0.05 M bis-tris–0.1 M NaCl (pH 6.5 at 5°C) with PDS and IHP as above. Oxygen equilibrium curves were performed in the same buffers except in the absence of PDS and at 10°C.

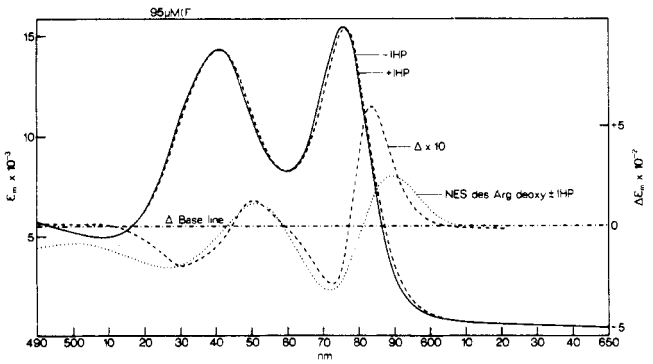

FIGURE 3: Comparison of visible difference spectra given by 95 μM (Fe) solutions of oxyhemoglobin A and a similar solution of deoxyhemoglobin NES des-Arg ± IHP (0.1 M Hepes (pH 7.0, 3°C) ± 0.2 mM IHP for oxy; for deoxy conditions as in part I (Perutz et al., 1974a)).

The difference spectrum in the visible is not the same as that observed in the R → T transition of deoxyhemoglobin since the former has a peak at 584 nm and the latter at 589 nm (Figure 3). On the other hand, the visible difference spectrum observed by Adams and Schuster is similar to and merely weaker than our T minus R difference spectrum of oxyhemoglobin Kansas, which suggests that IHP produces small changes in tertiary structure similar to those accompanying the R → T transition. Carbonmonoxyhemoglobin A also shows a slight spectral change in the visible on addition of IHP, but no indication of an R → T transition.

We conclude that IHP converts the quaternary structure of nitrosyl-, but not of oxy- or carbonmonoxyhemoglobin A, to the T-state. An explanation for this strange inconsistency will be suggested below.

Changes in the Soret and Visible Absorption Bands Accompanying the R → T Transition. The visible difference spectra of oxy- and carbonmonoxyhemoglobin Kansas with and without IHP are shown in Figures 4 and 5. The R → T difference spectrum of oxyhemoglobin Kansas is similar to, but stronger than, the IHP-dependent difference spectrum

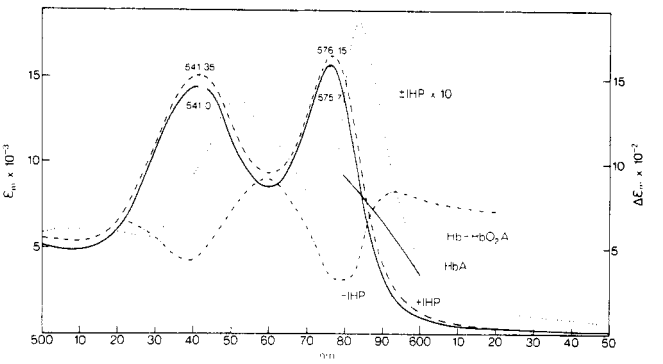

FIGURE 4: Visible difference spectrum of a 3.6 mM (Fe) solution of oxyhemoglobin Kansas in 0.1 M bis-tris (pH 6.5) ± 1.8 mM IHP at 3°C. The difference spectrum of deoxy minus oxyhemoglobin, shown for comparison, indicates that IHP caused some dissociation of oxygen, giving rise to the shoulder at 559 nm. The full line marked Hb A shows part of the spectrum of deoxyhemoglobin A and illustrates the red shift of the isosbestic point which would be observed in mixtures of oxyhemoglobin A (T state) and deoxyhemoglobin as compared with similar mixtures containing oxyhemoglobin in the R state.

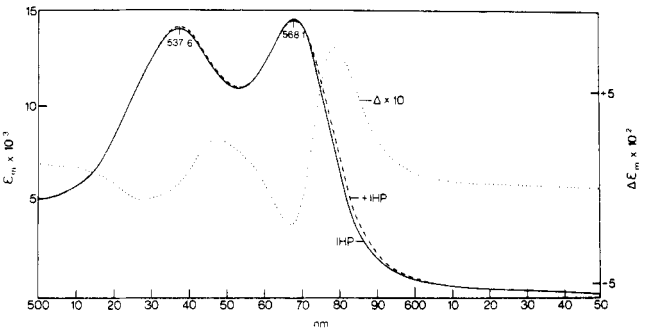

FIGURE 5: Visible difference spectrum of carbonmonoxyhemoglobin Kansas ± IHP. Conditions as in Figure 4.

of oxyhemoglobin A recorded by Adams and Schuster. In oxy, the α and β bands are red-shifted by about 0.4 nm and a shoulder develops at 583 nm; in carbonmonoxy, the red shifts are smaller, and a weaker shoulder develops at 579 nm. No change was detected in the near-infrared band of oxyhemoglobin. The Soret band could not be observed because of the high hemoglobin concentration required, but in carbonmonoxyhemoglobin of trout, component IV, which changes its quaternary structure from R to T on lowering the pH from 8.0 to 6.0, B. Giardina, F. Ascoli, and M. Brunori (personal communication) observed a red shift of the Soret band by about 1 nm.

