Quaternary structure has little influence on spin states in mixed-spin human methemoglobins

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Photosynthetic Oxygen Evolution: Changes in Magnetism of the Water-Oxidizing Enzyme

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Abstract: Changes in magnetic susceptibility produced by single-turnover flashes of light have been measured for the first time for four of the oxidation states, so-called S states, produced during oxygen evolution in Photosystem II (PSII) complexes of spinach. The data reveal new insights into the structure and bonding of the manganese cluster responsible for catalysis of water oxidation. In samples that have been dark adapted for 15 min or longer to favor population of the "resting" S_1 state, a train of six flashes increases the paramagnetism on flashes 1, 3, and 5, while no or small increases are observed on flashes 2, 4, and 6. Advancement to the S_1 state does not restore the dark level of S_1 magnetism. This is due to two effects: formation of net paramagnetism from O_2 release on the $S_4 \rightarrow S_0$ reaction (scavengeable by glucose oxidase) and a large increase in magnetism for the S_1(resting) $\rightarrow S_2$ reaction, which is not restored without dark readaptation. Comparison of these data with models proposed for the structure of the manganese site reveals that models in which oxidation of substrate water occurs prior to S_4 or oxidation of magnetically isolated Mn ions cannot account for the susceptibility changes observed. The large increase of 17 μ_B^2/PSII observed for the S_1(resting) $\rightarrow S_2$ oxidation is opposite in sign to the decrease in paramagnetism reported for oxidation of synthetic Mn dimers containing the μ_2-oxo-di-μ_2-carboxylato and di-μ_2-oxo-μ_2-carboxylato bridges undergoing the oxidation Mn_2(III,III) $\rightarrow$ Mn_2(III,IV). Consequently, these complexes must not provide complete structural representations of the bridging geometry or ligand types in the enzyme. The increase in susceptibility can be understood in terms of reduced antiferromagnetic coupling within a higher nuclearity cluster of three or four magnetically interacting Mn ions. This nuclearity is consistent with earlier EPR data.

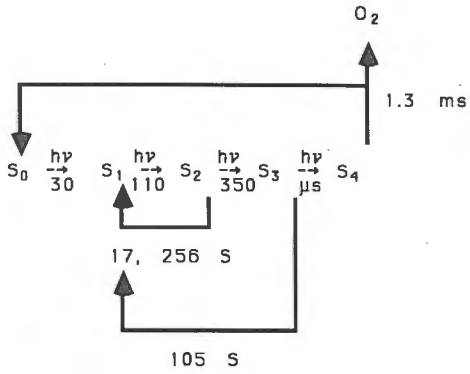
Photosynthesis in plants and algae produces the bulk of atmospheric oxygen through the oxidation of water. The oxygen gas so produced sustains all aerobic life as we know it on earth. A common biochemical apparatus appears to be responsible for catalyzing this fundamental process, from the most primitive cyanobacteria ("blue-green algae", dating back (2–3) × 10^9 years) to all known plants past and present. The biochemical apparatus has two parts, a photoactive reaction center membrane–protein complex termed Photosystem II (PSII) and a tightly associated water-oxidizing complex.[1-3] The reaction center protein complex binds the special chlorophylls and other chromophores needed for light absorption and charge separation. The primary photochemical reaction produces a strong oxidant that is not capable of directly catalyzing the four-electron oxidation of water (eq 1).

$$2H_2O \xrightarrow{\text{light + chloroplasts}} O_2 + 4e^- + 4H^+ \qquad (1)$$

Instead this is accomplished by a complex comprised of four manganese ions believed to be associated in the form of a ligand-bridged cluster of 2–4 ions that serve as a storehouse for the oxidizing equivalents and as the apparent substrate binding site. Essential roles are played also by chloride and calcium ions, but these have been less well characterized. The process appears to involve a concerted four-electron oxidation reaction, as suggested not only by the recovery in the yield of O_2 evolved every fourth flash in response to a train of short pulses of light[4,5] but also by flash-induced changes in manganese oxidation state as seen by EPR[6] and the exchangeability of isotopically labeled substrate water with free water in all oxidation states prior to the final state that releases O_2.[7]

There are five intermediate oxidation states in the catalytic cycle, called S states, S_0 through S_4, after Kok (Scheme I).[5] These are characterized by (1) an initial population of S_1/S_0 = 75%/25% in dark-adapted chloroplasts, (2) release of O_2 only on the $S_4 \rightarrow S_0$ reaction, and (3) a gradual loss of synchronization in the populations caused by random misses (10%) and double hits (5%) during flash photolysis. The half-times of the forward photochemical reactions and of the deactivation reactions in the dark are given in Scheme I.[8-10] Because S_1 is stable in the dark, while S_2 and S_3 decay to S_1 over tens of seconds, all S states except S_4 can be prepared readily with short pulses of light.

Scheme I



Knowledge about the structure and bonding of the manganese ions has come primarily from spectroscopic techniques and is far from complete. EPR spectroscopy on the S_2 state has established that the Mn spins are electronically coupled to give an $S = {}^1/_2$ ground state, while the other S states are EPR silent.[11] We therefore have sought to obtain information about the oxidation states and bonding of the manganese ions in *each* of the S states by measuring changes in their magnetic susceptibility. As a nonresonance technique, magnetic susceptibility can detect all

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Quaternary Structure Has Little Influence on Spin States in Mixed-Spin Human Methemoglobins†

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ABSTRACT: A key feature of the Perutz stereochemical model for cooperativity in hemoglobin is a strong coupling between quaternary structure and the spin state of the heme iron [Perutz, M. F. (1979) *Annu. Rev. Biochem. 48*, 327–386]. While this coupling appears to be present for carp azide methemoglobin, it should also be present for all liganded forms of human methemoglobin that exhibit a thermal high-spin ⇌ low-spin equilibrium. To test this hypothesis, we have measured the changes in spin equilibria upon conversion of six mixed-spin forms of human methemoglobin from the R (high-affinity) to the T (low-affinity) quaternary structure by addition of inositol hexaphosphate. These experiments were done with a sensitive superconducting magnetic susceptibility instrument on solutions at 20 °C in 20 mM maleate buffer, pH 6. The data show zero or small increases in high-spin content upon switching from R to T, changes that are equivalent to a relative stabilization of the high-spin form by only 0–300 cal mol^{-1} heme^{-1}. These changes in energy are far less than the 1200 cal mol^{-1} heme^{-1} predicted from the Perutz stereochemical model [Cho, K. C., & Hopfield, J. J. (1979) *Biochemistry 18*, 5826–5833]. That is, these data do not support a view that the low affinity of the T state is due to restraints acting through the iron–proximal histidine linkage. The mechanistic implications of these results and the differences between species and ferric ligands are discussed.

