Influence of Globin Structure on the State of the Heme. II. Allosteric Transitions in Methemoglobin†

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ABSTRACT: Aquomethemoglobin crystallizes in the R structure which is also the dominant form in solution. However, the displacement of the heme-linked histidine from the plane of the porphyrin ring in aquomethemoglobin is intermediate between the low-spin forms in which the heme group is known to be planar and the high-spin deoxyhemoglobin where its deviation from planarity is greatest. At constant pH, ionic strength, and concentration of organic phosphate, the equilibrium between the allosteric forms of hemoglobin appears to be governed mainly by the displacement of the proximal histidine from the plane of the porphyrin ring; one would therefore expect solutions of aquomethemoglobin to contain an appreciable fraction of molecules in the T state in dynamic equilibrium with the bulk of molecules in the R state. It was shown in part I (Perutz, M. F., Ladner, J. E., Simon, S. R., and Ho, C. (1974b), *Biochemistry 13*, 2163) that in deoxyhemoglobin the transition of quaternary structure from R to T is accompanied by characteristic changes in ultraviolet absorption and circular dichroism. Exactly the same changes can be produced by the addition of 1 mol of inositol hexaphosphate (IHP) to 1 mol of tetramer of aquo- or fluoromethemoglobin. Moreover, the combination with IHP diminishes the rates of reaction of their sulfhydryl groups by factors comparable to those observed on deoxygenation of oxyhemoglobin. By all these criteria IHP appears to change the allosteric equilibrium of high-spin methemoglobin derivatives in favor of the T state. Our evidence indicates that IHP binds to the same site in met- as in deoxyhemoglobin, *i.e.*, between the amino termini of the β chains. IHP also induces changes in the ultraviolet spectra and sulfhydryl reactivities of low-spin hemoglobins, but these changes are fewer and weaker and are not accompanied by the change in sign of the circular dichroism at 287 nm which is associated with the R → T transition of deoxyhemoglobin. IHP also produces large changes in the ring-current-shifted resonances of several aromatic residues in aquomethemoglobin, and much smaller ones in cyanomet- and carbonmonoxyhemoglobin. Our results indicate that solutions of high-spin methemoglobin derivatives normally contain the R and T forms in dynamic equilibrium, the R form being favored by high pH and the T form by low pH and by organic phosphates. It is shown that such a scheme can qualitatively account for the pH dependence of the oxidoreduction equilibrium of hemoglobin and for the effects of chemical modifications on that equilibrium.

The ferric iron atoms in aquomethemoglobin are octahedrally coordinated to four nitrogens of the porphyrin, one nitrogen of the proximal histidine, and a water molecule. The latter is replaceable by several other ligands, including fluoride, azide, cyanide, and hydroxyl. Taube (1952) and Williams (1955) first suggested that some of these derivatives may contain hemes with high- and low-spin states in thermal equilibrium. This was verified experimentally by Beetlestone and George (1964), George *et al.* (1964), and Iizukeand Kotani (1969). At room temperature aquo- and fluoromethemoglobin are predominantly high spin and the others mainly low spin. The radius of the iron atom varies with its spin state from 0.55 Å in low-spin to 0.60 Å in high-spin ferric compounds (Williams, 1961; Hoard, 1966). In the former the ferric ion is just small enough to fit into the plane of the porphyrin ring (Hoard, 1968) and accordingly the heme in cyanomethemoglobin is planar (Hendrickson and Love, 1971). In aquomethemoglobin, on the other hand, the larger radius of the ferric ion forces it out of the plane by 0.3 Å, which is intermediate between its position in the oxy and deoxy forms (Perutz, 1970). If it is true that under constant solvent conditions the equilibrium between these two forms is governed mainly by the displacement of the proximal histidine from the plane of the porphyrin ring, then one would expect methemoglobin to be capable of taking up either form.

Observations are conflicting. Properties such as crystal structure, sulfhydryl reactivity, circular dichroism (CD) in the ultraviolet (uv) region, or total number of Bohr protons released on oxidation all point to methemoglobin having a tertiary and quaternary structure which is closely similar to those of oxy- or carbonmonoxyhemoglobin. In fact, recent X-ray analyses have shown that the differences in structure between carbonmonoxy- and methemoglobin are very small and are mainly confined to the immediate vicinity of the hemes (E. J. Heidner, unpublished data). One would therefore expect the oxygenation and oxidation reactions to exhibit similar cooperative behavior, but this is not observed. In the oxygenation reaction, the slope of the oxygen equilibrium curve in its middle range varies little between pH 7.0 and 9.0, *e.g.*, the data of Roughton (1964) for human hemoglobin imply a decrease in n from 3.0 to 2.5 in going from pH 7.0 to 9.0. The older data gave rise to the widely held misconception of the free energy of heme–heme interaction being independent of pH in that range. However, Roughton (1963) had already shown that this is not true for sheep hemoglobin, because the first of the four equilibrium constants in the Adair equation rises with rising pH, while the fourth remains roughly constant. This has recently been confirmed also for human hemoglobin (Tyuma *et al.*, 1973), and implies that the free energy of heme–heme interaction falls with rising pH. In the oxidation reaction on the other hand, it rises from pH 6.0 to 8.5 (Antonini

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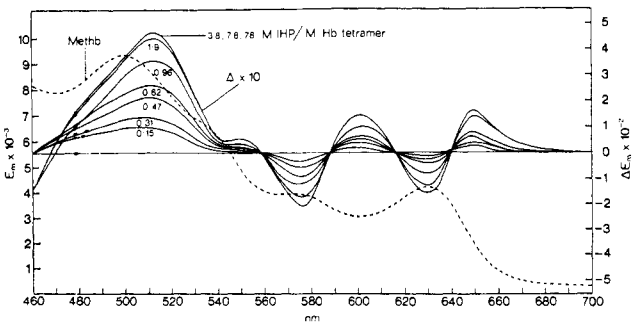

FIGURE 1: Broken curve, absorption spectrum of aquomethemoglobin solution in 0.2 M Bis-Tris (pH 6.6), μ = 0.14, and 25°; full curves, difference spectra, 10× magnified, between the original solution and solutions to which increasing amounts of IHP, neutralized to the same pH, have been added. All values of ϵ in this and subsequent figures are in moles of heme.

et al., 1964). In the oxygenation reaction, in both kinetic and equilibrium studies, the release of Bohr protons has been found to be proportional to oxygen uptake (Wyman, 1964; Antonini *et al.*, 1965; Gray, 1970; Olson and Gibson, 1973), while in equilibrium studies of the oxidation of deoxy- to methemoglobin at acid pH, the release of Bohr protons lagged behind the oxidation of the heme irons (Brunori *et al.*, 1965).

We have discovered that solutions of methemoglobin contain two major allosteric forms in equilibrium. In the absence of organic phosphates at pH 6.0–9.0, the oxy or R structure is dominant (Perutz, 1946). (There is crystallographic and nuclear magnetic resonance (nmr) evidence for a structural transition in methemoglobin with a pK of 7.0 or just above, but this is merely a small modification of the R structure (Cullis *et al.*, 1961; Huestis and Raftery, 1972; E. J. Heidner, unpublished data).) Below pH 7.2 stoichiometric combination of aquomethemoglobin with one mole of inositol hexaphosphate converts methemoglobin to another structure which has a sulfhydryl reactivity, and an absorption and circular dichroism in the aromatic region of the uv spectrum, close to those of the deoxy or T form. These findings have led to a simple explanation for the apparent inconsistencies between the various properties of methemoglobin, which will be proposed in the Discussion.

It will be shown in part III (Perutz *et al.*, 1974a) that the conversion of the methemoglobin structure by IHP[1] is accompanied by a transition of the spin equilibrium of the hemes to higher spin and by red shifts of all high-spin absorption bands in the visible and near-infrared regions, which implies a lengthening of the iron–nitrogen bonds.

Methods

Solutions of methemoglobin were prepared as described by Perutz (1972). Spectra were recorded on a Cary 14 spectrophotometer. Circular dichroism was recorded with a Cary 60 spectropolarimeter and Model 6001 attachment, modified by repositioning an improved electrooptic modulator (Isometric Corp.) outside the sample compartment, immediately behind the polarizer. Data were collected and analyzed as described (Simon and Cantor, 1969). Kinetics of the reaction with *p*-HgBzO were followed at 255 nm (Antonini and Brunori, 1969) by S. R. S. using a Durrum-Gibson stopped-flow spectrophotometer with a xenon light source. A. R. F. used a stopped-flow spectrophotometer of his own design monitoring *p*-HgBzO binding at 255 nm. Kinetics of the reaction with 2,2-dithiobis(pyridine) were followed at 343 nm in 1-cm cuvets in a Gilford 2400 spectrophotometer. The same instrument was used to follow the binding of cyanide at 405 nm and of azide at 422 nm. Proton nmr spectra in the aromatic region were obtained on a Varian HA-100 spectrometer at 94.1 or 100.0 MHz. Aquomet β chains were prepared as described by Kilmartin (1973) in 0.2 M sodium phosphate (pH 6.5)–10^{-3} M EDTA and used in that buffer. Carbonmonoxyhemoglobin Hirose was purified on Bio-Rex 70 200–400 mesh (Bio-Rad Laboratories, Richmond, Calif.) using 0.05 M sodium phosphate (pH 7.2) (3.02 g/l. of Na_2HPO_4 and 1.42 g/l. of $NaH_2PO_4 \cdot 2H_2O$).