The spectral changes observed on addition of IHP to nitrosylhemoglobin are far larger than those found in any other hemoglobin derivative (Figures 6 and 7). The Soret band is blue-shifted by 1.5 nm and drops in intensity by 23%. The α and β bands are blue-shifted by about 2 nm and drop in intensity by about 5%. Positive difference peaks appear at 495, 518, and 603 nm. These are similar in position to the absorption bands of fluoromethemoglobin and of the high spin form of hydroxymethemoglobin, but we have observed no increase in paramagnetic susceptibility on addition of IHP to nitrosylhemoglobin.

Rein et al. (1972) were the first to discover the dramatic effect of IHP on the electron spin resonance (ESR) spectrum of nitrosylhemoglobin and suggested that it was due to a change in conformation of the protein. They went on to show that a lowering of the pH from 7.0 to 5.0 produced the same effect as IHP and concluded that "an increasing pro-

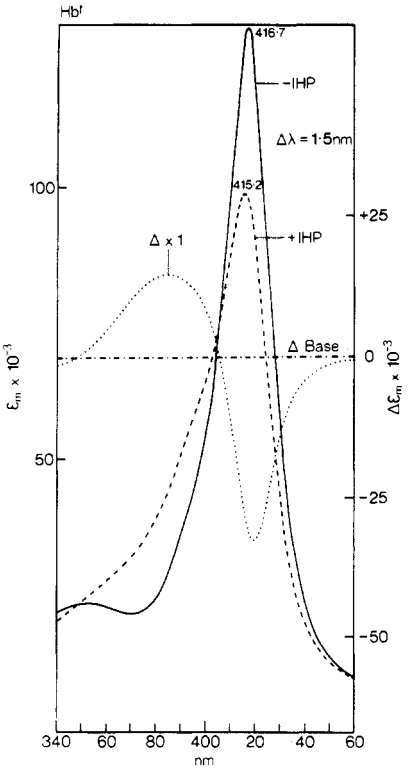

FIGURE 6: Difference spectrum of the Soret band of 30 μM solution of nitrosylhemoglobin A in 0.05 M bis-tris + 0.1 M NaCl (pH 6.5) ± 0.1 mM IHP in 4-mm path length cuvette at 20°C.

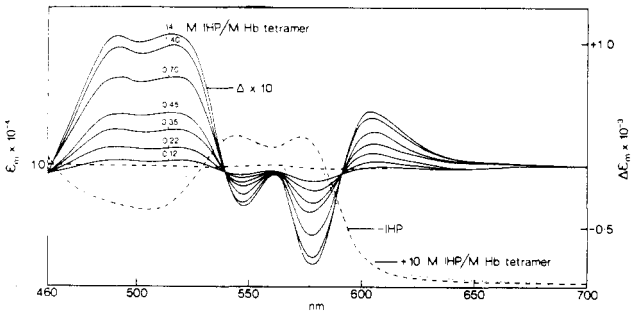

FIGURE 7: Visible difference spectra of solutions of nitrosylhemoglobin A ± different proportions of IHP, showing isosbestic points, reduction in the intensities of the α and β bands, and the emergence of new ones similar to high spin bands of ferric derivatives.

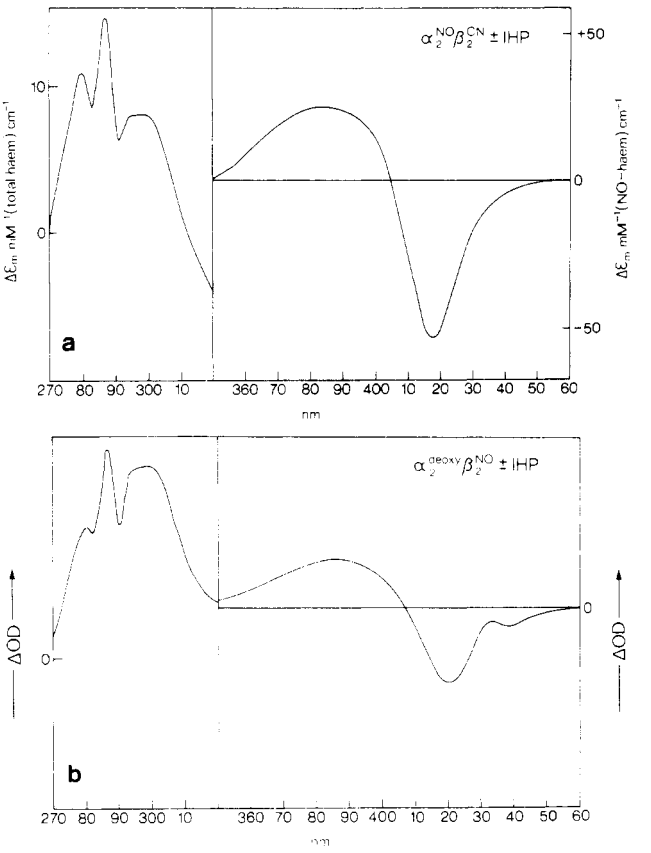

FIGURE 8: Uv and Soret difference spectra of nitrosyl hybrids at 0°C in 0.1 M bis-tris (pH 6.5) ± 170 μM IHP. (a) 7.5 μM (tetramer) $\alpha_2^{NO}\beta_2^{CN}$. (b) 15 μM (tetramer) $\alpha_2^{deoxy}\beta_2^{NO}$. The small difference peaks at 433 and 440 nm are due to the deoxy α chain (see Figure 3 of part I (Perutz et al., 1974a) and Sugita (1975)). The visible difference spectra of the two hybrids were like that of $\alpha_2^{NO}\beta_2^{NO}$ ± IHP shown in Figure 7.