A continuing goal of hemoglobin research is to evaluate the extent to which cooperativity is due to quaternary structure dependent restraints acting through the iron–proximal histidine linkage, as postulated by Perutz (1972), and to determine where the cooperative energy is stored. According to the Perutz stereochemical model for cooperativity, the T[1] (low-affinity) quaternary structure opposes the motion of the iron and proximal histidine from a position with the iron about 0.6 Å on the proximal side of the porphyrin in the high-spin deoxy form to an in-plane position in the low-spin oxy form. A number of studies by a variety of spectroscopic techniques have looked for this so-called "tension on the heme" and tried to evaluate the strain and cooperative energy localized at the heme. These studies have shown that changes in quaternary structure produce only qualitatively small changes at the heme for ferrous deoxyhemoglobins. With these spectroscopic techniques it is difficult to evaluate the energy changes, but estimates generally give less than 300 cal mol^{-1} heme^{-1} of strain energy at the heme, i.e., only a small fraction of the 3.6 kcal mol^{-1} heme^{-1} of cooperative energy [see Hopfield (1973), Perutz (1979), and Rousseau et al. (1984)]. However, the strain and energy should primarily be located in the *weakest* bonds (Hopfield, 1973). Thus, the results on ferrous hemoglobins are consistent with the Perutz mechanism if the ferrous heme is relatively "stiff" and the strain and energy reside in weaker bonds elsewhere, e.g., at the subunit interface.

In contrast to the ferrous case, the ferric (met) hemoglobins offer the opportunity of examining the influence of quaternary structure on a very "flexible" heme. In so-called "mixed-spin" methemoglobins, the high-spin $S = {}^5/_2$ and low-spin $S = {}^1/_2$ states lie very close in energy, so that a thermal spin-state equilibrium exists with both states populated by thermal excitation at room temperature. This spin-state equilibrium gives an additional degree of freedom for accommodating strain at the heme. Furthermore, since the changes in heme structure for a transition from high-spin to low-spin ferric are thought to be similar to (but less than) those in going from deoxy to liganded in the ferrous case, the Perutz model predicts that a switch in quaternary structure from the R (high-affinity) to the T form will destabilize the low-spin state and shift the spin equilibrium strongly toward the high-spin state. By measuring this shift in spin equilibrium, the changes in energy can be evaluated.

Therefore, the mixed-spin methemoglobins provide both a "soft" heme where strain energy can be stored and the opportunity to directly measure this energy by measuring changes in the spin equilibrium. To quantitatively describe this phenomenon, we can define an equilibrium constant between high-spin and low-spin states, K(spin), given by

$$K(\text{spin}) = [\text{high spin}]/[\text{low spin}] = 3 \exp[-\Delta E/(kT)] \quad (1)$$

where ΔE is the difference in energy between high-spin and low-spin states and the factor of 3 accounts for the greater spin degeneracy of the high-spin state. Measuring K(spin) also determines $\Delta G(\text{spin}) = -RT \ln K(\text{spin})$. If we can measure K(spin) for both the R and T quaternary structures, then the degree to which the T structure destabilizes the low-spin state is given by

$$\Delta\Delta G(\text{spin}) = RT \ln \{K(\text{spin,T})/K(\text{spin,R})\} \quad (2)$$

The Perutz model predicts that $\Delta\Delta G(\text{spin}) \sim 1.2$ kcal/mol (Hopfield, 1977; Hopfield & Cho, 1979).

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[1] Abbreviations: Hb, hemoglobin; metHb(s), methemoglobin(s) with various ferric ligands; T, low-affinity quaternary structure of Hb, e.g., that of deoxyhemoglobin; R, high-affinity quaternary structure of Hb, e.g., that of oxyhemoglobin; IHP, inositol hexaphosphate; NES, *N*-ethylsuccinimide; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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There have been several studies of the influence of changes in quaternary structure on the spin equilibria of metHbs, both by direct magnetic measurements and by indirect spectroscopic evidence. In nearly all of these studies, inositol hexaphosphate (IHP), a powerful allosteric effector that binds preferentially to the T structure, has been used to switch the quaternary structure from R to T. The results for human metHbs are conflicting and have not been calibrated in terms of energy but generally indicate that addition of IHP produces only small shifts of the spin equilibria toward the high-spin state (Perutz et al., 1974, 1978; Gupta & Mildvan, 1975; Messana et al., 1978; Noble et al., 1983). More attention has been focused on carp metHbs, which show larger effects of IHP. For carp azide metHb, the data are consistent with a value of $\sim$1 kcal/mol for $\Delta\Delta G$(spin) (Perutz et al., 1978; Messana et al., 1978; Noble et al., 1983).

These changes in magnetism of carp metHbs have been interpreted as strong evidence for the Perutz stereochemical model of cooperativity (Perutz, 1979). However, if this is indeed the mechanism of cooperativity, then similar values of $\Delta\Delta G$(spin) should be observed for human as well as carp metHbs, and for *all* mixed-spin forms that can be switched from R to T. The aim of this study is therefore to provide a much more stringent test of the Perutz model by determining $\Delta\Delta G$(spin) for human metHb with a wide range of ligands. This has been done by measuring the effect of IHP on the magnetic susceptibility of solutions of metHbs at 20 °C with a highly sensitive superconducting magnetic susceptometer. For purposes of comparison, we have also undertaken a limited study of the metHbs from carp, hemoglobin Kansas, and NES-desArg human hemoglobin. The results do not support the idea that restraints on the iron–proximal histidine linkage are the principal mechanism for regulating oxygen affinity in human hemoglobin.