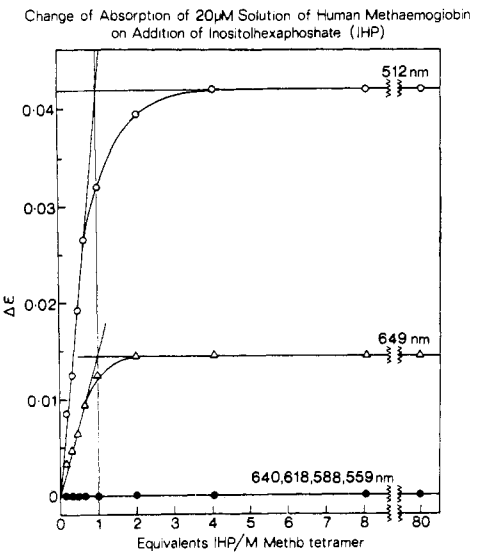

FIGURE 2: Peak heights of difference spectra in Figure 1 at 512 and 649 nm, plotted against the concentration of IHP. The curves were calculated for a binding constant of 1.4 μM.

Results

Stoichiometry and Strength of Binding. The addition of one molar equivalent of IHP per tetramer at pH 6.5 alters the entire absorption spectrum, from the ultraviolet to the infrared region, of the two high-spin derivatives aquo- and fluoromethemoglobin, and produces lesser spectral changes in the low-spin derivatives, azide and cyanide methemoglobin.

The difference spectrum of aquomethemoglobin in the visible contains three prominent peaks at 512, 600, and 649 nm, and shows isosbestic points at 559, 588, and 640 nm, indicating a transition between two alternative states (Figure 1). A plot of the intensities of the maxima at 512 and 649 nm against IHP concentration shows that 1 mol of IHP binds stoichiometrically to 1 mol of methemoglobin tetramer with a dissociation constant in 0.2 M Bis-Tris of pH 6.5 and 25° of 1.4 μM (Figure 2).

Binding Site. IHP is known to bind to both deoxy- and oxyhemoglobin. The dissociation constant from oxyhemoglobin has been estimated as 1.4 μM (Gray and Gibson, 1971) while that to deoxy has proved too small to measure. X-Ray analysis has shown that the binding site of IHP to deoxyhemoglobin is the same as for diphosphoglycerate, *i.e.*, it lies between the amino termini of the β chains and includes histidine-2 and -143, and lysine-82β (Arnone, 1972; Arnone and Perutz, 1974). The binding site of IHP to the oxy form is still unknown. In the presence of 0.1 M NaCl 2,3-diphosphoglycerate or ATP binds strongly to the deoxy, but only

[1] IHP, inositol hexaphosphate; *p*-HgBzO, *p*-mercuribenzoate; NES, *N*-ethylsuccinimide; BME, bis(maleimidomethyl) ether.

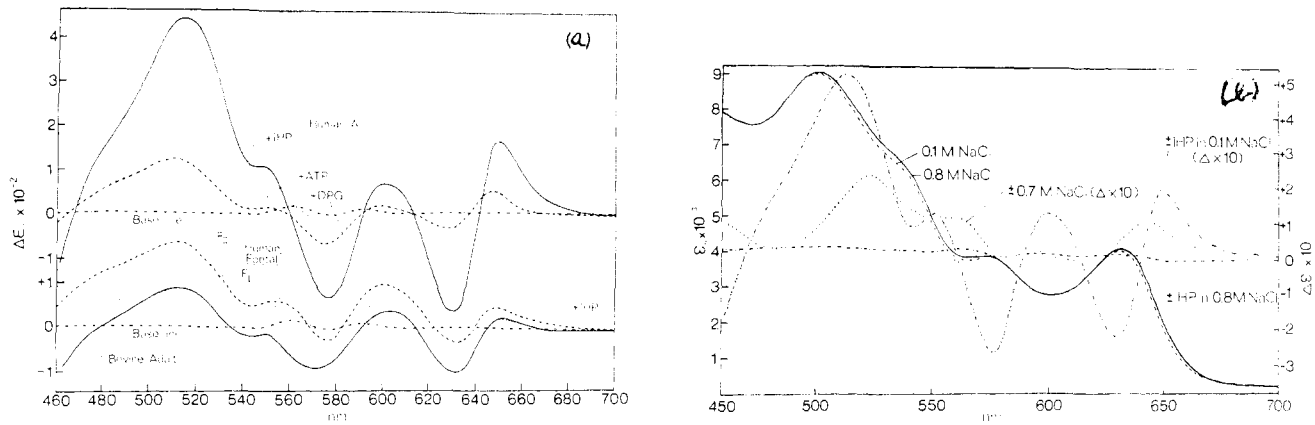

FIGURE 3: Spectral studies. (a) Comparison of difference spectra observed on addition of 1 mol of IHP/tetramer to 38 μM (tetramer) solutions of different methemoglobins in 0.05 M Bis-Tris of pH 6.5 + 0.1 M NaCl. (b) (——) Absorption spectrum of aquomethemoglobin in 0.05 M Bis-Tri (pH 6.5) + 0.1 M NaCl; (- - - - -) same + 0.8 M NaCl; (· · · · · ·) shows the difference between them 10× magnified; (-·-·-·) difference spectrum 10× magnified on adding 4 equiv of IHP/tetramer in 0.1 M NaCl; (-·-·-·-·-) same in 0.8 M NaCl.

very weakly to the oxy form (Bénesch *et al.*, 1971). We have found that addition of diphosphoglycerate or ATP to methemoglobin produces spectral changes similar to, but weaker than, those produced by IHP, and that their dissociation constants are much larger (~1 mM) (Figure 3 and Table I).

Binding of organic phosphates to bovine deoxyhemoglobin is weakened by the deletion of residue 2β which increases the distance between the α-amino groups so that phosphates cannot reach them both (Perutz, 1970). In the F_{II} fraction of human fetal deoxyhemoglobin it is weakened by the replacement of histidine-143 by serine, and in the F_I fraction it is further weakened by the acetylation of the α-amino groups of the β chains (Bunn and Briehl, 1970). Table I shows that the dissociation constants of IHP from bovine methemoglobin and from fetal methemoglobin F_{II} are much larger than from human adult methemoglobin. The spectral changes are similar in kind but smaller in amplitude (Figure 3a). The spectral changes produced by a given concentration of IHP in F_I are smaller than in F_{II} but the dissociation constant proved too large to measure. Evidently the binding properties of IHP to different methemoglobins run parallel to those of the corresponding deoxyhemoglobins which indicates that IHP binds to the same sites in both derivatives.

Ultraviolet Absorption Spectra. Addition of one or more molar equivalents of IHP per tetramer to aquo- or fluoromethemoglobin produces uv difference spectra with peaks in the aromatic region at 279, 287, 294, and 302 nm (Figure 4a). Figure 4b shows these difference peaks to be identical, both in position and amplitude, with those observed on addition of IHP to a solution of deoxyhemoglobin Kempsey, where the difference spectrum was proved to be due to a transition from the R to the T structure. They are also identical in position with the peaks observed in a difference spectrum of deoxyoxyhemoglobin A (see part I; Perutz *et al.*, 1974b). Only the large difference peak at 322 nm which corresponds to a shoulder on the Soret band of the ferrous heme is absent from the difference spectra of the ferric forms. Suspecting that the uv difference spectra of aquo- and fluoromethemoglobin might signify a change in quaternary structure, we wondered if they were due to changes in the environment of aromatic residues at the $\alpha_1\beta_2$ boundary where large shifts are known to occur during the R $\rightleftharpoons$ T transition (Perutz, 1970; Perutz and Ten Eyck, 1971). These residues include Tyr-C7(42)α, Trp-C3(37)β, and Phe-C7(41)β. The only mutant available in which any of these residues is replaced is hemoglobin Hirose (C3(37)β-Trp → Ser) (Yanase *et al.*, 1968). We found

TABLE I: Dissociation Constants of Organophosphates and Methemoglobins, and Magnitudes of Accompanying Spectral Changes.

Methemoglobin	Organophosphate	T (°C)	pH	Buffer (M)	Ionic Strength (μ)	K_{diss} (μM)	% Increase in Absorption at 515 nm on Addn of Excess P
Human aquo	IHP	20	6.5	Bis-Tris (0.05)	0.025	~0.1	6.4
	IHP	25	6.6	Bis-Tris (0.2) + NaCl (0.04)	0.14	1.4 ± 0.4	5.4
	IHP	20	6.5	Bis-Tris (0.05) + NaCl (0.2)	0.225	1.5 – 3.0	4.7
	IHP	20	6.5	Bis-Tris (0.05) + NaCl (0.4)	0.425	~40	4.1
	IHP	25	6.5	Phosphate (0.1)	0.12	3.6 ± 1.3	*a*
	DPG[c]	25	6.6	Bis-Tris (0.2) + NaCl (0.02)	0.14	0.2 – 2.0 × 10^3	1.9
Bovine aquo	IHP	25	6.6	Bis-Tris (0.2) + NaCl (0.02)	0.14	8.5 ± 2.0	3.3
Human fetal F_{II} aquo	IHP	25	6.6	Bis-Tris (0.2) + NaCl (0.02)	0.14	16.4 ± 1.1	3.3
Human cyano	IHP	25	6.6	Bis-Tris (0.2) + NaCl (0.02)	0.14	1.9[b]	
Human azido	IHP	25	6.6	Bis-Tris (0.2) + NaCl (0.02)	0.14	2.2[b]	

[a] Followed at the Soret band at 409 nm. [b] Calculated respectively from dissociation constant of cyanide and azide in presence and absence of IHP. [c] DPG = diphosphoglycerate.

that aquomethemoglobin Hirose gave a difference spectrum in the visible which was similar to that observed in aquomethemoglobin A, but that the difference peaks in the uv at 294 and 302 nm were absent (Figure 4a).[2]

The uv difference spectra of azide and cyanomethemoglobin are weaker than those of the high-spin derivatives and contain only the peaks 279 and 287 nm. Addition of more than a molar equivalent of IHP fails to increase the intensity of the difference spectra.