tonation of hemoglobin produces a conformational change with the same effect on the NO group as the allosteric effectors". We find that lowering the pH from 6.5 toward 5.0 has an effect on the Soret band which is 16 times weaker than that of IHP, that no significant uv difference spectrum is produced, and that no negative CD peak appears at 287 nm. Therefore the similarity of the ESR spectrum in the T state and at acid pH in nitrosylhemoglobin A may be caused by tertiary changes or by a change to a quaternary structure other than deoxy.

Hybrid Nitrosylhemoglobins. To discover the relative contribution of the α and β subunits to the large spectral changes of nitrosylhemoglobin on addition of IHP, we undertook a study of hybrids. We interpreted the results of Henry and Banerjee (1973) and Sugita (1975) to show that the α subunits make the major contribution to the changes in ESR and optical spectra on transition to the T-state, but that there remained some doubt whether the β subunits also contribute significantly. Since exchange of hemes and NO is known to be rapid at room temperature, we did all our experiments at 0°C where the rate of change in intensity of the Soret band was slowed down to no more than 20%/hour even in $\alpha_2^{deoxy}\ \beta_2^{NO}$, which is the most rapidly exchanging hybrid, and we recorded our difference spectra within 10 min of mixing.

Addition of IHP to $\alpha_2^{+CN}\beta_2^{NO}$ and to $\alpha_2^{NO}\beta_2^{deoxy}$ caused no significant changes in either the uv or visible spectra, presumably because the former remained in the R state even in the presence of IHP and the latter in the T state even in its absence. Addition of IHP to $\alpha_2^{NO}\beta_2^{+CN}$ caused large spectral changes (Figure 8a). The amplitudes of the uv difference peaks indicate that the bulk of the molecules in solution have undergone the R → T transition. The amplitude of the difference peak in the Soret band, extrapolated to the moment of mixing the two components, corresponds to a 34% reduction in the contribution of the α^{NO} subunits (the electronic spectra of cyanomethemoglobin are insensitive to the R → T transition). The visible difference spectrum was the same as that of pure nitrosylhemoglobin shown in Figure 7. The difference spectra produced by IHP in solutions of $\alpha_2^{deoxy}\beta_2^{NO}$ were much weaker, perhaps because only a fraction of the molecules underwent the R → T transition. To gauge the response of the Soret band contributed by the β subunits, we made the assumption that the amplitude of the uv difference spectrum near 287 nm is proportional to the concentration of hybrids that have switched quaternary structure. We then adjusted the con-

centration of our solutions so that this amplitude should be roughly equal to that in Figure 8a. This needed twice the concentration compared to $\alpha_2^{NO}\beta_2^{+CN}$, but produced a difference peak in the Soret band of only half the amplitude (Figure 8a). Therefore, if our assumption is right, the response to the R → T transition of nitrosyl hemes in the β subunits is only about half as strong as that in the α subunits; if our assumption is wrong and all the molecules have undergone the R → T transition, then the response is only one-quarter.

Effect of Sulfhydryl Reagents. PMB and *N*-ethylmaleimide (NEM) both raise the oxygen affinity of hemoglobin A, but this effect is made up of two antagonistic components. The dominant one consists of a lowering of the allosteric constant L which destabilizes the quaternary R structure and raises the affinity, the minor one in a lowering of the intrinsic oxygen affinity of the β subunits in the R state (Riggs and Wolbach, 1956; Benesch and Benesch, 1961; Riggs, 1961; Antonini et al., 1965; Kilmartin et al., 1975; Imai, 1973). We wondered whether this lowering was brought about by the stereochemical mechanism depicted in Figure 11 of part II (Perutz et al., 1974b), implying a raising of the tension of the heme. Difference spectra of oxyhemoglobin ± PMB or NEM did indeed produce the difference peak at 584 nm seen in Figures 3 and 4, only weaker. PMB also produced a small blue shift of the Soret band of nitrosylhemoglobin. NEM, on the other hand, had a small effect on nitrosylhemoglobin which was the exact reverse of IHP, as if it caused a small fraction of molecules in the T state to revert to the R state. The presence of such a fraction is also indicated by the resonance Raman spectrum of nitrosylhemoglobin (see below). Removal of Tyr-145 and His-146β with carboxypeptidase A had no significant effect on the spectra.