Materials and Methods

Magnetic susceptibility measurements of metHb solutions at 20 °C were made with a superconducting magnetic susceptometer constructed in our laboratory. Volume susceptibilities were measured by the procedures described previously (Philo & Fairbank, 1977; Philo et al., 1984) at an applied field of 0.23 T. The precision of this instrument is typically ±0.001% of the diamagnetism of water, but for protein solutions the reproducibility is limited by protein purity, denaturation, and errors in determining the protein concentration to about ±0.01% of the diamagnetism of water. In each case, the diamagnetism of the buffer alone was measured, both with and without added IHP. The susceptibility of each protein sample was monitored for 1 h or more to check for time-dependent behavior. The raw data for the protein and buffer solutions were converted to the molar susceptibility of the protein by using eq 1 of Philo et al. (1984). These molar susceptibilities were then converted to molar paramagnetism by subtracting a diamagnetic reference derived from measurements of apohemoglobin and metal-free protoporphyrin IX. This diamagnetic reference is equivalent to using either oxyhemoglobin or (carbonmonoxy)hemoglobin as the reference, since we have recently shown them to be diamagnetic under these conditions (Philo et al., 1984).

Human oxyhemoglobin A, unfractionated with respect to minor components, was prepared by the ammonium sulfate precipitation method (Rossi-Fanelli et al., 1961) followed by removal of the sulfate and organic phosphates on a Sephadex G-25 column equilibrated with 0.05 M Tris-HCl–0.1 mM EDTA, pH 7.5 buffer. Oxyhemoglobin Kansas [Asn-β102(G4) → Thr] was prepared following the procedures of Williams & Tsay (1973) for preparation of the hemolysate and those of Bonaventura & Riggs (1968) for chromatographic purification of the Hb Kansas component. NES-des-Arg-hemoglobin, which has Arg-α141(HC3) enzymatically removed and *N*-ethylsuccinimide bound at Cys-β93(F9), was a gift from S. Simon. These oxyhemoglobins were either immediately converted to metHb or frozen and stored under liquid nitrogen. Oxyhemoglobins were converted to metHb by adding a 2–3-fold molar excess of potassium ferricyanide, followed by removal of the ferricyanide on a Sephadex G-25 column equilibrated with 20 mM maleate buffer, pH 6. Carp metHb was prepared from frozen red cells following the procedures of Cerdonio et al. (1980). These metHb stock solutions were concentrated further either with an Amicon pressure concentrator or with a Millipore CX-10 immersible ultrafiltration unit, centrifuged to remove any precipitated material, and stored in the cold. The various liganded metHb derivatives were made by adding concentrated solutions of the appropriate reagent-grade salt adjusted to the desired pH. Matched samples with and without IHP were made by adding either concentrated buffered IHP or an equivalent amount of buffer. The choice of a maleate buffer system and a low pH ($\sim$6) was based on our desire to maximize IHP binding and conversion to the T state, and also to avoid effects of chloride ion. Previous kinetic studies of human metHb have shown chloride-dependent effects (Dreyer & Ilgenfritz, 1979), and the properties of carp hemoglobin are known to be particularly chloride sensitive (Cerdonio et al., 1983).

Concentrations of the samples were determined by conversion to the cyanomet form and using the standard extinction coefficient of 11.0 mM^{-1} cm^{-1} at 540 nm (Tentori & Salvati, 1981). In earlier measurements the samples were diluted before the absorption spectra were recorded; in later experiments the spectra were recorded without dilution in a 1-mm path-length cell to avoid any errors from dilution. The visible absorption spectra of each sample was recorded, and ±IHP difference spectra were formed by digital subtraction. These ±IHP difference spectra were similar to those reported previously (Perutz et al., 1974, 1978).

Results

We have examined the effects of IHP on the paramagnetism of a wide variety of liganded forms of human metHb. The results are summarized in Table I. With none of these ligands do we find large changes in the spin-state equilibrium upon addition of IHP, and indeed in some cases, the data show no change at all. Furthermore, there is no consistent pattern between the size of the IHP-induced change and the average position of the spin equilibrium for that ligand. Sufficient IHP was added to give 0.4–1.5 mM free IHP, which ensures complete saturation with IHP under our sample conditions.

The uncertainties shown for the molar paramagnetism are estimates based on contributions from a number of sources of error. These include the instrumental uncertainty in the data for both the protein solution and the buffer, the uncertainty in diamagnetic reference, and a term reflecting possible degradation of the protein, which we derive from the observed stability of the solution susceptibility over 1 h. Finally, in many cases the largest source of uncertainty is due to errors in determining the protein concentration. We estimate this error as ±0.5% when no dilution was involved and ±1.5% when a dilution was made.

The data for two of the ligands merit some special discussion. In addition to binding to the heme, cyanate also carbamylates the terminal valines of the α and β chains (Garner et al., 1975). Thus, these data are for cyanate metHb, which

Table I: Effect of IHP on Paramagnetism of Methemoglobins[a]