Effects of pH, Ionic Strength, and Chemical Modifications on Difference Spectra. The positions and strengths of the peaks in the difference absorption spectra produced by IHP in aquomethemoglobin are strongly pH dependent. The difference spectra weaken above pH 7.0 and fade out at pH 8.0, even in the presence of a large excess of IHP. These and other changes in the difference spectra between pH 5.0 and 7.5 will be discussed in detail in part III (Perutz *et al.*, 1974a). Increasing ionic strength raises the dissociation constant of IHP from methemoglobin (see Table I) and weakens the maximum spectral change observed on addition of an excess of IHP. This weakening arises from a spectral change which high concentrations of neutral salt produce in methemoglobin. Figure 3b shows that 0.8 M NaCl produces red shifts and slight increases in intensity of the bands at 500 and 630 nm, but does not produce the positive peak at 600 nm and the negative peaks characteristic for IHP. In 0.8 M NaCl even a large excess of IHP no longer produces a difference spectrum.

IHP produces no difference spectra in free aquomet β chains, nor in derivatives whose allosteric equilibrium is so strongly biased towards the R structure that their reaction with oxygen is noncooperative, such as BME,[3] NES-des-Arg- or des-His-Tyr-aquomethemoglobin. It does produce weakened difference spectra in derivatives that lack some of the salt bridges needed to stabilize the T structure such as des-Arg-, des-Arg-Tyr-, and des-His-aquomethemoglobin. It also produced a weak difference spectrum in methemoglobin M Iwate (87α-His → Tyr) (Miyaji *et al.*, 1963) which has the quaternary deoxy structure in the crystal and in solution (Greer, 1971; Shulman *et al.*, 1973); presumably IHP can alter the tertiary structure of its β chains.

Circular Dichroism. While the work at Cambridge showed that the changes in absorption in the aromatic region of the uv seen on addition of IHP to high spin methemoglobin derivatives or to deoxyhemoglobins in the R state are the same, parallel work at Stony Brook demonstrated that the changes in CD produced by this reaction in ferrous and ferric derivatives are also quite similar. It was shown in part I (Perutz *et al.*, 1974b) that the two quaternary structures show a characteristic difference in circular dichroism between 275 and 295 nm which can be clearly distinguished from differences associated with changes in state of ligation of the hemes. All the liganded forms of hemoglobin which crystallize in the R form show weak positive ellipticity in this region with two slight dips near 285 and 290 nm. Deoxyhemoglobin A,

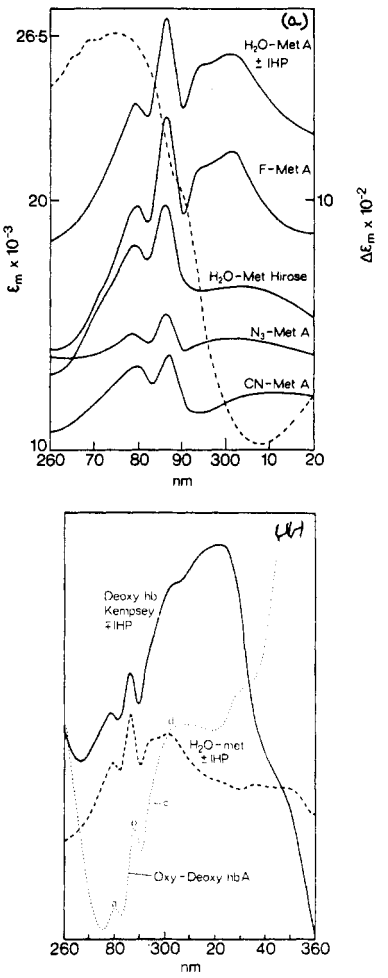

FIGURE 4: Spectral studies. (a) Broken line, uv absorption spectrum of a 15 μM (tetramer) solution of aquomethemoglobin; full lines, difference spectra, 10× magnified, of various methemoglobin derivatives with and without 2.5 M IHP/tetramer. The concentration of aquomethemoglobin Hirose was 13 μM. (b) Comparison of uv difference spectra of 15 μM solutions of deoxyhemoglobin Kempsey and aquomethemoglobin A with and without 2.5 M IHP/tetramer, and of oxy- minus deoxyhemoglobin A.

however, has a prominent band of negative ellipticity with a single minimum at 287 nm. Deoxygenated hemoglobin derivatives which retain the R quaternary structure fail to reveal this prominent single negative band. Instead, two weaker negative bands, centered at 285 and 290 nm, are found. This R-type CD spectrum has now been seen in deoxyhemoglobin Rainier, des-Arg-deoxyhemoglobin above pH 8.0, and, as reported in part I, in NES-des-Arg-deoxyhemoglobin and deoxyhemoglobin Kempsey.

Figure 5a shows the CD spectra of high- and low-spin methemoglobin derivatives in the presence and absence of IHP, with the spectra of oxy- and deoxyhemoglobin for comparison. It is apparent that IHP induces a change in CD in the high-spin derivatives, aquomethemoglobin and fluoromethemoglobin, but not in the low-spin derivatives, cyanomet- and azidomethemoglobins. The difference CD spectrum calculated for aquomethemoglobin in the absence and presence of IHP is identical in shape with those obtained for NES-des-Arg-deoxyhemoglobin and deoxyhemoglobin Kempsey in the absence and presence of the organic phosphate (Figure 5b) although it is somewhat increased in magnitude. Above pH 7.2, the effect of IHP on the CD of aquomethemoglobin is markedly diminished (Figure 6), and at pH 8.0 the spectra

[2] It has since been proved that the bands at 279 and 287 nm are due to the interaction of Tyr-C7-42α and Arg-C6-40β. It has also been found that addition of IHP to nitric oxide hemoglobin produces the same strong uv difference spectrum as to aquo- and fluoromethemoglobin, even though it is a low-spin compound. The stereochemical explanation for this effect will be discussed in part III (Perutz *et al.*, 1974a).

[3] BME (bis(maleimidomethyl) ether) is a bifunctional reagent which cross-links Cys-F9-93β to His-FG4-97β and inhibits all cooperative effects by displacing Tyr-HC2-145β from its pocket between helices F and H. It has much the same properties as des-Tyr-145-His-146 hemoglobin (Simon, 1967; Moffat, 1971; Moffat *et al.*, 1971; Simon *et al.*, 1971).

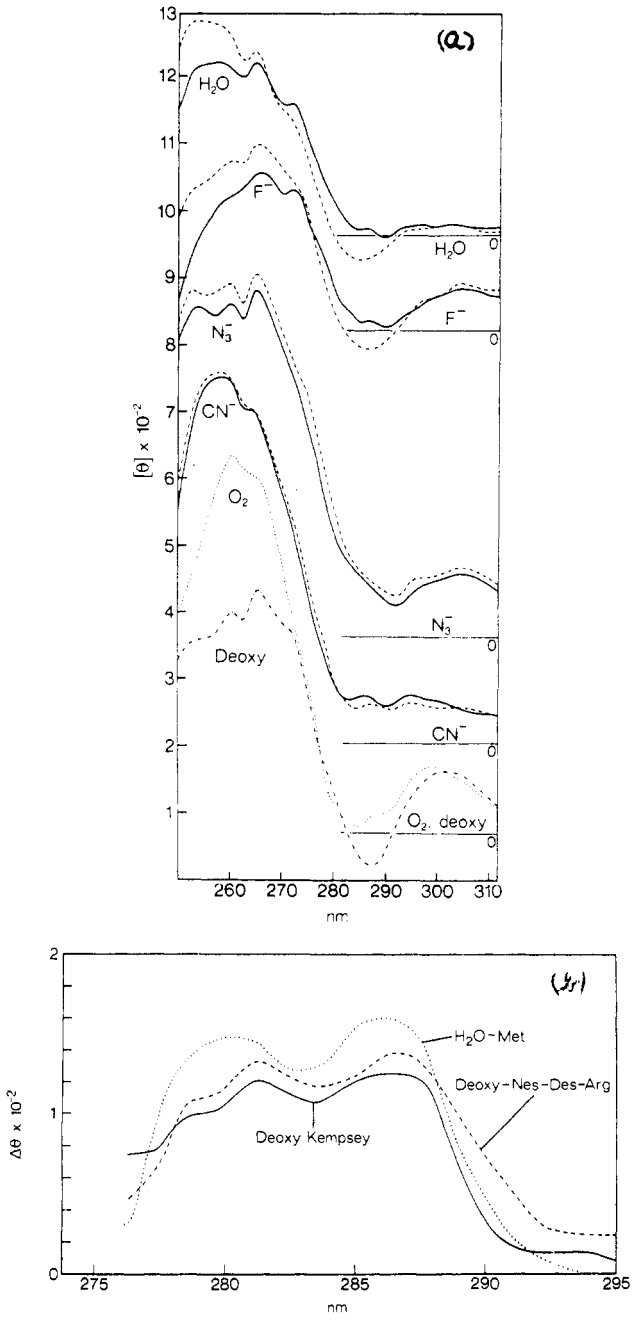

FIGURE 5: Spectral studies. (a) CD spectra of 62 μM (tetramer) solutions of different hemoglobin derivatives in 0.05 M Pipes buffer of pH 7.0 and 25°. The full curves refer to methemoglobin derivatives without IHP; the broken ones to the same derivatives with 4 M IHP/tetramer. The dotted curve refers to oxy- and the dot-dashed one to deoxyhemoglobin. The horizontal lines give zero ellipticity for each derivative. The differences near 260 nm are due mainly to the heme, those near 285 nm mainly to the tryptophans and tyrosines. (b) Difference CD spectrum of aquomethemoglobin (−IHP) − (+2 × 10^{-4} M IHP), with difference spectra of deoxy-NES-des-Arg-hemoglobin and Kempsey hemoglobin for comparison: aquomethemoglobin (- - - - -); NES-des-Arg-deoxyhemoglobin (— — —); deoxyhemoglobin Kempsey (——). All measurements were made at pH 7.0 in 0.1 M Bis-Tris buffer.

in the presence and absence of the organic phosphate are essentially identical. The CD spectral changes induced by IHP in high-spin methemoglobin derivatives cannot be duplicated by inorganic orthophosphate, and are not eliminated by addition of 0.1 M NaCl to the IHP-containing buffers, indicating that nonspecific binding of organic phosphates does not contribute to the spectra.

