

The Azido Human Methemoglobin Controversy: Is There Evidence for a Quaternary Structure-Spin State Linkage or Not?

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Hemoglobin is the red pigment in blood which reversibly binds oxygen. Since the beginning of this century the reaction was known to have some peculiar characteristics. The most striking feature could be seen when a plot was made of the percentage of oxygen-bound hemoglobin versus the oxygen concentration (expressed in partial pressure (p_{O_2})). The curve was sigmoidal such that the binding constant appeared to increase with oxygen concentration. A simple bimolecular reaction would have only one binding constant, and a plot of oxygen bound versus p_{O_2} would have the shape of a rectangular hyperbola. Myoglobin which is found in the muscles exhibits this latter type of behavior.

Myoglobin is a hemoprotein made up of one heme (iron-porphyrin) group enveloped in a single, folded protein chain. Hemoglobin is similar to myoglobin except that it is made up of four myoglobin-like subunits (two α and two β units) held together in a tetrameric arrangement via noncovalent interactions. Since the only major difference between myoglobin and hemoglobin is the tetrameric quaternary structure of the latter, it was postulated that the sigmoidal shape of the hemoglobin binding curve was due to some type of linkage between the heme groups. This linkage has been termed "heme-heme interaction" or "cooperativity".

Progress in unraveling the secrets to the mechanism of cooperativity was quite slow until the crystallographic work of Max Perutz and colleagues.^{1,2} They observed some key structural differences between the liganded and unliganded hemoglobin. These differences gave rise to a mechanistic theory³ of heme-heme interaction based on the following structural features. The four heme groups are not in direct contact with one another, and thus the linkage must be transmitted through the globin. Each heme sits flatly between two helices (E and F) in a folded pocket of the globin chain with the heme being bound to the F-helix. The opposite side of the heme provides a free binding site for the oxygen molecules. Unbound hemoglobin or deoxyhemoglobin (Hb) differs in its quaternary structure from oxyhemoglobin (HbO₂) by a slight rotation of the subunit α - β dimers relative to one another. These distinct

quaternary states were labeled T (tense) and R (relaxed), respectively, after the allosterism model of Monod et al.⁴ There are also tertiary structural differences between the deoxy and oxy states, including a change in distance of the iron atom relative to the porphyrin plane as hemoglobin goes from the unbound to the bound state. In deoxyhemoglobin the iron atom is about 0.5 Å out of the mean plane of the porphyrin nitrogens, to the side of the F-helix, and it moves into the plane upon ligand binding. In going from the deoxy (T) to the oxy (R) state, a number of salt bridges and hydrogen bonds that link the subunits are broken. Finally, the T structure has a lower affinity for oxygen than the R structure.

Perutz's model for heme-heme interaction can then be summarized as follows: the binding of oxygen to Hb brings about a movement of the iron atom, and consequently the F-helix, toward the plane of the porphyrin, which triggers tertiary structural changes to enhance the breakage of the salt bridges and enable the T quaternary state to shift to the R state, effecting a consequent increase in oxygen affinity.

The free energy of heme-heme interaction, which is the difference in the free energy of oxygen binding to the T and R structures, has been calculated to be 3.6 kcal/mol of heme³ (at pH 7.4, 20 °C, in the presence of 0.1 M NaCl and 10 mM 2,3-diphosphoglycerate). If Perutz's mechanism were to hold, then a good deal of the cooperative energy could be stored at the heme. The static information from X-ray diffraction data was, however, incapable of yielding thermodynamic quantities. Thus a dynamic model was sought which could lead to thermodynamic measurements of the tension at the heme. The nearest dynamic models were the mixed-spin complexes of methemoglobin (MetHb). Methemoglobin is hemoglobin in which the iron atom has been oxidized to the ferric state. Oxygen does not bind methemoglobin, but a great variety of singly-charged anions as well as some uncharged ligands containing lone pairs of electrons do. The most common include cyanide, azide (N₃⁻), thiocyanate, cyanate, nitrite, water, imidazole, and fluoride. A number of the complexes of MetHb with these ligands exhibit a very peculiar feature: they are thermal mixtures of high- and low-spin states. In the high-spin state iron has