Discussion

Nature of the Changes in Quaternary Structure Induced by IHP in Nitrosylhemoglobin. Nitrosylhemoglobin without IHP has uv absorption and CD spectra, exchangeable proton resonances and sulfhydryl reactivities characteristic for the quaternary oxy structure. Addition of IHP changes the spectra to those characteristic for the quaternary deoxy structure and reduces the half-time of the reaction of the sulfhydryl groups with 4,4′-dithiobispyridyl 17-fold. It may be objected that this half-time is still ten times shorter than that of deoxyhemoglobin with IHP, and that therefore the two quaternary structures must be different.

This argument typifies a widespread misunderstanding of the hemoglobin system for which we must perhaps blame ourselves, because in past publications we have failed to emphasize sufficiently the distinction between changes in quaternary and tertiary structure, and the associated thermodynamic states. We define the two alternative quaternary structures by the distances between the iron atoms and the dovetailing of the $\alpha_1\beta_2$ contacts. In the R structure Fe(α_1 − α_2) = 36 Å, and Fe(β_1 − β_2) = 33 Å; in the T structure the corresponding distances are 35 and 40 Å. In the R structure Thr C3(38)α_1 occupies the notch at Val, FG5(98)β_2 (Figure 15 of Perutz, 1969); in the T structure Thr C6(41)α_1 fills the notch instead (Figure 6 of Fermi, 1975). These gross features express the relative arrangement of the four subunits in the tetramer and are largely independent of their tertiary structure, which may be modified according to the presence or absence of ligands, the nature of the ligands, the valence and spin state of the iron, by pH, organic phosphates, and also by genetic mutations or chemical modification.

X-ray crystallographic studies have shown that all liganded forms of hemoglobin, ferrous and ferric, high and low spin, crystallize in a form that is isomorphous with oxyhemoglobin and that the molecules have the quaternary R structure. If some of the bonds between the subunits that normally stabilize the quaternary deoxy structure are inhibited, then deoxyhemoglobin also crystallizes in the R structure, even though the tertiary structures of the subunits differ markedly from those of liganded ones (Perutz and Ten Eyck, 1971). Conversely, aquomethemoglobin A and carbonmonoxyhemoglobin Kansas have been maintained in the quaternary T structure in crystals (Anderson, 1973, 1975), and fluoromethemoglobin A has recently been crystallized in the quaternary T structure (G. Fermi and M. F. Perutz, unpublished). These studies have left no doubt that the two quaternary structures can accommodate all the different tertiary ones.

In solution the two quaternary structures can be distinguished by spectroscopic criteria, such as those used in this study, which reflect mainly the structure of the $\alpha_1\beta_2$ contact. On the other hand, *thermodynamic properties* such as ligand affinities, sulfhydryl reactivities, tetramer–dimer dissociation constants, affinities for haptoglobin, etc., must be interpreted with caution, because they *vary with the equilibrium* between R and T as expressed by the allosteric constant L. This constant depends on the tension imposed by the different tertiary structures and on the many other factors involved in the reciprocal interaction between tertiary and quaternary structures and their surrounding ions. Hence the difference between the sulfhydryl reactivities of deoxy- and nitrosylhemoglobin does not speak against the latter having the quaternary T structure (Baldwin, 1975).

Nature of the Changes at the Heme Induced by IHP. Why are the spectral changes induced by the R → T transition in nitrosylhemoglobin so much stronger than in any other hemoglobin derivative? A chance observation suggested a possible answer. Solutions of nitrosylhemoglobin were stable in air for several hours, but those with IHP became oxidized within minutes of taking the stopper off. Such rapid oxidation was likely to occur if the solution contained an appreciable fraction of free heme in equilibrium with hemoglobin, due to some loosening of the bond between the iron atom and the globin. Another clue came from the changes in the ESR spectrum reported by Rein et al. (1972) which indicated that IHP alters the distribution of unpaired spin density in nitrosylhemoglobin so that interaction with a single nitrogen atom becomes dominant. Suspecting that these factors might also affect the ir stretching frequencies of the NO bonds, we asked Dr. W. S. Caughey to examine these. The results, reported in the accompanying paper, confirmed our suspicions, but are more intriguing than anything we could have expected. They show that in the presence of IHP about one-half of the hemes remain six-coordinated, while the other half becomes five-coordinated, the bond from the iron atom to N_ϵ of the proximal histidine having either been broken or stretched very dramatically. Our first reaction, based on various results by other workers, was that the five-coordinated hemes might be in the α and the six-coordinated ones in the β subunits. We then started to examine the nitrosyl hybrids and soon convinced ourselves that the two forms must be in equilibrium in both α and β subunits, with the equilibrium in α being biased toward the five- and in β toward the six-coor-