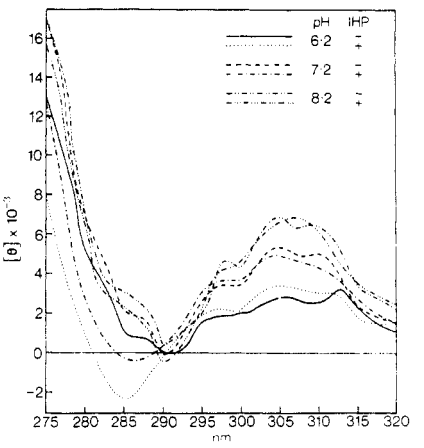

FIGURE 6: CD spectra of aquomethemoglobin, with and without 4 M IHP/tetramer in 0.05 M Bis-Tris or Pipes buffers of different pH. Molar ellipticities in this figure are in terms of $\alpha\beta$ dimers.

Sulfhydryl Reactivity. The effect of IHP on the sulfhydryl reactivity of methemoglobin was investigated in parallel and independent studies at Stony Brook and at Cambridge, using somewhat different methods and arriving at essentially the same results. At Stony Brook the apparent first-order rate constants were measured as a function of pH without IHP and in the presence of 4 mol of IHP/mol of hemoglobin tetramer, using 50 μM (tetramer) solutions of hemoglobin in 50 mM Pipes buffer with 0.1 M NaCl and 20 μM *p*-HgBzO (Table II). Below pH 7.0 IHP reduces the rate constants of aquo- and fluoromethemoglobins by large factors and those of the cyano- and azidomethemoglobins and of carbonmonoxyhemoglobin by small factors. Table II and Figure 7 both show that the effect of IHP disappears above pH 7.2. Figure

TABLE II: Effect of IHP on Reaction of Hemoglobins with *p*-HgBzO.[a]

Hb Deriv	pH	App First-Order Rate Constant (sec^{-1}) − IHP	App First-Order Rate Constant (sec^{-1}) + 2×10^{-4} M IHP	Ratio
Aquomet-	6.8	12.6	0.7	18.00
	6.9	15.8	0.6	26.33
	7.0	11.8	1.9	6.21
	7.1	12.7	4.2	3.02
	7.2	14.9	14.6	1.02
	7.4	12.8	12.6	1.01
Fluoromet-	6.6	26.48	0.57	46.45
	6.8	30.77	1.79	17.18
	7.0	36.34	3.47	10.47
	7.2	42.78	10.99	3.89
	7.4	40.33	25.88	1.55
	7.6	44.71	30.01	1.48
CO-	6.8	19.75	10.14	1.94
	7.0	15.95	12.21	1.30
	7.2	27.32	17.61	1.55
Cyanomet-	7.0	13.37	8.48	1.57
Azidomet-	7.0	19.18	9.27	2.06

[a] All reactions were carried out in 0.05 M Pipes buffers, containing 0.1 M NaCl. *p*-HgBzO concentration was 2×10^{-5} M; hemoglobin concentration was 5×10^{-5} M, 20°.

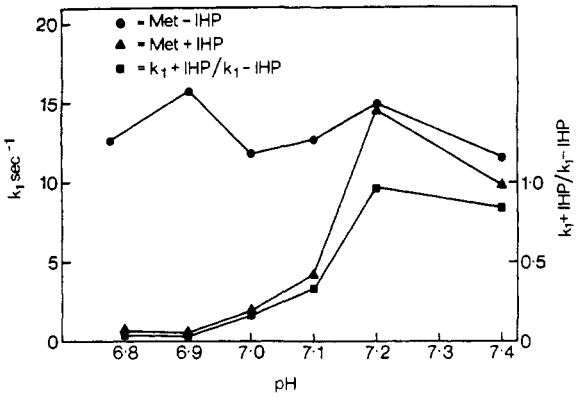

FIGURE 7: pH dependence of the apparent first-order rate constants of the reaction of *p*-HgBzO with aquomethemoglobin, with and without IHP, and of the ratio between them. Experimental conditions as in Table II.

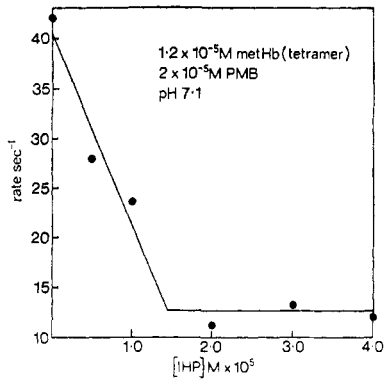

FIGURE 8: Second-order rate constant of the reaction of sulfhydryl groups of cysteine-93β of a 12 μM (tetramer) solution of aquomethemoglobin with a 20 μM solution of *p*-HgBzO in 0.05 M Pipes buffer + 0.1 M NaCl at pH 7.1 and 25°, plotted against the concentration of IHP.

8 shows the effect of IHP on the sulfhydryl reactivity to be stoichiometric, produced by the combination of 1 mol of IHP with 1 mol of hemoglobin tetramer. The 26- to 18-fold reduction in rate constants of methemoglobin below pH 7.0 may be compared with the 44- to 15-fold reducton in the rate constants observed by Antonini and Brunori (1969) on deoxygenation of oxyhemoglobin, though under slightly different experimental conditions, which precludes comparisons of the absolute values.

At Cambridge the second-order rate constants of the reaction with *p*-HgBzO were compared without IHP and with an 83-fold molar excess of IHP using 3 μM (tetramer) solutions of hemoglobin in 0.1 M Bis-Tris of pH 6.60 with KCl added to bring the ionic strength to μ = 0.2, and reacting with 25–50 μM *p*-HgBzO at 25°. There are known to be certain difficulties in determining the second-order rate constants of this reaction. One of these arises from the nonlinear increase of the reaction rate with *p*-HgBzO concentration (Antonini and Brunori, 1969). However, in the lower range of *p*-HzBzO concentrations (15–60 μM) linearity was maintained, and our results confirmed this. A further difficulty lies in the high absorption of the Bis-Tris buffer. This was overcome by using a 1-cm path instead of the 2 cm in the Durrum-Gibson machine, and by employing a home-built machine which is more sensitive and has a lower noise level than commercially available ones.

Table III shows the results. At pH 6.60, IHP diminishes the second-order rate constants of the reaction of *p*-HgBzO with aquo- and fluoromethemoglobin by factors of 23 and 74, respectively. The absolute values found for aquomethemoglobin in the absence and presence of IHP are comparable to those reported by Maeda and Ohnishi (1971) for oxy- and deoxyhemoglobin. IHP also reduces the second-order rate constants of the low-spin oxy- and carbonmonoxyhemoglobins, but by much smaller factors. Azidomethemoglobin shows an effect which is intermediate between the high- and fully low-spin derivatives. The very large effect of IHP on the reaction rates of fluoromethemoglobin seen in Tables II and III deserves comment. Note that IHP reduces the reaction rates of aquo- and fluoromethemoglobin to about the same values and that the difference arises because the reaction rates of fluoromethemoglobin *in the absence of IHP* are about twice as large as those of aquomethemoglobin. It will be shown in the Discussion that the higher reaction rate is consistent with the small changes in tertiary structure of the β chains that occur as a function of differences in the spin state of the heme (E. J. Heidner, 1973, unpublished data).

TABLE III: Reaction of *p*-Mercuribenzoate with SH-93β of Human Hemoglobins in Presence and Absence of IHP.[a]

Hemoglobin	k_2,[b] M^{-1} sec^{-1} (No IHP)	k_2', M^{-1} sec^{-1} (+250 μM IHP)	k_2/k_2'
Met	1.3×10^6	4.1×10^4	32
HbCO	5.3×10^5	1.6×10^5	3.3
HbO_2	8.0×10^5	1.6×10^5	5.0
HbO_2[c]	1.5×10^6		
Hb	3.3×10^4		
Azidomet	1.5×10^6	1.4×10^5	10
Fluoromet	2.5×10^6	3.4×10^4	74

[a] Performed in 0.1 M Bis-Tris (pH 6.60), 25°, μ = 0.20 (added KCl), 3 μM tetramer. Monitored at 255 nm. [b] Average of values for 25 and 50 μM *p*-HgBzO. Rate is nonlinear in *p*-HgBzO concentration at higher values (Antonini and Brunori, 1969). [c] 20°, pH 7.2 (Maeda and Ohnishi, 1971).

Since the reaction with *p*-HgBzO is very fast, we thought it desirable to check the results with a slower reacting sulfhydryl reagent. Ampulski *et al.* (1969) introduced 4,4′-dithiobis-(pyridine) as a reagent for heme proteins. We employed the slightly more soluble 2,2′-dithiobis(pyridine) with and without a 30-fold molar excess of IHP. Table IV shows the second-order rate constants at pH 6.61 and 7.85. At the lower pH, IHP reduces the rate with aquomethemoglobin 9-fold, with oxy- 4.2-fold and with carbonmonoxyhemoglobin 2.4-fold; at the higher pH, it produces no changes. Figure 9 is a plot of the rates of reaction with and without IHP and of their ratios against pH and shows that the effect of IHP vanishes above pH 7.0, in agreement with the results obtained with *p*-HgBzO at Stony Brook.

Effect of IHP on Heme Ligand Binding. We have measured the equilibrium curves of cyanide and azide with methemoglobin in the presence and absence of IHP. We found that the addition of IHP produced only small increases in the dissociation constants for those ligands, amounting to differences in free energy of binding of a few hundred calories per mole. However, there are large effects on the enthalpy of binding (J. G. Beetlestone, unpublished). Hill's constant remained at unity within the limits of error regardless of the presence of IHP (Table V).