Hb type	ligand	[ligand] (mM)	[IHP] (mM)	pH	[heme] (mM)	quaternary structure	χ_m ($\times 10^6$ emu/mol)	moment (μ_B)	% high spin	$\Delta\Delta G$(spin) (cal/mol)
human	F^-	500		6.0	2.44	R	14390 ± 200	5.81	96	
		500	1	6.0	2.44	T	14110 ± 200	5.75	94	−200 ± 200
		500		6.6	1.90	R	14210 ± 200	5.77	95	
		500	1	6.0	1.90	T	14340 ± 200	5.80	96	120 ± 200
	H_2O			6.0	1.2–2.6	R	13300 ± 80[b]	5.58	88	
			0.75	6.0	1.65	T	13870 ± 200	5.70	92	280 ± 120
			1	6.0	1.49	T	13940 ± 100	5.72	93	320 ± 70
	CH_3COO^-	305		6.0	1.63	R	13060 ± 200	5.53	86	
		305	1	6.0	1.63	T	13190 ± 200	5.56	87	50 ± 110
		1000		6.2	0.75	R	13270 ± 200	5.58	87	
		1000	1	6.2	0.75	T	13190 ± 200	5.56	87	−40 ± 110
	$HCOO^-$	500		6.0	1.63	R	12830 ± 200	5.48	84	
		500	1	6.0	1.63	T	13140 ± 200	5.55	87	100 ± 100
		1000		6.0	0.75	R	12400 ± 200	5.39	81	
		1000	1	6.0	0.75	T	12260 ± 200	5.36	80	−40 ± 80
	CNO^-	18		6.0[c]	1.90	R	12400 ± 300	5.39	81	
		18	2	6.0	1.10	T	12870 ± 300	5.49	84	140 ± 130
		100		6.1[c]	1.90	R	12070 ± 200	5.28	77	
		100	1	6.1	1.90	T	12080 ± 200	5.28	77	0 ± 70
		100		6.1	0.75	R	11760 ± 200	5.21	75	
		100	1	6.1	0.75	T	12240 ± 200	5.36	80	120 ± 70
	SCN^-	100		6.0	2.29	R	10350 ± 200	4.93	65	
		100	2	6.0	2.29	T	10930 ± 200	5.06	70	120 ± 60
		100		6.1	1.90	R	12010 ± 100	5.31	78	
		100	1	6.1	1.90	T	12870 ± 100	5.49	84	250 ± 40
		100		6.1	1.49	R	9910 ± 100	4.82	62	
		100	1	6.1	1.49	T	11470 ± 100	5.19	74	310 ± 30
	N_3^-	18		6.0	1.90	R	3240 ± 200	2.76	12	
		18	2	6.0	1.90	25% T?	3330 ± 200	2.79	12	40 ± 130
		20		6.1	1.87	R	3320 ± 200	2.79	12	
		20	1	6.1	1.87	25% T?	3530 ± 200	2.88	14	80 ± 130
		20		6.1	1.49	R	3420 ± 100	2.83	13	
		20	1	6.1	1.49	25% T?	3600 ± 100	2.91	14	70 ± 55
	CN^-	3		6.0	1.90	R	2680 ± 200	2.51	(0)[d]	
		3	2	6.0	1.90	R	2360 ± 200	2.35	(0)	
		5		6.0	1.90	R	2370 ± 200	2.36	(0)	
		5	1	6.0	1.90	R	2490 ± 200	2.42	(0)	
		5		6.1	1.54	R	2490 ± 100	2.42	(0)	
		5	1	6.1	1.54	R	2640 ± 100	2.49	(0)	
		4.8		6.1	3.09	R	2500 ± 50	2.42	(0)	
NES-des-Arg	SCN^-	100		6.1	0.59	R	12800 ± 300	5.48	84	
		100	0.5	6.1	0.59	R	11830 ± 300	5.27	77	−270 ± 140
Kansas	SCN^-	100		6.1	0.28	T	14100 ± 400	5.75	94	
		100	0.5	6.1	0.28	T	13900 ± 400	5.71	92	−130 ± 300
carp	H_2O			6.3	2.41	R	12210 ± 100	5.34	79	
			2	6.1	2.41	T	12870 ± 100	5.49	84	200 ± 40
	SCN^-	100		6.9	2.06	R	11370 ± 100	5.16	73	
		100	1	6.9	2.06	T	12820 ± 100	5.48	84	390 ± 40
		100		6.5	2.06	R and T?	12320 ± 100	5.37	80	
		100	1	6.5	2.06	T	12900 ± 100	5.50	85	400 ± 40[e]
	N_3^-	20		5.9	2.06	R and T?	4530 ± 70	3.26	21	
		20		6.8	2.77	R	3880 ± 50	3.02	16	
		20	1	6.0	2.06	T	7130 ± 70	4.09	41	735 ± 20

[a] 20 mM maleate buffer, 20 °C. [b] Average of eight samples. [c] Initial value, rises with time. [d] Assumed to be zero. [e] $\Delta\Delta G$(spin) with respect to pH 6.9, no IHP.

is at least partially carbamylated. Furthermore, the pH of these solutions rises with time. Because of these effects, the buffers and hemoglobin solutions were made fresh and measured within ~1 h, and the data are for the initial values. Nonetheless we cannot state with any certainty the degree of carbamylation or the exact pH for these data, and the higher paramagnetism for the data at lower cyanate concentration probably reflects differences due to these effects. We have also observed significant variation between different preparations of thiocyanate metHb, differences that do not extend to samples with other ligands derived from the metHb stock. These differences do not seem to be simply denaturation or degradation, since these samples are quite stable. The differences in paramagnetism between samples are correlated with differences in the absorption spectra. On the other hand, the changes in both paramagnetism and absorption spectra induced by IHP are much more similar for the different preparations. We should also mention that we measured nitrite metHb but were unable to interpret the results. Both the paramagnetism and the visible absorption spectra for this derivative are quite time dependent, at least under our sample conditions. We believe this time dependence reults from chemical alteration of the porphyrin group by the nitrite, forming a new green compound. The kinetics of this process is altered by IHP, which further complicates any attempt to interpret IHP-induced differences.

The assignments of quaternary structures shown in Table I have been made by others (Perutz et al., 1978; Neya et al., 1983) and are based largely on spectroscopic criteria. There are two cases where the assignment is unclear. For the cyanate

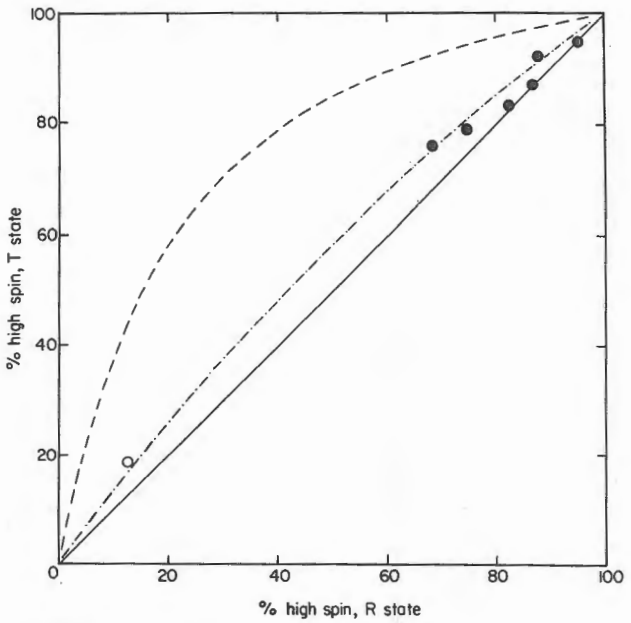

FIGURE 1: Percentage of high-spin state for the R vs. T conformations. The full curves illustrate the theoretical behavior for values of $\Delta\Delta G$(spin) of 1000 (--), 200 (-·-), and 0 (—) cal/mol, respectively. The solid circles represent averages of the data for human fluoro-, aquo-, formato-, acetato-, and cyanato-, and thiocyanatomethemoglobin from Table I, assuming that IHP has produced a complete conversion from R to T. The open circle shows an extrapolation of the data for human azidomethemoglobin, assuming that IHP produced a 25% conversion to the T state (Neya et al., 1983). The experimental uncertainty in these data corresponds approximately to the size of the data points.