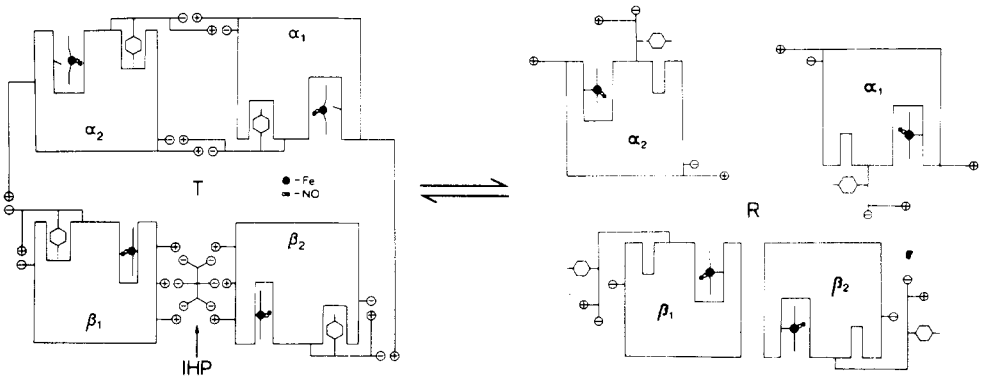

FIGURE 9: Equilibrium of human nitrosylhemoglobin A in the R and T states. Subunits in the R state are shown free and those in the T state bonded by salt bridges of the C-terminal residues and of IHP (Perutz, 1970). This diagram is oversimplified because it illustrates only two of the equilibrium states: in fact, a minor fraction of α hemes in the T state may be six-coordinated and of the β hemes five-coordinated. Moreover, the iron-nitrogen bonds in the α hemes could be greatly stretched rather than broken, and some of the salt bridges in the T state could be broken.

dinated hemes (Figure 9).[2] We shall first consider whether the conclusions derived from the ir spectra are supported by other evidence and then seek for explanations of the various phenomena observed here.

Scheidt and his colleagues have determined the crystal structures of two synthetic nitrosyl heme complexes: the six-coordinated nitrosyl-$\alpha,\beta,\gamma,\delta$-tetraphenylporphinato(1-methylimidazole)iron(II), NO-TPP-Fe-Im, and the five-coordinated nitrosyl-$\alpha,\beta,\gamma,\delta$-tetraphenylporphinatoiron(II), NO-TPP-Fe (Piciulo et al., 1974; Scheidt and Frisse, 1975). Comparison of their optical absorption spectra showed the Soret band to be blue-shifted by 10 nm and reduced in intensity by over 40% in the five-, as compared to the six-coordinated complex. For comparison, IHP induces a 23% reduction in intensity of the Soret band in $\alpha_2^{NO}\beta_2^{NO}$ and a 34% reduction in $\alpha_2^{NO}\beta_2^{CN}$. Our blue shifts of about 1.5 nm are much smaller than those observed in the synthetic compounds; this may be due to different band shapes contributed by the two components of the hybrid; alternatively, it may mean that the Fe-N_ϵ bond is very much stretched but not entirely broken. Whichever is the correct interpretation we shall hereafter refer to these hemes as five-coordinated.

Nitrosylhemoglobin without IHP shows a rhombic ESR spectrum with weak hyperfine splitting in the g_z region, suggestive of nine lines due to contact of the unpaired electron with the nitrogen atoms of the NO and the N_ϵ of the proximal histidine. A similar spectrum is given by NO-TPP-Fe-Im or NO-TPP-Fe-pyridine. Nitrosylhemoglobin with IHP gives a rhombic ESR spectrum with hyperfine splitting into three very strong lines in one direction, due to contact of the unpaired electron with the NO nitrogen, and only weak splitting in the other two directions. A similar spectrum is given by NO-TPP-Fe (Rein et al., 1972; Wayland and Olson, 1974; Kon, 1975; Maxwell and Caughey, 1976). Both Wayland and Olson, and Kon, drew attention to the similarities mentioned above, and Kon showed that hyperfine splitting is also produced by treating hemoglobin with the denaturing agent sodium lauryl sulfate which evidently causes the heme to be split from the globin. What the ESR spectra fail to reveal is the fact that only about half the hemes in nitrosylhemoglobin with IHP are five-coordinated.

Szabo and Barron (1975) examined the change in the resonance Raman spectrum of nitrosylhemoglobin on addition of IHP and observed a weakening of a depolarized band at 1633 cm^{-1}, coupled with a strengthening of what had been merely a weak shoulder at 1643 cm^{-1}, so that the two bands assumed equal intensity. In the light of the present results the band at 1633 cm^{-1} should correspond to the six- and that at 1643 cm^{-1} to the five-coordinated nitrosyl heme; the weak shoulder at 1643 cm^{-1} in the absence of IHP implies that a small fraction of the five-coordinated form may be in equilibrium with the six-coordinated one. In the hybrid $\alpha_2^{NO}\beta_2^{+CN}$ + IHP the band at 1643 cm^{-1} was dominant (L. Barron, private communication).

Cause of Changes in Quaternary Structure. Why does IHP change the quaternary structure of nitrosyl, but not of oxy- or carbonmonoxyhemoglobin, to the T state? ESR data indicate that in the absence of IHP a substantial fraction of the unpaired electron density is transferred from the NO to the d_{z^2} orbital of the iron (Kon, 1968; Chien, 1969; Yonetani et al., 1972). Mingos (1973) has pointed out that this would weaken the bond from the iron to N_ϵ of the proximal histidine, because the σ bonding orbital between these atoms is already filled by the lone pair electrons of N_ϵ, so that the unpaired electron has to go into the σ antibonding orbital (Figure 10). Crystallographic evidence for this effect has been presented by Piciulo et al. (1974) who showed the distance Fe-N_ϵ in NO-TPP-Fe-Im to be 2.18 Å compared to 1.98 Å in bis(dimethylglyoximato)diimidazoleiron(II) (Bowman et al., 1972), indicating that the trans effect of the nitrosyl ligand lengthens the bond by about 0.2 Å. The trans effects of O_2 and CO would be expected to be much weaker, so that the Fe-N_ϵ can stretch or break under tension, and the quaternary structure change to T, in nitrosyl but not in the other two low spin ferrous compounds. We have argued in part III (Perutz et al., 1974c) that the fraction of the total free energy of heme-heme interaction which is stored at the hemes themselves will be inversely related to the force constants of the iron-nitrogen bonds. Carbonmonoxy- and nitrosylhemoglobin seem to represent the two extreme cases of this phenomenon: in the former the force constant is so large that the tension in the T state appears to produce only a negligible distortion, while in the