Proton Magnetic Resonance in the Aromatic Region. Proton resonances of some of the aromatic residues, including

TABLE IV: Reactivity of Sulfhydryl Groups with 2,2′-Dithiobis(pyridine) in the Presence and Absence of IHP.[a]

Hemoglobin	pH	k_2, M^{-1} sec^{-1}	$\frac{k_2 - IHP}{k_2 + IHP}$
MHb	6.61	12.6	9.0
MHb + IHP	6.61	1.4	
HbO_2	6.61	6.3	4.2
HbO_2 + IHP	6.61	1.5	
HbCO	6.61	3.2	2.4
HbCO + IHP	6.61	1.3	
MHb	7.85	55	1.0
MHb + IHP	7.85	55	
HbO_2	7.85	33	1.0
HbO_2 + IHP	7.85	33	
HbCO	7.85	21	1.0
HbCO + IHP	7.85	22	

[a] μ = 0.15, 25°, 250 μM IHP, 16 μM β chains, 2–8 × 10^{-4} M dithiobis(pyridine), monitored at 343 nm.

histidines, are shifted away from the main peak of the aliphatic globin resonances, which allows them to be resolved. In aquomethemoglobin these appear between 7 and 9 ppm downfield from the reference line of hexamethyldisiloxane. If IHP in aquomethemoglobin binds to histidine-2 and -143, as it does in deoxyhemoglobin, then the proton resonances of these residues should be affected. If IHP changes the quaternary structure, then the environments of several other aromatic residues would be altered, and this should result in further spectral changes. We have studied the effects of IHP on the aromatic resonances of aquo- and cyanomethemoglobin and of carbonmonoxyhemoglobin (Figure 10). In aquomethemoglobin IHP causes the peaks at 7.07 and 7.18 ppm to merge into a broad peak at 7.12 ppm, and a new peak to appear at 7.43 ppm. In the histidine region, peaks at 7.98, 8.26, and 8.43 ppm move slightly downfield and new ones appear at 7.91 and 8.67 ppm. The latter is almost certainly a histidine peak, but its identity is unknown. Some of the downfield movements may be due to the increase in

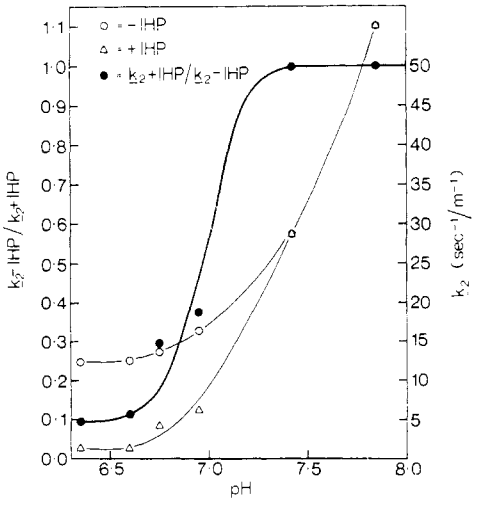

FIGURE 9: pH dependence of the reactivity of cysteine-93β of an 8 μM (tetramer) solution of aquomethemoglobin with 2,2-dithiobis(pyridine) at 25°, μ = 0.2 (KCl added), in the presence and absence of 250 μM IHP. 0.2 M Bis-Tris buffer below pH 7.0 and Tris buffer above pH 7.0.

TABLE V: Binding of CN^- and N_3^- to Human Methemoglobin in the Presence and Absence of IHP.[a]

Ligand	IHP	K_{diss} (μM)	ΔG Binding (kcal mol^{-1})	Calcd as Hill Plot	
				n	K (μM)
CN^- [b]	0	3.10 ± 0.02	−7.51	1.0	3.1
	5 × 10^{-3}	4.26 ± 0.04	−7.32	1.0	4.26
N_3^- [c]	0	8.33 ± 0.41	−6.94	1.01	7.6
	5 × 10^{-3}	13.13 ± 0.74	−6.66	0.99	14.4
	5 × 10^{-2} [d]	11.56 ± 0.87	−6.73	0.92	27.6

[a] 25°, μ = 0.2, pH 6.60, 0.2 M Bis-Tris, added 0.1 M KCl unless stated. [b] CN^-, 4 μM–1 mM, 1.7 μM tetramer followed at 405 nm. [c] Azide, 3 μM–32 mM, 3.8 μM tetramer followed at 422 nm. [d] Ionic strength ~0.5.

paramagnetic susceptibility which occurs on addition of IHP to aquomethemoglobin (see part III).

Cyanomethemoglobin shows smaller changes than aquomethemoglobin in the aromatic region between 7 and 8 ppm, partly due perhaps to the absence of any change in paramagnetic susceptibility in this derivative, but striking ones in the histidine region between 8 and 9 ppm. A peak at 7.52 shifts downfield to 7.64, overlapping another peak, and one at 8.14 seems to move upfield to 8.07 ppm and to become broader. In the histidine region, a prominent peak at 8.69 ppm disappears; it may move downfield to 8.94 ppm forming a shoulder on another peak. In carbonmonoxyhemoglobin the only noticeable changes occur in two of the histidine resonances: peaks at 8.34 and 8.53 ppm move upfield to 8.26 and 8.45 ppm. They also become broader. There is no evidence of changes in the aromatic region.

Effect of IHP on the Oxidation Reaction. The spectral changes and the reduction in sulfhydryl reactivity induced by IHP in methemoglobin at acid pH led us to the conclusion that IHP changes the quaternary structure of aquomethemoglobin to the T form. Since cooperativity in the reaction with heme ligands is generally conditional on a change of quaternary structure, we expected that the oxidation reaction would become noncooperative if the T → R transition were inhibited by IHP. This question has been investigated by Kilmartin (1973) who found that IHP lowers Hill's constant, causing the shape of the oxidation–reduction equilibrium curve to become hyperbolic below pH 7.5 and the alkaline Bohr effect to be decreased. The redox potential at half-oxidation at pH 7.0 is increased from 148 to 187 mV.

Discussion

Stoichiometry and Binding Site. Our results show that human methemoglobin A combines stoichiometrically with 1 mol of IHP/mol of tetramer, and that below pH 7.0 this reaction is accompanied by a change of structure. The absence of spectroscopic changes on addition of IHP to either free met β chains, BME-methemoglobin or NES-des-Arg-methemoglobin excludes the possibility that these changes are due to nonspecific effects by the polyvalent anions on the hemes. The increase in the dissociation constant of IHP from methemoglobin with increasing ionic strength of the solvent points to the interaction being ionic. The variations of the dissociation constants in human adult, human fetal, and bovine methemoglobin indicate that the binding site is

the same as in human deoxyhemoglobin, where it lies on the twofold axis between the β chains and is made up of basic groups contributed by Val-1, His-2, Lys-82, and His-143. Especially convincing is the difference of the spectral response and binding constant between human fetal F_{II} and F_I because they are known to differ chemically only in the acetylation of the α-amino groups of the β chains.

Absorption Spectra and CD in the Uv Region. It was shown in part I that the deoxygenation of hemoglobin A is accompanied by characteristic changes in absorption and circular dichroism in the uv region. The difference absorption spectrum of deoxy- minus oxyhemoglobin shows positive peaks at 279, 287, 294, and 302 nm and the difference CD spectrum a sharp negative peak at 287 nm. These features were found to be missing unless deoxygenation was also accompanied by a change of quaternary structure of the globin; they could be produced in the absence of any heme ligand by converting the quaternary structure of certain modified or abnormal deoxyhemoglobins from R to T. These observations showed that they could serve as diagnostic tests of the R → T transition. The addition of IHP to the high-spin aquo- and fluoromethemoglobins produces difference absorption spectra that are identical with, and difference CD spectra that are closely similar to, those produced in the conversion of deoxyhemoglobin from the R to the T structure. We therefore conclude that IHP induces a similar change in the quaternary strucure of these methemoglobins. This change involves large atomic movements at the $\alpha_1\beta_2$ contact. One of the aromatic residues lying in the contact is tryptophan-C3(37)β. The absence of the difference peaks at 294 and 302 nm in hemoglobin Hirose, in which this tryptophan is replaced by a serine, provides further evidence in favor of the connection between the appearance of the difference spectra and the change in quaternary structure. Crystals of deoxyhemoglobin Hirose are isomorphous with those of deoxyhemoglobin A which shows that the absence of the peaks at 294 and 302 nm cannot be attributed to a failure to form the T structure.

Addition of IHP to the low-spin azido- and cyanomethemoglobins produces weak peaks at 278 and 287 nm in the difference absorption spectrum, but none at 294 and 302 nm, nor does it produce any negative peak at 287 in the CD spectrum. IHP appears to modify the structures of these low-spin derivatives in some manner as yet unknown, but without changing their quaternary structure to the T state.