metHb samples, the binding of IHP and the conversion to the T structure are inhibited by the carbamylation. For azide metHb there is some disagreement about the assignment, with Perutz et al. (1978) assigning this as an R-state species and Neya et al. (1983) suggesting about a 25% conversion to the T structure.

The last two columns in Table I show the free-energy difference between the low-spin and high-spin states derived from these data and the difference in this free energy between pairs of samples with and without added IHP. For some of the ligands that bind very weakly (e.g., acetate), it is difficult to be sure that the protein is 100% saturated with ligand, especially when IHP is added. However, the paramagnetism of these species is not very different than that of the aquomet form, so a small percentage of aquometHb would hardly affect the data. The paramagnetism of formate metHb does seem to be lower at the higher ligand concentration, but this is more likely to be due to an effect of ionic strength than to a difference in ligand saturation. (Note that we have verified that IHP binding is complete even under these high ionic strength conditions.) No value of $\Delta\Delta G$(spin) is shown for the cyanide derivative because it assumed to be completely low spin.

How do these data compare to the results expected if a switch to the T structure lowers the relative energy of the high-spin state by a constant amount? Figure 1 shows the theoretical values of high-spin content for the R and T states for $\Delta\Delta G$(spin) values of 1000, 200, and 0 cal/mol. Also plotted in Figure 1 are the data for human metHbs, assuming that IHP has produced a 100% switch from R to T for fluoro-, aquo-, formato-, acetato-, cyanato-, and thiocyanatometHb and a 25% switch to T for azidometHb. Clearly, the data do not behave as expected for any constant value of $\Delta\Delta G$(spin) and are entirely incompatible with a $\Delta\Delta G$(spin) of the order of 1 kcal/mol.

We have also briefly examined two human hemoglobins with allosteric properties differing from Hb A. These data were an attempt to assess the effect of IHP on the spin equilibrium of a predominantly high-spin form when there is no change in quaternary structure. Hemoglobin Kansas has its allosteric equilibrium tipped strongly toward the T state (Ogawa et al., 1972). We therefore believe that thiocyanate metHb Kansas is in the T structure both with and without IHP, and we find no IHP-induced change in its magnetism. NES-des-Arg-hemoglobin has its allosteric equilibrium tipped toward the R state (Kilmartin et al., 1975), so we believe thiocyanate NES-desArg-metHb is in the R structure both with and without IHP. In this case, the addition of IHP seems to produce a decrease in high-spin content. The very small ±IHP absorption difference spectra we observe for both these metHbs are also consistent with no change in quaternary structure.

Since these changes in magnetism for human metHbs are so much smaller than those reported for carp metHbs (Messana et al., 1978; Noble et al., 1983), we have also undertaken a limited study of carp metHbs for purposes of comparison. These data are also included in Table I. In previous studies of carp metHbs the largest effects of quaternary structure on spin-state equilibria have been reported for the azide derivative. As in previous studies, we find large variations in the paramagnetism of carp azide metHb with pH and IHP. At low pH, both the carp azide and thiocyanate metHb are likely to be at least partially in the T state. Therefore, we have evaluated the $\Delta\Delta G$(spin) between a higher pH without IHP and at low pH with IHP. The changes for carp aquometHb and SCN-metHb seem to be significantly smaller than those for the azide form.

Discussion

Before discussing the implications of these results for an understanding of structure/function relations in hemoglobin, it is first important to discuss how these results compare to earlier studies and the possible sources of error in the computed energies. As mentioned in the introduction, there have been several earlier studies of this same problem, with a wide variation in the results. The effect of IHP on the magnetism of the fluoride, formate, acetate, and cyanate forms of human metHb have not been previously reported. Our data for the human cyanate and thiocyanate derivatives are in fairly good agreement with the 0% and 11% changes in paramagnetism, respectively, observed by the NMR method by Perutz et al. (1978). Our data for human and carp azide metHb are in excellent agreement with the recent magnetic studies of Noble et al. (1983) and also agree fairly well with the values inferred indirectly from infrared absorption spectra by Perutz et al. (1978).

The largest discrepancies between the different studies are for aquomethemoglobin. We find a small but non-zero increase in high-spin content upon addition of IHP to human aquometHb. Much larger changes were originally reported by Perutz et al. (1974), but no change was reported in two separate results by the NMR method (Perutz et al., 1978; Gupta & Mildvan, 1975). The largest discrepancy is with the data of Noble et al. (1983), who report that IHP converts both the human and carp aquometHb completely to the high-spin state. We believe these latter results must be in error, since we are aware of no other magnetic or spectroscopic study that shows human aquometHb to be completely high-spin, even in the presence of IHP. Indeed, while we would be the last ones to suggest that optical spectra can be used to quantitatively measure spin equilibria, the visible absorption spectrum of aquometHb + IHP clearly shows the presence of a substantial

low-spin component, as does the resonance Raman spectrum (Rousseau et al., 1980).