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five unpaired electrons ($S = 5/2$) and in the low-spin state one unpaired electron ($S = 1/2$). The iron is about 0.2 Å out of the porphyrin plane in the high-spin state and moves into the plane in the low-spin state. Since the movement of the iron is much smaller in the MetHb analogue, Cho and Hopfield estimated the maximum tension at the heme to be about 1.2 kcal/mol in this system.⁵

To obtain a thermodynamic value of the tension at the heme, it was necessary to find a way of measuring the spin equilibrium of the mixed-spin complexes in both the R and T quaternary states. From the high-spin-low-spin equilibrium constant is obtained a ΔG . The change in the spin equilibrium constant upon alteration of the quaternary state will yield a $\Delta(\Delta G)$ which is a measure of the tension at the heme. It was found that in solution the complexes of MetHb existed mainly in the R state,⁶ and thus if a way could be found to push them to the T state, then the strain energy could be calculated.

The next problems were to select the ligands and find ways of altering the R-T equilibrium. Of the ligands mentioned above, all react to form mixed-spin complexes with MetHb at room temperature except cyanide, which is pure low spin, and fluoride, which is predominantly high spin.⁷ From the viewpoint of stability the azide complex is the best choice because it has a large binding constant and is stable over a wide pH and temperature range.^{8,9}

Theoretically, finding a way to alter the R-T equilibrium was not so difficult because hemoglobin is an allosteric protein: i.e., the equilibrium between its quaternary states can be altered by exogenous substances which bind to the molecule at positions removed from its active site. Hydrogen ions, chloride ions, and CO₂ are examples of such allosteric effectors for hemoglobin. When Perutz tested his model in the 1970s, the most powerful known allosteric effector of Hb was inositol hexaphosphate (IHP).

The experimental concept was straightforward: add IHP to the mixed-spin MetHb complex, and observe what happens to the spin-state parameters. The main spin-state parameters are paramagnetic susceptibility (which senses the number of unpaired electrons) and visible absorption spectroscopy (because methemoglobin exhibits absorption bands characteristic of both the high- and low-spin states).

The key relationships and terminology for analysis by paramagnetic susceptibility can be summarized as follows. The molar paramagnetic susceptibility, χ , is related to the effective magnetic moment, μ_{eff} , by the relationship

$$\chi_M = \frac{\mu_{\text{eff}}^2}{8.06T} \quad (1)$$

The effective magnetic moment is related to the total spin quantum number, S , by $\mu_{\text{eff}} = 2(S(S + 1))^{1/2}$ (assuming there is no orbital contribution to the

magnetic moment). An orbital contribution will raise the value of μ_{eff} for a given spin state. For reference, the spin-only magnetic moment and molar paramagnetic susceptibility for one unpaired electron at 20 °C are 1.73 μ_B and 1269×10^{-6} emu, respectively. For five unpaired electrons the values are 5.92 and 14810×10^{-6} , respectively. High-spin iron(III) complexes do not exhibit orbital contribution and have magnetic moments very near 5.92. Low-spin complexes, however, frequently manifest orbital contribution, and values range from 1.73 to 2.6.¹⁰

The high-spin-low-spin equilibrium constant can be expressed in terms of the fraction of low-spin component, α , as

$$K = \frac{\alpha}{1 - \alpha} \quad (2)$$

The low-spin fraction is then related to the effective magnetic moment by

$$\alpha = \frac{\mu_h^2 - \mu_{\text{eff}}^2}{\mu_h^2 - \mu_l^2} \quad (3)$$

where μ_h and μ_l are the magnetic moments for the pure high- and low-spin states of the given complex.

The fraction of low spin, α , can also be determined from visible absorption spectroscopy if the molar absorptivities of the pure high- and low-spin states can be determined at a given wavelength. The corresponding equation is

$$\alpha = \frac{\epsilon_h - \epsilon}{\epsilon_h - \epsilon_l} \quad (4)$$

Testing of the Perutz Mechanism and the Evolution of the Azido Human Methemoglobin Controversy

The first attempts by Perutz and co-workers to test the mechanism were published in 1974.⁶ They looked mainly at the effect of IHP on the azido, fluoro, and aquo complexes of human MetHb. Evidence suggested that IHP induced an R-T shift only in the aquo and fluoro complexes. The spectral and magnetic properties were altered qualitatively in accordance with the theory, but some of the quantitative values were later found to be conflicting due to some spurious results from one of the labs.