Sulfhydryl Reactivity. X-Ray studies have revealed two different stereochemical effects that can alter the reactivity of cysteine-93β. The first kind is exemplified by the transition from carbonmonoxy- to methemoglobin which is accompanied by an increase in the second-order rate constant of the reaction with *p*-HgBzO by a factor of two. E. J. Heidner (unpublished data) has shown that this is due to small changes in tertiary structure in the immediate vicinity of the heme which include a slight rotation of helix F. This rotation moves the SH group from an exposed position in aquomethemoglobin to a more shielded one in carbonmonoxyhemoglobin (Figure 11). The rotation is governed by the displacement of the heme-linked histidine from the plane of the porphyrin ring, so that among liganded derivatives, which all have the quaternary oxy structure, the sulfhydryl reactivity should be highest for fluoromethemoglobin which is pure high spin and lowest for carbonmonoxyhemoglobin which is pure low spin. Tables III and IV show that this is generally true. Note, however, that the reactivity of oxyhemoglobin is intermediate between carbonmonoxy and aquomet, suggesting that the

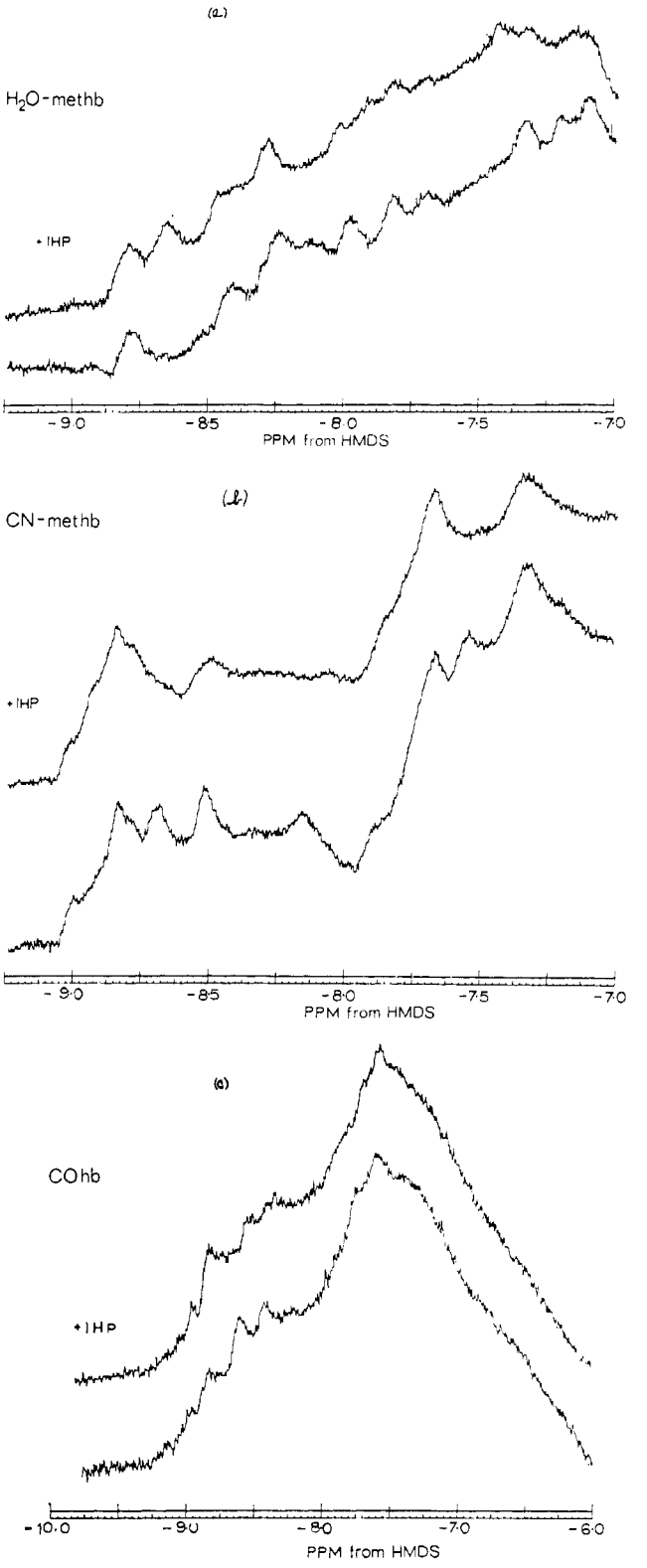

FIGURE 10: Proton magnetic resonance spectra of 3 mM (tetramer) D_2O solutions of hemoglobin in 0.05 M Bis-Tris of pD 6.2 + 0.1 M NaCl at 31° with and without 3 M IHP/tetramer; 2–3 ml were placed in 12-mm sample tubes with Teflon plugs to prevent vortexing. The instrument was field-frequency locked to hexamethyldisiloxane contained in a concentric capillary. Spectra were time averaged for up to 100 scans at sweep rates of 1–2 Hz/sec using a Biomac computer; for time averaging the Varian sweep oscillator was replaced by a Wavetek voltage-to-frequency converter driven by a voltage ramp from the Biomac: (a) aquomethemoglobin; (b) cyanomethemoglobin; (c) carbonmonoxyhemoglobin.

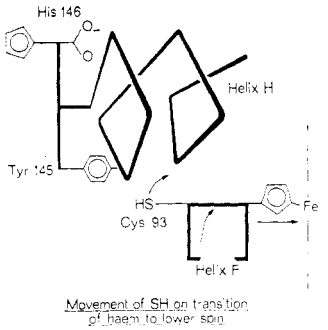

FIGURE 11: Effect of changes in spin state of the heme on the F helix and on the freedom of the sulfhydryl group of cysteine-93β to react with ligands. Quaternary R structure.

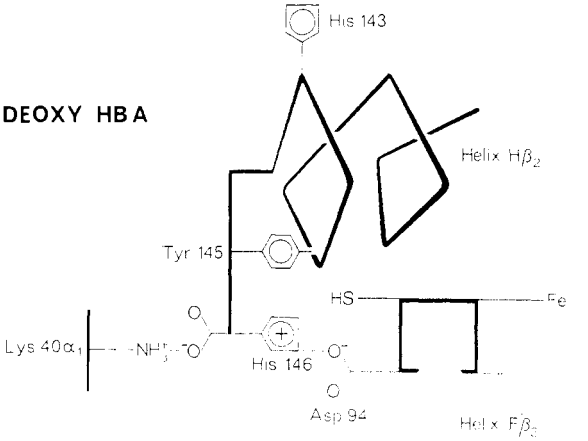

FIGURE 12: Screening of the sulfhydryl group by the salt bridges of histidine-146β in the quaternary T structure.

distance of the heme-linked histidine from the plane of the porphyrin ring is also intermediate, even though both have the R structure. Note also that azidomethemoglobin has a higher reactivity than expected for a low-spin derivative.

The second, more drastic, effect on the sulfhydryl reactivity is linked to the change to the quaternary deoxy structure. Perutz (1970) has suggested that this is due to the screening of the sulfhydryl groups in the quaternary T structure by His-HC3(146)β which is joined by salt bridges to Asp-FG1(94)β and to Lys-C6(40)α (Figure 12).

Investigators have asked if it really is the salt bridges that are responsible for the low reactivity of the sulfhydryl groups in deoxyhemoglobin rather than the change in tertiary structure of the β chains that occurs on deoxygenation. Studies of two mutant and one modified hemoglobin have answered this question. Des-His-146β-hemoglobin lacks the residues that normally screen the sulfhydryl groups; in hemoglobin Hiroshima the histidines are replaced by aspartates that cannot make the salt bridge to Asp-94β. Both these hemoglobins form the normal deoxy structure (Perutz and Ten Eyck, 1971; Perutz *et al.*, 1971); difference Fourier syntheses at 3.5-Å resolution show no structural disturbances away from the site of the removed or replaced histidine. Their sulfhydryl reactivities are listed in Table VI. Under conditions where, on reduction, the second-order rate constant of hemoglobin A with *p*-HgBzO is diminished 73-fold, that of des-His is diminished only 2.4-fold; similarly, under conditions where, on reduction, the first-order rate constant of hemoglobin A with 4,4′-dipyridine disulfide is diminished 7.3-fold, that with hemoglobin Hiroshima is diminished only 1.9-fold. This proves that the major part of the diminution in sulfhydryl reactivity on reduction is due to the salt bridge formed by histidine-146β, but it still leaves open the possibility that these salt bridges might be formed by deoxygenated β chains in the R as well as in the T structure. This point has been clarified by measuring the SH reactivity of deoxyhemoglobin Kempsey which was shown in part I to have an R-type quaternary structure and to be converted to the T structure by IHP. T. B. Bradley, H. F. Bunn, and Q. H. Gibson (unpublished data) determined the apparent first order rate constants of its reaction with *p*-HgBzO. Table VI shows that in the absence of IHP deoxyhemoglobin Kempsey reacts faster than oxy-, while in its presence it reacts 12.5 times more slowly. This experiment proves that formation of the salt bridges that lower the sulfhydryl reactivity occurs on transition to the quaternary, not the tertiary, deoxy structure. It appears that the screening effect of histidine-146β depends on its being locked in position by two salt bridges, the one with Asp-94β as well as that with Lys-40α. In the R structure the ϵ-amino group of that lysine is 13 Å away from the position where it could form a bridge with the α carboxyl of His-146; the rotation of the α chain which is part of the R $\rightarrow$ T transition is needed to bring the two polar groups into contact (Figure 12).

IHP diminishes the second-order rate constant of the reaction of *p*-HgBzO with aquomethemoglobin 32-fold and with fluoromethemoglobin 74-fold, bringing these constants close to the values observed in deoxyhemoglobin A. The experiments and stereochemical arguments set out above leave little doubt that such low SH reactivities would not be observed unless IHP changed the quaternary structure of these derivatives from R to T. The twofold difference between the two factors arises from the small differences in tertiary struc-

TABLE VI: Rates of Reaction of Abnormal and Modified Hemoglobins with Sulfhydryl Reagents.

Ref		Reagent	-Order Rate Constant	Oxy	Deoxy	Oxy/Deoxy
a	Hb A	*p*-HgBzO	$k_2 \times 10^{-6}$ M^{-1} sec^{-1}	5.1 ± 0.4	0.07 ± 0.005	73
	Des-His-146	*p*-HgBzO	$k_2 \times 10^{-6}$ M^{-1} sec^{-1}	6.0 ± 0.5	2.5 ± 0.3	2.4
b	Hb A	4-PDS	k_1 sec^{-1}	12.8	1.75	7.3
	Hb Hiroshima	4-PDS	k_1 sec^{-1}	28.8	15.0	1.9
c	Hb A	*p*-HgBzO	k_1 sec^{-1}	~80	~1	~80
	Hb Kempsey	*p*-HgBzO	k_1 sec^{-1}	100	120	0.83

[a] 50 mM potassium phosphate buffer (pH 7.0); 5 μM heme and 15 μM *p*-HgBzO after mixing, 20°, 254 nm (Geraci and Sada, 1972). [b] 0.1 M phosphate (pH 7.0); 4-PDS = 4,4′-dithiobis(pyridine); 40 μM heme; 160 μM 4-PDS for oxyHb and 1.7 mM 4-PDS for deoxyHb. All concentrations before mixing (Imai *et al.*, 1972). [c] 50 mM phosphate (pH 7.0); 20 μM heme and 50 μM *p*-HgBzO before mixing, 20°, 255 nm (T. B. Bradley, H. F. Bunn, and Q. H. Gibson, unpublished data).

ture of the R forms referred to above. So far we have been unable to account for the 10-fold decrease in rate constant found on addition of IHP to azidomethemoglobin and for the 4.3-fold reduction of the second-order rate constant of 4,4′-bis-(dithiopyridine) after addition of IHP to oxyhemoglobin. These cannot be due to an R → T transition since the CD at 287 nm remains positive and the uv spectrum of Trp 37β remains unperturbed. However, the magnitude of the changes in reactivity point to changes in tertiary structure which should be investigated.