Also, with regard to the interpretation of changes in spin equilibria in general, and for aquometHb in particular, we would like to correct some erroneous statements in the literature that may cause confusion here. In their 1978 papers, Perutz et al. and Messana et al. noted that their susceptibility data for human aquometHb at room temperature corresponded to 75% high spin, i.e., to the case $|\Delta E| \ll kT$ in eq 1, giving rise to a temperature-independent spin equilibrium with the 3-fold ratio of high-spin to low-spin components being determined only by the spin degeneracy. When ΔE is positive and comparable to kT, an increase in temperature will increase the high-spin fraction due to increased thermal excitation. But if ΔE is positive and small compared to kT, further thermal excitation cannot increase the high-spin population. Therefore, they argued that for aquometHb adding IHP should not be expected to increase the high-spin content. We believe this argument is incorrect. There is nothing unique or singular about the case for 75% high spin that forces it to always remain 75%. Their analysis essentially ignores the case when $\Delta E < 0$ and confuses the inability to increase the high-spin content by changing the *temperature* with the inability to change the high-spin content by changing the *structure*. We can see no theoretical reason why ΔE must always be zero for aquometHb or any other metHb that happens to have $\Delta E \sim 0$. Moreover, the high-spin fraction of aquometHb near room temperature is actually much larger than 75%, as shown by more recent data from the same laboratory (Noble et al., 1983) as well as this study.

One of the principal difficulties in translating these magnetic data into values for $\Delta\Delta G$(spin) is knowing precisely the endpoint values for the paramagnetism of a completely high-spin or low-spin form. For derivatives with substantial populations of both states, the energies are not very sensitive to the exact value used. However, for derivatives that are nearly completely in one spin state a small change in the assumed end point can lead to a large change in the calculated equilibrium constant and energy. For a completely high-spin state we can be quite certain that the magnetic moment should be 5.92 μ_B (the effect of zero-field splitting is negligible at these temperatures). We should note that fluoride metHb is *not* a suitable high-spin standard since both magnetic (Scheler et al., 1957; Havemann & Haberdizl, 1958; Beetlestone & Goerge, 1964) and resonance Raman data (Rousseau et al., 1980; Scholler & Hoffman, 1979) show that it is not completely high spin at room temperature. The low-spin standard is more troublesome. Cyanide metHb is believed to be completely low spin because its magnetic moment is temperature invariant (Iizuka & Kotani, 1969) and it shows no high-spin EPR signals, but its magnetic moment contains an unusually large contribution from unquenched orbital angular momentum. Thus, it is unlikely that the moment of the low-spin fraction of other derivatives is as large as that of cyanometHb. We have tried to use EPR data as a guide for the correct values. The sum of the square of the principal g values for the low-spin EPR spectrum sets a lower limit for the true magnetic moment [second-order terms can raise this value; see Harris (1966)]. The EPR data for a number of low-spin forms of metHb and metmyoglobin all imply values for the low-spin moment of 2.00 $\pm$ 0.02 μ_B (Griffith, 1957; Hori, 1971; Scholler & Hoffman, 1979). Therefore, we have chosen to assign a value of 2.00 μ_B to the low-spin fraction in this work.

Significance for Hb Structure/Function. The key result of this study is that these direct magnetic measurements fail to show evidence for the strong linkage between spin state and quaternary structure in human metHbs that is predicted by the Perutz stereochemical model. What is the significance of this for our understanding of the mechanism of cooperativity? First, it is worth reviewing the evidence that such a linkage should exist. X-ray crystallographic analysis has shown the binding of oxygen leads to a $\sim$0.5-Å movement of the heme iron from an out-of-plane position in oxyhemoglobin, a movement that is transmitted through the proximal histidine to the F helix. Perutz (1972) postulated that this motion served two functions in the cooperative process: (1) to serve as the "trigger" to initiate the change in quaternary structure and (2) to provide a means by which the protein can regulate oxygen affinity by exerting "tension" or restraints through the iron–proximal histidine bond. The connection between the spin state of the iron and this motion of the iron is based on structural studies of both heme proteins and model compounds which show that, in general, high-spin ($S = 2$ or $^5/_2$) ferrous or ferric hemes have the iron out of the heme plane and relatively long Fe–pyrrole nitrogen bonds, while low-spin ($S = 0$ or $^1/_2$) hemes have shorter Fe–N bonds and the iron lies in or very near the heme plane. For purposes of this discussion, we wish to emphasize that we are not assuming that changes in spin state necessarily imply motion of the iron relative to the heme plane.

The correlation between spin states and heme stereochemistry suggests that for ferric hemoglobins a transition from a high-spin to a low-spin state should produce structural changes at the proximal side of the heme that are similar to, but less than, the structural changes produced by oxygen binding in the ferrous form. Therefore, if the T quaternary structure constrains motion of the iron and proximal histidine toward the porphyrin, then a change from the R to the T structure should favor the high-spin state of the ferric forms, an effect expressed quantitatively as $\Delta\Delta G$(spin).

What degree of coupling is energetically significant? If indeed the iron–proximal histidine linkage is the main coordinate through which the protein modulates oxygen affinity, then the value of $\Delta\Delta G$(spin) should represent a substantial fraction of the $\sim$3.6 kcal mol^{-1} heme^{-1} energy of heme–heme interaction in oxygen binding. The value of $\Delta\Delta G$(spin) may be regarded as the product of the difference in proximal histidine–porphyrin separation between high-spin and low-spin states and the average force exerted by the protein along this coordinate. On the basis of the relative motions for the ferrous vs. ferric crystal structures, Cho & Hopfield (1979) estimated that the Perutz mechanism should give 1.2 kcal/mol for $\Delta\Delta G$(spin).

Some recent crystallographic and resonance Raman studies of the regulation of oxygen affinity have emphasized the possible role of the tilt of the proximal histidine and steric repulsion between the imidazole carbon and pyrrole nitrogen (Baldwin & Chothia, 1979; Friedman et al., 1983), rather than tension transmitted through the iron–proximal histidine linkage as originally proposed by Perutz (1972). It is important to note that for the purposes of this discussion such mechanisms are equivalent, since the tilt of the proximal histidine only manifests itself during a change from a non planar to a planar heme. Thus, a strong coupling between spin state and quaternary structure is also expected even if tilt of the proximal histidine is the mechanism for generating constraints opposing oxygen binding.

These new results for human metHbs give values of $\Delta\Delta G$(spin) of only 0–300 cal/mol, i.e., *not* significant with respect to the heme–heme interaction energy. Thus, these results fail

to support mechanisms in which oxygen affinity is regulated primarily by constraints opposing motion of the iron and proximal histidine toward the porphyrin plane when oxygen is bound. Our results and conclusions contrast markedly with those of Perutz and co-workers (Perutz et al., 1974, 1978), who have argued that the larger magnetic changes reported for carp metHbs are strong evidence for significant "tension on the heme". While the differing magnetic behavior of carp and human metHbs in both relevant and interesting, certainly if the motion of the iron–proximal histidine linkage plays a central role in cooperativity for human as well as for carp hemoglobin, then we should observe significant values for $\Delta\Delta G$(spin) for human metHb, which we do not.