At about the same time, Tan and Noble reported that carp Hb could be more easily switched from the R to the T state by lowering the pH and/or addition of IHP.¹¹ This led to studies by Perutz et al.¹² and Messana et al.¹³ on the effect of IHP on numerous complexes of the methemoglobins from carp, trout IV, and human. They consistently found larger changes in the spin-state parameters for fish MetHb than for human. IHP induced a 1000 cal/mol change in the spin equilibrium for carp MetHbN₃⁻ whereas the corresponding value in human was only 100–200 cal/mol. Likewise the IHP-induced visible spectral change in trout MetHbN₃⁻ was

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5 times that of human. Thus the fish hemoglobins gave good quantitative support to the Perutz mechanism whereas the small changes in human MetHb proved to be enigmatic and were attributed to alterations in the tertiary structure only.

In 1979 Cho and Hopfield⁵ published studies in which the R-T shift was brought about not by IHP but by flash photolysis of a mixed hybrid ferrous-ferric hemoglobin containing carbon monoxide and a mixed-spin ligand. They found changes in the spin equilibrium of the azido, thiocyanato, and cyanato complexes of 700, 300, and 300 cal/mol of heme, respectively. The overall average led them to conclude that either the iron spin state was not the only factor in determining its position with respect to the heme or the strain energy is only 50% of that expected from simple models.

In 1981 Neya et al.¹⁴ published NMR evidence that IHP does induce at least a partial R-T transition in human MetHbN₃⁻, which they later found to be about 25%.¹⁵ They also found that the T conformer had a greater fraction of the high-spin state. However, the observation that both the low-spin state and the T conformer were favored by a decrease in temperature led them to conclude that simple coupling between the quaternary structure and the spin state was unlikely.¹⁵ In 1983 Noble et al.⁹ gave more magnetic susceptibility evidence that the effect of IHP on the azido spin equilibrium was greater in carp than in human. The changes in the square of the effective magnetic moments were 7.4 and 1.0, respectively. By 1985 there was no doubt that the R-T quaternary shift induced a large change in the spin equilibrium at the heme for carp MetHbN₃⁻, but its effect on human MetHbN₃⁻ and other complexes such as aquo MetHb was unclear. This led Philo and Dreyer¹⁶ to carry out a thorough study on the effect of IHP on the spin equilibrium of numerous complexes of human MetHb with a very sensitive susceptometer. They found $\Delta(\Delta G)$ values from 0 to 300 cal/mol of heme and concluded that the data did not support a view that the affinity of the T state is due to restraints acting through the iron-proximal histidine linkage. Their average value for the azide complex was a mere 65 cal/mol, which adjusts to 230 cal/mol, taking into account a 25% R-T shift.¹⁵ The evidence seemed convincing, but it is strange that the azido value was less than half that obtained by Cho and Hopfield.⁵

The above result led Perutz et al.³ to repeat the susceptibility studies on human MetHbN₃⁻. They also found no significant change for this complex, although they found that a rather large (45%) change was observed with the nitrito complex which Philo and Dreyer did not report.

As the controversy seemed to come to an unresolved impasse, Noble et al.¹⁷ reported in 1989 that the combination of IHP with another allosteric effector, bezafibrate, brought about greater changes in the spin-state parameters of various complexes of human MetHb than either effector alone. They calculated $\Delta(\Delta G)$ values of 500 and 660 cal/mol for thiocyanate and cyanate, respectively, from the magnetic susceptibility

data. No magnetic data were given, however, for the azide complex. The visible absorption change in the azide complex induced by the combination of the effectors was about 1.7 times that of IHP alone. However, the absolute values of these changes were small, being one-fifth the magnitude of the changes in the nitrito complex. Although their result implied that an increase in the extent of the R-T shift brings about greater changes in the spin parameters, the inability to make reliable quantitative magnetic measurements on the human MetHbN₃⁻ complex enhanced the enigma.

Human MetHbN₃⁻, however, was not to remain stubborn forever. In 1990 Lalezari et al.¹⁸ reported a new and more powerful allosteric effector, L345. This effector induced a visible spectral shift in the azide complex comparable to that observed in trout IV with IHP,¹² implying a large shift in spin state. No magnetic data, however, were reported.