Reactions with Azide and Cyanide. If IHP converted aquomethemoglobin to the T structure, one would expect it to oppose the oxidation of deoxy- to methemoglobin and the replacement of the iron-bound water molecule by ligands that form predominantly low-spin complexes such as cyanide or azide. The redox potential of oxidation of deoxy- to methemoglobin is indeed raised as expected (Kilmartin, 1973) but the free-energy changes accompanying the replacement reactions in the presence and absence of IHP differ by only a few hundred calories. This seems paradoxical at first, but may be explained by the marked effect which IHP appears to have on the R structure. Indications of this effect are found in the changes which IHP produces in the aromatic regions of the electronic absorption spectra and of the proton magnetic resonance spectra of cyanomethemoglobin; in the reduction of the sulfhydryl reactivity of azidomethemoglobin; and in the rise of the paramagnetic susceptibility of azidomethemoglobin (see part III). If IHP does indeed distort the R structure substantially, then the equilibria of the replacement reactions (1) with, and (2) without, IHP might not be very different. This point is closely related to the sulfhydryl reactivities discussed above and clearly also needs further study.

$$[Hb^+H_2O]^R \underset{H_2O}{\overset{CN}{\rightleftharpoons}} [Hb^+CN^-]^R \qquad (1)$$

and

$$[Hb^+H_2O + IHP]^T \underset{H_2O}{\overset{CN}{\rightleftharpoons}} [Hb^+CN^- + IHP]^R \qquad (2)$$

R′ indicates a distorted R structure.

Cooperativity in the Oxidation and Reduction of Hemoglobin. We shall now attempt to interpret the cooperative effects exhibited in the oxidation and reduction of hemoglobin on the basis of a simple allosteric theory. The following observations are important. (1) In phosphate or borate buffers, Hill's constant in the oxidation–reduction equilibrium curves rises from about 1.3 at pH 6.1 to 2.6 at pH 9.4, and the free energy of heme–heme interaction rises from 300 cal at pH 6.3 to 1300 at pH 8.6 (Antonini *et al.*, 1964). (2) The release of Bohr protons is linear with oxidation at alkaline pH 8.0, but lags behind oxidation between pH 7.0 and 6.2 (Brunori *et al.*, 1965). (3) After reaction of the cysteines-93β with iodoacetamide or cystine, the value of Hill's constant no longer decreases with falling pH, but remains constant at n = 2 from pH 9.0 to 6.0. At pH 9.0 the potentials for half-oxidation ($E_{1/2}$) of SH-blocked hemoglobins are similar to those of the untreated protein, but at pH 6.0 $E_{1/2}$ for the SH-blocked ones becomes more negative (which means that they are more readily oxidized). Blocking of the SH groups also makes the release of Bohr protons proportional to oxidation at pH above 6.5 (Brunori *et al.*, 1967). On the other hand, reaction of *p*-HgBzO with free β chains does not affect $E_{1/2}$ at any pH (Banerjee and Cassoly, 1969). (4) Removal of the two C-terminal residues of either the α or the β chains, or both, abolishes cooperativity and makes $E_{1/2}$ more negative, *i.e.*, facilitates oxidation (Brunori *et al.*, 1964). (5) Hill's constant becomes less than unity in the reduction equilibrium of a methemoglobin–haptoglobin complex. The potentiometric curves can be accounted for by the presence of two oxidation–reduction systems present in equimolar amounts, which are thought to represent the α and β chains (Brunori *et al.*, 1968). (6) In 1 M glycine buffer, free α chains have a value of $E_{1/2}$ = +0.052 V which is invariant between pH 6.0 and 8.0 and is similar to that of myoglobin. Free β chains have a value of $E_{1/2}$ = +0.113 V between pH 6.0 and 6.6, after which $E_{1/2}$ decreases, to become equal to that of free α chains at pH 8.0 (Banerjee and Cassoly, 1969). (7) If methemoglobin and deoxyhemoglobin are mixed in solutions containing a dye to promote electron exchange, the β chains gain electrons at the expense of the α chains, However, contrary to what would be expected on the basis of Banerjee and Cassoly's results with free chains, the fraction α-ferric/β-ferric in tetramers remains the same at pH 6.1 aud 8.7 (MacQuarrie and Gibson, 1971). The inconsistency between the two sets of results can be explained if we examine the absorption spectra of the free methemoglobin chains published by Banerjee and Cassoly (1969). At pH 5.9 the spectrum of the free α subunits shows a small increase in the fraction of low-spin component, but that of the β subunits shows that a major fraction had been converted to hemichromes, six-coordinated low-spin ferriporphyrin compounds which represent a first step toward the denaturation of the protein (see part III, Discussion). At pH 9.5, on the other hand, the spectra of both α and β subunits were not very different from the spectrum of tetrameric alkaline methemoglobin. Therefore, the pH dependence of the redox potential of the β subunits may have been an artifact due to hemichrome formation at acid pH.

We now come to interpret these observations in allosteric terms. According to allosteric theory cooperative interactions are defined by three variables: $\alpha = [F]/K_R$, the ligand concentration divided by the microscopic equilibrium constant of the ligand with the protein in the R state; $c = K_R/K_T$, the ratio of the microscopic equilibrium constants of the ligand with the protein in the R and T states; and $L = [T]/[R]$, the equilibrium constant between the T and R states of the protein in the absence of ligand. On full saturation with ligand of a tetrameric protein this equilibrium constant becomes $L^* = Lc^4$. Any modification of hemoglobin is liable to change both L and c, but we shall assume here for the sake of simplicity that c remains constant at 0.01 for the oxygenation reaction and 0.1 for the oxidation reaction, so that L^* for aquomethemoglobin would always be 10^4 times greater than for oxyhemoglobin, because of the greater displacement of the proximal histidine from the plane of the porphyrin. This would give a maximum of $n = 3$ at $L = 10^4$ for oxygenation and of $n = 2$ at $L = 100$ for oxidation. A. Szabo (unpublished data) has estimated $c = 0.08$ for oxidation, giving a maximum of $n = 1.93$. Figure 13a illustrates the dependence of Hill's constant n and of the ligand affinities, $p(O_2)_{1/2}$ or $E_{1/2}$, on log L (Rubin and Changeux, 1966; Edelstein, 1971). n is related to log L by bell shaped curves, because cooperativity is at its maximum if the allosteric transition occurs halfway through the reaction, and drops if it occurs either too early or too late as a result of L being either too low or too high. $p(O_2)_{1/2}$ and $E_{1/2}$ both rise with increasing L because both oxidation and oxygenation are opposed by the constraints of the T state.

Factors that stabilize the T structure, such as hydrogen ions and organic phosphates, increase L, while those that

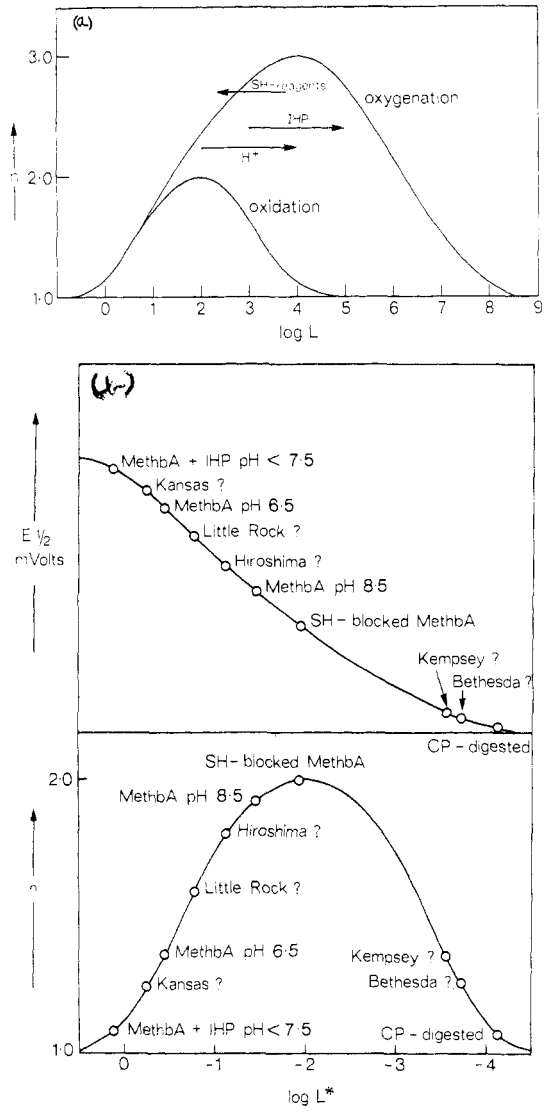

FIGURE 13: (a) Dependence of n on L for the oxygenation and oxidation reactions. (b) Suggested dependence of redox potential $E_{1/2}$ and cooperativity (n) of the oxidation reaction of various hemoglobin on the allosteric constant of their aquomet form L^*. The diagram is purely illustrative and some of the relationships implied, say between Hiroshima and Little Rock or Kempsey and Bethesda, may be opposite to those given here. (At pH 6.0 the values of $E_{1/2}$ for different derivatives are hemoglobin A + 0.175 V, SH-blocked hemoglobin A + 0.150 V, carboxypeptidase A digested hemoglobin A + 0.08 V (Brunori *et al.*, 1967; Antonini and Brunori, 1971).) In this diagram the same value of $c = 0.1$ is assumed to apply to all derivatives. This is almost certainly incorrect, and possible variations in c will have to be taken into account in more detailed studies.