While our data do not provide support for the regulation of oxygen affinity through motion of the iron–proximal histidine linkage, these results also do not necessarily contradict such a mechanism. There are several possible ways in which the existence of a large, quaternary structure dependent force acting along the iron–porphyrin separation coordinate can be reconciled with small values for $\Delta\Delta G$(spin). However, if our data are correct, such a reconciliation can only be achieved by discarding much of the evidence that such forces exist.

Let us examine the possibilities in more detail. One possibility that we cannot absolutely exclude is, of course, that our data are incorrect. However, it is important to note that this is not the first time that only small changes in spin populations have been seen upon IHP addition to human metHbs. The significance of these earlier data (Perutz et al., 1978) was not recognized because the NMR data could not be translated into changes in energy and because the aquometHb data were misinterpreted, as discussed previously.

Another possible explanation for the small values of $\Delta\Delta G$(spin) might be that IHP does not truly convert these human metHbs to the T state. Certainly it is possible that some of these derivatives are not switching from 100% R state without IHP to 100% T state with IHP, which would cause us to underestimate $\Delta\Delta G$(spin). However, such an effect should vary systematically with the average spin-state distribution produced by a given ligand. That is, in a plot such as Figure 1 one would expect the data for predominantly high-spin derivatives to indicate a significant $\Delta\Delta G$(spin), while as the low-spin content increased and the transition to T is less favored the data would move toward the diagonal. This is not the pattern we observe. Moreover, it is unlikely such an effect could reduce all the $\Delta\Delta G$(spin) values so far below the $\sim$1 kcal/mol expected from the Perutz model. There has been some question as to whether metHbs ever adopt a true T structure in solution (Hensley et al., 1975), although there is both crystallographic and spectroscopic evidence that they do [see Perutz (1979)]. Also, studies of oxygen binding to a single ferrous heme per tetramer formed by pulse radiolysis of metHb strongly suggest that a true T state exists prior to the radiolysis pulse when IHP is present (Chevion et al., 1979). Furthermore, if the assignments of quaternary structures are in fact incorrect, then studies of metHbs must be regarded as largely irrelevant for understanding the mechanism of cooperativity. If we abandon the data derived from metHb derivatives, virtually the only remaining direct evidence for tension on the heme is the reported rupture of the iron–proximal histidine bonds in the α chains of T-state nitrosylhemoglobin.

Another possibility that would seriously affect the interpretation of these data is that IHP might induce a specific tertiary effect forcing the β chains toward the low-spin state, which could then mask an overall shift toward high spin produced by the change in quaternary structure. If this were true, IHP should produce a decrease in paramagnetism of human azide metHb, which is not observed. Studies of the absorption spectral changes induced by IHP binding to human aquometHb were interpreted as showing an *increase* in high-spin content of the β chain, with no change in the α chain (Olson, 1976). Our data for metHbs NES-des-Arg and Kansas were also an attempt to assess the effect of IHP when no change of quaternary structure occurs. The data for SCN-metHb NES-des-Arg do show a decrease in paramagnetism when IHP binds, while those for SCN-metHb Kansas do not. These measurements are single experiments at very low concentrations (due to the limited material we had available), and we cannot therefore place a great deal of reliance on them. This question may deserve further investigation, but the available data do not suggest that IHP has a specific tertiary effect that masks the quaternary effects. Moreover, if it is argued that such effects are important, then all data that involve the use of IHP are brought into question, which again would eliminate most of the data supporting the existence of a significant tension on the heme.

Small values for $\Delta\Delta G$(spin) would also be observed if the difference in heme geometry between high-spin and low-spin states is much smaller than expected. That is, if the change in heme geometry is very small, even if the T state generates large forces along the iron–proximal histidine linkage, these forces will have little effect on the spin-state equilibrium. Do changes in spin state actually change the structure of the ferric hemes? The idea that a high-spin iron must necessarily lie out of the porphyrin plane was strongly influenced by crystallographic studies of metmyoglobin and metHb and of *five-coordinate* iron porphyrins, all of which showed the iron to be 0.3–0.5 Å out of plane. Refinement of the aquometHb structure has given substantially lower Fe–porphyrin separations: 0.07 Å in the α chain; 0.21 Å in the β chain (Ladner et al., 1977). Moreover, several examples are now known of six-coordinate high-spin ferric porphyrins with a *planar* iron and an expanded porphyrin core (Scheidt & Gouterman, 1983). Resonance Raman spectra of high-spin metHbs indicate that they also have expanded porphyrin cores (Scholler & Hoffman, 1979; Spiro et al., 1979). Thus it might be argued that there is almost no motion of the iron for changes in spin state in metHbs. However, changes in the length of the iron–proximal histidine bond are as important for this discussion as motion of the iron itself. The high-spin state should give a longer Fe–N_ϵ bond as well as longer Fe–$N_{pyrrole}$ bonds. Indeed, crystallographic data on a six-coordinate 3-chloropyridine adduct of octaethylporphyrin that is in a thermal spin equilibrium clearly show a planar iron for both spin states *but* have been interpreted as showing a 0.27 Å longer Fe–pyridine bond in the high-spin state. Moreover, while the Fourier-difference maps of aquometHb vs. cyanometHb crystals could not clearly resolve any motion of the iron relative to the porphinato nitrogens, an overall shift of the F helix toward the porphyrin in the low-spin forms was seen, presumably indicative of a shorter Fe–N_ϵ bond (Moffat et al., 1979). Other indirect evidence that suggests that spin-state changes produce a significant change in structure is the large value of the change in entropy (Iizuka & Kotani, 1969), the "freezing in" of the spin-state equilibrium at low temperatures (Messana et al., 1978), and the rapid increase in the amplitude of iron vibrations in metmyoglobin crystals at temperatures above 210 K (the temperature range where thermal excitations to the low-spin state begin) (Parak et al., 1981). While these data do not prove that spin-state changes in these metHb dervatives actually produce changes in the proximal histidine–porphyrin

separation, they certainly suggest that the high-spin state can easily accomodate a longer separation either via a longer Fe–N_ϵ bond or by motion of the iron out of the plane, or a combination of both. Therefore, if the T structure *does* generate large forces acting along the iron–proximal linkage, then indeed the heme should be able to accomodate this strain by shifting to the high-spin form, giving a much larger value of $\Delta\Delta G$(spin) than we have observed.