[2] Since we submitted this paper Nishikura and Sugita have determined the effect of IHP on the Soret bands of hybrid hemoglobins containing protoheme in one pair of subunits and mesoheme in the other. The Soret bands of the two hemes lie at different wavelengths, so that the effect of IHP on the α subunits can be distinguished from that on the β subunits. The results show the reduction in intensity of the Soret band to be associated almost exclusively with the α subunits, which suggests that the weak effect on the β subunits in our Figure 8b may be an artefact due to heme exchange.

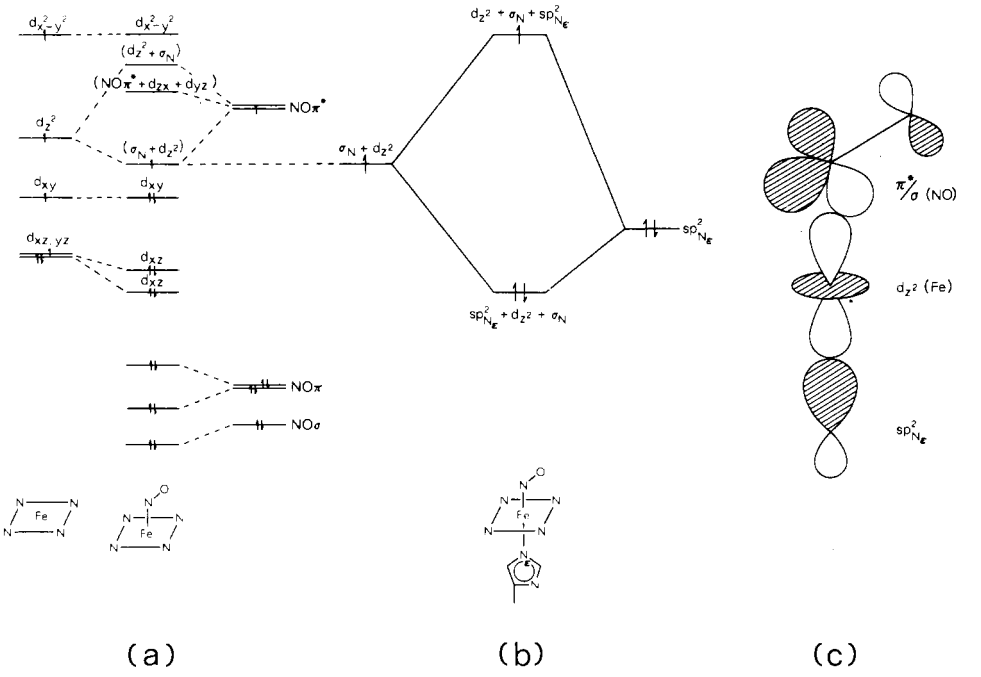

FIGURE 10: (a) Energy levels of Fe d electrons and NO σ and π electrons in nitrosylheme (adapted from Wayland and Olson, 1974). (b) Interaction of sp^2 (N_ϵ) with d_{z^2} (Fe) + π^* (N). The splitting may be exaggerated. (c) Orbitals of N_ϵ of the proximal histidine, iron, and NO. Only the σ antibonding $sp^2 + d_{z^2}$ orbitals are shown. The Fe d_{xz} and d_{yz} orbitals are omitted.

latter it is so small that the tension suffices to break the Fe–N_ϵ bond.

Interpretation of Shifts of the Soret and Visible Absorption Bands. X-ray analysis by Scheidt and his colleagues has shown that in nitrosyl complexes of ferrous tetraphenylporphines the NO is inclined to the porphyrin plane, the Fe NO angle being 142° in NO-TPP-Fe-Im and 149° in NO-TPP-Fe; the iron is displaced from the plane of the ring on the side of NO; this happens because the iron–nitrogen (NO) bond is so short (1.743 Å) and strong that the NO nitrogen approaches the porphyrin nitrogens to within less than the normal van der Waals distance (2.7 instead of 3.0 Å). If the iron were to remain in the plane of the porphyrin, these distances would have to be compressed even further; instead of doing this, the system yields by pulling the iron atom out of the plane. The displacement of the iron is greater in the five-coordinated NO-TPP-Fe (Scheidt and Frisse, 1975) than in the six-coordinated 1-methylimidazole complex of the same compound (0.211 as compared to 0.07 Å) (Piciulo et al., 1974). Since the effect of this displacement on the electronic spectra is similar to that of adding IHP to nitrosylhemoglobin we must expect the iron atom in nitrosylhemoglobin also to be displaced toward the NO and the displacement to be trebled on rupture of the Fe–N_ϵ bond.