destabilize the T structure, such as reagents blocking the reactive sulfhydryl groups, decrease L. Figure 13a shows that the same factor may influence the cooperativities of the oxygenation and oxidation reactions in opposite ways. Suppose that in the oxidoreduction equilibrium the top of the bell-shaped curve is reached at say pH 8.5 where n therefore assumes its maximum value. As the pH drops, the fraction of high-spin component rises and the salt bridges favoring the T structure gain in strength. The combination of the two effects raises L, which raises $E_{1/2}$ and causes n to be lowered to the right of the maximum in Figure 13a. In the oxygenation reaction, on the other hand, the same rise in L will bring n nearer to the top of the curve. IHP raises L much more strongly than H^+; as a result n in the oxidation reaction is reduced to unity. This prediction has been verified by Kilmartin (1973) and independently by J. G. Beetlestone (unpublished data). In the oxygenation reaction, on the other hand, IHP merely moves n over the peak of the curve and slightly down the right-hand slope (K. Imai, unpublished data).

We now consider why blocking of the sulfhydryl groups lowers the cooperativity of the oxygenation reaction but raises that of the oxidation reaction. Blocking of the reactive sulfhydryl groups opposes the salt bridge between His-146β and Asp-94β (Perutz, 1970). In deoxyhemoglobin His-146β has a pK of 8.1 (Kilmartin *et al.*, 1973). Above pH 8.1, therefore, most of the histidines are discharged even in the T structure, so that the salt bridges would be open no matter whether the sulfhydryl groups are blocked or not (Kilmartin *et al.*, 1973). On the other hand, as the pH drops below 8.1 blocking of the sulfhydryl groups increasingly destabilizes the T structure, thus lowering L. This affects n in the oxidoreduction and the oxygenation reaction in opposite ways. In the former, where n is normally displaced to the right of the maximum in Figure 13a, it lifts n up to the top of the curve. In the latter, where n is normally at the top of the curve, blocking of the sulfhydryl groups lowers n by displacing it to the left of the maximum. In both cases the ligand affinity must rise because of the rise in L. This explains why blocking of the reactive sulfhydryl groups raises the cooperativity of the oxidoreduction equilibrium, but lowers the cooperativity of the oxygen equilibrium.

Removal of the two C-terminal residues of either chain drastically reduces L and increases c, as it destabilizes the quaternary T structure (Perutz and Ten Eyck, 1971; Kilmartin and Hewitt, 1971). Consequently cooperativity in all reactions with heme ligands is abolished and the ligand affinity is increased. Haptoglobin combines with $\alpha\beta$ dimers and prevents their reaggregation into tetramers on deoxygenation or reduction (Hamaguchi, 1967; Kagiyama *et al.*, 1968). Since the $\alpha\beta$ dimer is noncooperative heme–heme interaction disappears (Kellett and Gutfreund, 1970; Anderson *et al.*, 1971; Kellett, 1971; Hewitt *et al.*, 1972).

The mechanism outlined above accounts qualitatively for all the observations and leads to several further predictions about the oxidoreduction equilibrium illustrated in Figure 13b. This equilibrium should be noncooperative at all pH's in hemoglobins with very high values of L, such as Kansas (G4(102)β-Asn → Thr) (Bonaventura and Riggs, 1968), or with very low values of L such as Kempsey (G1(99)β-Asp → Asn) (Reed *et al.*, 1968) or Bethesda (HC2(145)β-Tyr → His) (Hayashi *et al.*, 1971). Hill's constant at acid pH should be larger than normal in hemoglobins with moderately raised oxygen affinities, such as des-His-146β, Hiroshima (HC3(146)β-His → Asp) (Perutz *et al.*, 1971), or Little Rock (H21(143)β-His → Gln) (Bromberg *et al.*, 1974). It should also be larger than normal in the presence of azide or cyanide since these reagents should lower L.

Brunori *et al.* (1968) advanced a different interpretation for the pH dependence of the cooperativity. Since the reduction potentials of the α and β chains are markedly different at acid pH, but become equal at alkaline pH, they suggest that at acid pH the heterogeneity of the reaction lowers Hill's constant, while at alkaline pH the reaction becomes homogeneous, leading to a rise in n. This interpretation was disproved by MacQuarrie and Gibson (1971) when they found that the pH-dependent difference in the oxidoreduction potential of free α and β chains is absent in the tetramer. Furthermore, the interpretation of Brunori *et al.* fails to account for

the changes in ligand affinity and cooperativity which follow the blocking of the sulfhydryl groups, while allosteric theory provides a satisfactory explanation. Finally, we have seen that the published redox potentials of β subunits have become suspect because hemichromes formed during oxidation.

Tetramer–Dimer Equilibrium. Might some of the spectral changes have arisen as a result of the methemoglobin being partly dissociated into dimers in the absence of, and associated into tetramers in the presence of, IHP? This point was tested by comparing the uv difference spectra of aquo- and azidomethemoglobin in 15 μM (tetramer) solutions using a 1-cm light path and in 150 or 300 μM solutions using a 1-mm light path. The difference spectra were identical. We also compared the visible spectra of a 3.8 and a 38 μM solution of aquomethemoglobin, using the D = 0–0.2 slide-wire of the Cary spectrophotometer for the former and the D = 0–2.0 slide-wire for the latter. The two spectra were identical. Assuming the dissociation constant of methemoglobin to be similar to that of oxy, it should have been about 6 μM under our experimental conditions, so that there would be large differences in the degree of dissociation at the various concentrations used for these experiments. Our negative results indicate that the spectra of these derivatives in the quaternary R structure, without IHP, are closely similar to those of $\alpha\beta$ dimers.

Hensley *et al.* (1973) found that at pH 6.0 IHP reduced the tetramer–dimer dissociation constant of methemoglobin from 10 to 0.1 μM, while they estimate the dissociation constant of deoxyhemoglobin to be as low as 10^{-12} M. They argued that such a high dissociation constant was inconsistent with methemoglobin + IHP having the T structure, but in fact this is exactly what would be expected if the structure and properties of the system are considered. The T structure is "designed" for a displacement of the iron atoms relative to the plane of the porphyrin ring by probably more than 0.5 Å and the absence of a sixth ligand at the iron. In aquomethemoglobin that displacement is only 0.3 Å and a sixth ligand is present; in consequence the T state will be strained and its free energy will be higher than that of deoxyhemoglobin in the T state. This increase in free energy will be reflected in a higher dissociation constant. The strains can actually be seen in an electron density map of methemoglobin in the T state (Anderson, 1973). The point can be illustrated by another experiment in which we compared the effect of IHP on the deoxy and aquomet derivatives of NES-des-Arg-hemoglobin. IHP converts the former to the T state but not the latter. Evidently the additional free energy of stabilization for the T state which IHP provides through formation of extra salt bridges is sufficient to convert the deoxy form to the T state, even though it lacks the C-terminal salt bridges, but it is insufficient to have an appreciable effect on the allosteric equilibrium of the aquomet form where strains due to the "wrong" tertiary structure must also be overcome.

Conclusions

(1) IHP combines with human aquomethemoglobin in the ratio of 1 mol/mol of tetramer with a dissociation constant of 1.4 μM in 0.2 M Bis-Tris of pH 6.6, μ = 0.14 and 25°. The dissociation constant increases with rising pH and ionic strength. IHP appears to bind to the same site as in deoxyhemoglobin which means that it lies on the molecular dyad between the two β chains and forms salt bridges with Val-1, His-2 and -143, and Lys-82.

(2) Combination of aquo- and fluoromethemoglobin with IHP produces changes in the uv absorption spectrum of Tyr 42α and Trp 37β which are the same as those observed on deoxygenation of oxyhemoglobin A and on changing the quaternary structure of certain deoxyhemoglobins from R to T. IHP also induces a sharp negative peak at 287 nm in the CD spectrum of these high-spin methemoglobin derivatives. This peak was found to be specifically associated with the R → T transition in deoxyhemoglobin. IHP reduces the pseudo-first-order or the second-order rate constant of the reaction of aquo- and fluoromethemoglobins with *p*-HgBzO to a level normally characteristic for deoxyhemoglobin in the T state. It produces marked changes in the histidine and aromatic regions of the proton magnetic resonance spectrum. Finally, IHP causes the oxidoreduction equilibrium curve to become hyperbolic below pH 7.5 and markedly diminishes the intrinsic oxidation Bohr effect (*i.e.*, that part which is not due to changes in pK of basic groups reacting with IHP). These observations led us to conclude that IHP changes the quaternary structure of fluoro- and aquomethemoglobin from R to T.

(3) IHP also induces changes in the uv absorption spectrum of Tyr 42α, sulfhydryl reactivities, and nmr spectra of the low-spin azido- and cyanomethemoglobins, but these changes are weaker and are not associated with a change in the uv absorption spectrum of Trp 37β or a change of sign of the CD at 287 nm. We conclude tentatively that IHP slightly alters their R structure but we do not know yet in what way. IHP has little effect on the equilibrium between high- and low-spin heme ligands of methemoglobin.

(4) Our results suggest that solutions of high-spin methemoglobin derivatives contain molecules in the R and T states in a state dynamic equilibrium which varies with the spin equilibrium of the heme iron and the concentrations of hydrogen ions, organic phosphates, and neutral electrolytes. It is shown that such a scheme can account for the differences that have been observed between the cooperative effects of the oxygen and the oxidoreduction equilibria.

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