The interpretation of these data relies heavily on the assumption that the magnetism we observe reflects a *thermal* mixture of distinct high-spin and low-spin states, rather than a single electronic state which is a *quantum mechanical* mixture of such states, and furthermore assumes that the $S = {}^3/_2$ state plays no role here. The best evidence for a true thermal equilibrium is that the resonance Raman spectra of mixed-spin aquo-, hydroxy-, and azidometHb clearly show the simultaneous presence of both high-spin and low-spin forms (Asher et al., 1977; Spiro et al., 1979; Scholler & Hoffman, 1979) and that azidometHb shows distinct infrared stretching frequencies for the two spin-states (Perutz et al., 1978). It is difficult to absolutely exclude the possibility that a pure $S = {}^3/_2$ state or a quantum mechanical mixture of $S = {}^3/_2$ and $S = {}^5/_2$ may occur in these metHbs. Were such states to occur here, they would best be distinguished by EPR and Mössbauer spectroscopy, but we are of no such evidence.

In summary, it appears that the only way to reconcile these data with the Perutz stereochemical mechanism would be to assert that the solution structures of metHbs + IHP differ so much from the deoxyhemoglobin T structure that studies of metHb are irrelevant for our understanding of cooperativity. Such an assertion would not only conflict with many experimental data, but it also would force the rejection of most of the data in support of the Perutz mechanism.

Differences between Ligands. For human metHb, some ligands show values of $\Delta\Delta G$(spin) that are nearly zero (acetate, formate, and cyanate), yet both SCN^- and H_2O show small but significant values of the order of 300 cal/mol. If indeed about 25% of human azidometHb + IHP switches to the T state (Neya et al., 1983), then the true $\Delta\Delta G$(spin) value for metHbN_3^- is also about 300 cal/mol. Carp metHb also seems to have a different $\Delta\Delta G$(spin) for different ligands. A plausible explanation of the different magnetic changes for different ligands is that the spin-state changes are due at least in part to structural changes on the *distal* side of the heme. One such change could be hydrogen bonding between the ferric ligand and the distal histidine. Such hydrogen bonding is known to occur in the aquomet and oxy forms of both hemoglobin and myoglobin (Shaanan, 1982; Phillips & Schoenborn, 1981) and has been suggested in fluorometHb at low pH (Asher & Schuster, 1982). Such hydrogen bonding of ferric ligands to the distal histidine (possibly involving a shift of a hydrogen from the N_δ to the N_ϵ of the distal histidine) has often been proposed to explain the large pH dependence of the equilibria and kinetics of ligand binding to metHb and metmyoglobin (Czerlinski, 1966; Beetlestone & Irvine, 1971; Ver Ploeg et al., 1971). The kinetic data, in particular, show that the binding rates are greatly enhanced by a protonation step. Whether this represents proton binding to the free ligand or to a group on the protein remains ambiguous.

The effect of such hydrogen bonding on the spin equilibria obviously would depend on the strength of the bond and its location relative to the iron–ligand bond. Thus for acetate or formate, a putative hydrogen bond to the carbonyl oxygen (if sterically possible at all) would probably have little or no direct electronic effect on the spin-state equilibrium, whereas a hydrogen bond to an aquo ligand probably would. A less specific distal effect could arise from changes in steric interactions between the protein and the ferric ligand, if one spin-state allows more favorable steric interactions than the other. Thus it is possible that at least some of the changes in spin-state distributions reflect such distal effects of the R and T conformations rather than changes acting through the iron–proximal histidine linkage.

Role of Subunit Differences. These bulk magnetic measurements give no information about possible differences in spin equilibria between the α and β subunits. It is possible that the changes we see come exclusively from one type of subunit, which would increase the $\Delta\Delta G$(spin) values for that subunit. Perutz et al. (1978) suggested that the tension on the heme may be stronger in the α chain and that, therefore, the value for $\Delta\Delta G$(spin) for the α chain in carp azide metHb might be as large as 2 kcal/mol. However, it is inescapable that if one tries to increase the importance of tension on the heme for one type of subunit, then one must lessen it for the other. Therefore, invoking such subunit inequivalence for $\Delta\Delta G$(spin) necessarily implies that tension on the heme is not the main mechanism regulating oxygen affinity in at least one type of subunit.

Differences between Species. It is now possible to say with some certainty that there are significant difference in the magnetic behavior of human and carp metHbs. Clearly, it would valuable to learn what structural differences between species lead to these differences. It is possible that at the millimolar concentrations used for magnetic studies the carp metHb tetramers are associating to higher states of aggregation when they are the T state (Atha & Riggs, 1982), which might be related to the different magnetic behavior. If our speculation is correct that the spin-state changes may be related in part to changes in distal interactions, then the differences between carp and human metHbs may be due to differences in the importance of such distal effects.

Conclusions

In conclusion, these direct magnetic measurements have failed to show the strong coupling between spin states and quaternary structure that is predicted by the Perutz stereochemical mechanism for cooperativity. We believe that these results suggest strongly that restraints acting through the iron–proximal histidine linkage are not the dominant mechanism for the regulation of oxygen affinity in human hemoglobin. The alternative explanations of these data discussed above all lead to a discarding of other studies involving methemoglobins and/or IHP and, therefore, of nearly all evidence supporting the tension on the heme mechanism. At minimum, then, we believe these new data raise serious questions about the mechanism(s) through which oxygen affinity is controlled.

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Registry No. FluorometHb A, 54577-85-4; aquometHb A, 61008-19-3; formatometHb A, 96150-56-0; acetatometHb A, 96150-55-9; cyanatometHb A, 39340-60-8; thiocyanatometHb A, 72175-40-7; azidometHb A, 9072-23-5; thiocyanatometHb Kansas, 96150-57-1; cyanometHb A, 39340-60-8.

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