How are the accompanying blue shifts of the electronic spectra to be explained? In part I we had shown that the R → T transition produced blue shifts of charge transfer bands in ferrous hemoglobins, and in part III we had been able to account for these on the basis of a simple electrostatic effect due to increased iron–nitrogen bond distances in the T state. These arguments do not apply to the low spin Soret (γ) and visible (α and β) absorption bands which are due to $\pi \rightarrow \pi^*$ transitions of the porphyrin from the a_{1u} and a_{2u} to the e_g^* orbitals (Gouterman, 1961; Zerner et al., 1966). According to Gouterman's calculations the $a_{1u} \rightarrow e_g^*$ and $a_{2u} \rightarrow e_g^*$ orbital transitions have very similar transition dipoles so that their intrinsic intensities before mixing are also similar. Since the two transition moments belong to the same representation of the symmetry group (E_u), the two states are mixed by configuration interaction. The two new excited states correspond to the sum and difference of the two states before mixing; the higher energy state gives rise to the Soret band and the lower energy state generates the visible bands. When the iron atom is moved out of the porphyrin plane the energy of the a_{1u} orbital is not changed, since it has no electron density on the pyrrole nitrogens. On symmetry grounds e_g^* can interact only with the d_{xz} and d_{yz} orbitals of the iron, but it is so far separated from them in energy that the interaction is very weak. On the other hand, the a_{2u} orbital which is forbidden by symmetry to interact with the d_{z^2} orbital when the iron lies in the plane of the porphyrin, increasingly interacts with it, the further the iron is displaced from that plane. This interaction lowers the energy of a_{2u}, which causes a rise in the energy of the orbital transition $a_{2u} \rightarrow e_g^*$. When configurational mixing due to electron interaction is taken into account, this increase in the energy of the basic orbital transition results in a blue shift of both the Soret and visible bands (Figure 11). In contrast to the blue shifts, the dramatic decrease in the intensity of the Soret band cannot be simply understood.

The absorption bands at 495, 518, and 603 nm which appear in the difference spectrum on addition of IHP to nitrosylhemoglobin are similar to the charge transfer bands in ferric high spin hemoglobins, yet addition of IHP to nitrosylhemoglobin causes no rise in paramagnetic susceptibility, and both the five- and the six-coordinated nitrosyl complexes of TPP-Fe are low spin (Piciulo et al., 1974; Scheidt and Frisse, 1975). The origin of these bands is not clear.

We now come to the red shifts of the α and β bands observed on addition of IHP to oxy- and carbonmonoxyhemoglobin Kansas and the red shift of the Soret band observed in the R → T transition of carbonmonoxyhemoglobin of the trout. Crystallographic studies indicate that in these derivatives the iron lies exactly in the plane of the porphyrin ring

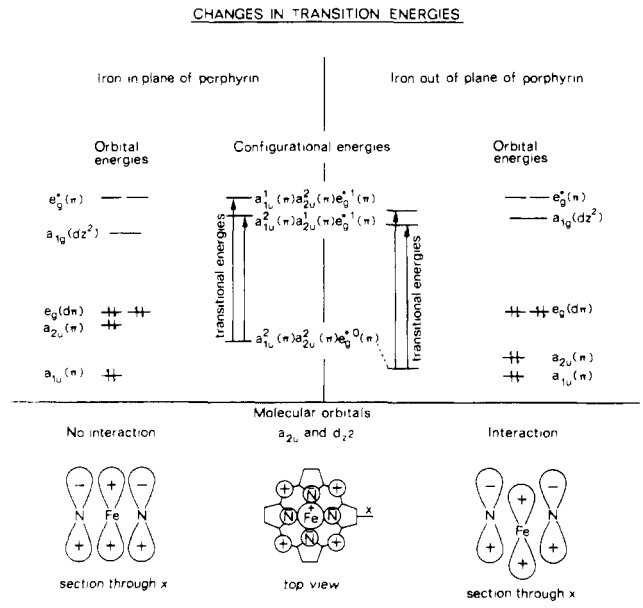

FIGURE 11: Increase in transition energies of $\pi \rightarrow \pi^*$ interactions of porphyrin with movement of the iron atom out of the porphyrin plane.

(Huber et al., 1970; Collman et al., 1974). We have seen that a displacement of the iron atom from the ring on transition to the T state would cause blue and not red shifts. Dr. Elizabeth J. Heidner has suggested to us that a red shift could occur if the R → T transition caused a distortion of the porphyrin ring which raised the energies of the occupied π levels but left the energies of the unoccupied $e_g(\pi^*)$ levels unaltered. However, this fails to explain why the spectral changes should be so much weaker in carbonmonoxy- than in oxyhemoglobin Kansas.

Effect of pH on the ESR Spectrum of Nitrosylhemoglobin. Rein et al. (1972) found that a lowering of the pH from 7.0 to 5.0 produced the same hyperfine splitting as IHP, yet we find no evidence in favor of a change to the quaternary T structure on lowering of pH toward 5.0, nor do we find the dramatic changes in the Soret band. Therefore the hyperfine splitting must have a different explanation. IHP fails to produce a significant difference spectrum at pH 8.0. At this pH several of the weak bases which form salt bridges with strong acids of other subunits, or of IHP, begin to lose their positive charges; consequently IHP no longer stabilizes the quaternary T structure (Salhany et al., 1975).

Kinetic Data for Nitrosylhemoglobin. When Cassoly (1974) first discovered the large electronic difference spectra caused by the addition of IHP to nitrosylhemoglobin, he also found that the addition of CO to a partially saturated solution of nitrosylhemoglobin caused the change in the nitrosyl spectra to be reversed. Howev·, the rate constants for both the forward and the reverse reactions were of the order of 1 sec^{-1}, slower by several orders of magnitude than the usual rates observed in transitions of the quaternary structure. Salhany et al. (1974, 1975) then discovered that Cassoly's slow spectral changes occur also after reaction of NO with deoxyhemoglobin A + IHP, or with deoxyhemoglobin Kansas, where the quaternary structure remains T both before and after reaction with NO. They suggested that it may be due to a change in tertiary structure of the subunits which follows the reaction with NO. Their value for the time constant of the forward reaction is $k = 0.65$ sec^{-1}. We now see that this represents the rupture or at least dramatic stretching of the Fe-N_ϵ bond and corresponds to an activation energy of $\Delta F^\ddagger = -RT \ln (kh/KT) = 71$ kJ/mol (R = gas constant, h = Planck's constant, K = Boltzmann's constant). For comparison, Bunn and Jandl (1966) found no detectable exchange of ^{59}Fe-labeled hemes between hemoglobins A and F for deoxy-, oxy-, carbonmonoxy-, or cyanomethemoglobins, but for aquomethemoglobin they did find an exchange with a time constant of 100 min. It would be interesting to compare the exchange rates of nitrosyl- and aquomethemoglobins with and without IHP.

Kinetic Data for Oxyhemoglobin. Gibson (1973) has measured the rate of dissociation from partially saturated solutions of oxyhemoglobin under conditions where a large fraction of the molecular species present are the mono- and dioxygen intermediates. At 2°C in 0.05 M phosphate buffer of pH 7.0 the deoxygenation reaction consists of two separate phases, a fast one of about 100/s and a slow one of 6/s. Their relative amplitudes depend on the wavelength of observation: at 585 nm the rapid dissociation dominates, but at 588 nm the slow one does. Gibson attributed the rapid rate to the β and the slow one to the α subunits, but our results suggest a simpler interpretation. Figure 4 shows that 585 nm is close to the isosbestic point of oxyhemoglobin in the R state and of deoxyhemoglobin, while 588 nm is close to the isosbestic point of oxyhemoglobin in the T state and of deoxyhemoglobin. This means that at 585 nm Gibson would have seen mainly the rapid dissociation from oxyhemoglobin in the T state and at 588 nm mainly the slow dissociation from oxyhemoglobin in the R state. This is consistent with his observation that the relative amplitude of the fast rate is greatest at the smallest initial oxygen saturations, and also with the effects of pH, IHP, and temperature which he observed.

Role of α and β Subunits. How does the quaternary structure of the globin in the T state lower the oxygen affinities of the α and β subunits? Our results point to two complementary mechanisms: tension at the hemes, which may be the dominant one in the α subunits, and steric hindrance at the ligand site which occurs in both subunits. If the T minus R difference spectrum in the Soret band of deoxyhemoglobin is taken as a measure of the tension (Figure 3 of part I, Perutz et al., 1974a), then Sugita's elegant experiments with hybrids of proto- and mesoporphyrin show that *in deoxyhemoglobin* the tension exists *only* in the α subunits (Sugita, 1975). This cannot be true of methemoglobin since on transition from R to T of hemoglobin M Milwaukee the band at 620 nm from the ferric β subunits undergoes the red shift normally associated with increased tension (Perutz et al., 1972, 1974c). Why should the tension differ in these derivatives if they have the same quaternary T structure? This happens because they have different tertiary structures, due to the steric effects of the heme ligands and the different displacements of the iron from the plane of the porphyrin ring: 0.65 and 0.2 Å toward the proximal histidine in deoxy- and methemoglobin, respectively, and 0.07 Å toward the distal histidine in nitrosylhemoglobin.

We now come to the steric obstructions at the ligand sites. In the β subunits C_γ of valine E11 has to give way before a ligand can bind to the iron (Perutz and Ten Eyck, 1971), while in the α subunits a water molecule bound to the distal histidine, but not to the iron itself, must be displaced (Fermi, 1975). Combination with ligands requires changes in tertiary structure which allow these obstructions to be cleared and the iron atoms to be moved into the planes of the porphyrin rings. These tertiary changes are opposed by the constraints of the quaternary T structure.

When Perutz (1970) advanced his Stereochemical Mechanism of the Cooperative Effects, he did not know of the existence of the tension at the hemes nor of the water molecule in the α heme pocket. His proposal that in the T state the α hemes have the higher ligand affinity rested solely on the steric hindrance of valine E11β which is now seen to be only one of several factors regulating the ligand affinities of the two subunits.

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