The last result highlights one of the key factors for the solution to the controversy; however, a number of questions deserve special attention. Why does IHP induce such small changes in the spin-state parameters of human MetHbN₃⁻? Are these small changes due merely to tertiary structural changes, or can they be attributed to quaternary shifts? Why does the nitrite complex of human MetHb exhibit such large changes in the spin-state parameters relative to the azide (to the extent of being as much as 5-fold greater)? Why were there such large differences in the values of $\Delta(\Delta G)$ from the various workers? Is there a good estimate of the $\Delta(\Delta G)$ value for human MetHbN₃⁻ induced by L345?

Factors Responsible for the Small Changes in the IHP-Induced Spin Parameters for Azido Human Methemoglobin

IHP binds to Hb at a site that is remote from the hemes.¹⁹ Thus the effect of IHP on the spin state in MetHb must be mediated through tertiary and/or quaternary structural changes that are felt at the heme. Therefore, the quantitative influence of IHP on the spin state involves, at the least, two distinct consecutive interactions: (i) the interaction of IHP on the R-T equilibrium (allosteric effect) and (ii) the interaction of changes in the R-T equilibrium on the spin state (thermodynamic linkage effect). In relation to the allosteric effect the results seem to imply that IHP induces only a partial shift, approximately 25%, in the R-T equilibrium; the combination of IHP and bezafibrate brings about a larger shift; and the single addition of L345 the largest. In carp, on the other hand, IHP seems to bring about a complete shift as the addition of bezafibrate effects no further changes in the spin parameters.²⁰ Thus the magnitude of an allosteric-effector-induced shift in the quaternary equilibrium is species dependent.

The evidence also implies that the extent of the quaternary shift is also ligand dependent, with high-spin complexes being more easily altered than low-spin complexes. This, however, cannot completely account

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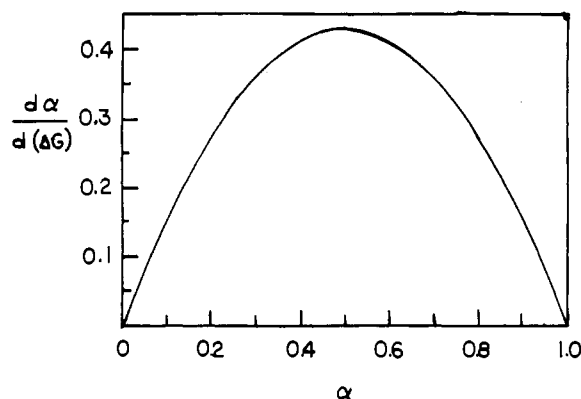


Figure 1. The instantaneous rate of change of the low-spin fraction, α , with work done on the spin system, $d(\Delta G)$, as a function of α at 20 °C.

for the 5-fold-greater change in the spin-state parameters of the nitrite complex of human MetHb relative to the azide. The missing factor can be understood if we derive a relationship between the interaction energy, $\Delta(\Delta G)$, the change in the spin state, $\Delta\alpha$, and the position of the equilibrium represented by α .

Letting α be the fraction of low-spin component, taking the natural logarithm of both sides of eq 2, and differentiating with respect to α yields

$$\frac{d \ln K}{d\alpha} = \frac{1}{\alpha} + \frac{1}{1-\alpha} = \frac{1}{\alpha - \alpha^2} \quad (5)$$

Substituting $\Delta G = -RT \ln K$ and rearranging yields

$$\frac{d\alpha}{d(\Delta G)} = -\frac{1}{RT}(\alpha - \alpha^2) \quad (6)$$

The significance of eq 6 is that at constant temperature the instantaneous rate of change of the low-spin fraction, $d\alpha$, with respect to the amount of work on the system, $d(\Delta G)$, is directly proportional to the quantity $\alpha - \alpha^2$. Thus $d\alpha/d(\Delta G)$ is a nonlinear, quadratic function of α as shown in Figure 1 with a maximum value at $\alpha = 0.5$ and which tends to zero as α goes to 0 or 1. When the same amount of work is done on the system, the degree of change of spin state will depend on the original position. When the equilibrium is equally balanced ($\alpha = 0.5$), the observed changes will be large, whereas, when the equilibrium is shifted toward the pure high- or low-spin positions, the changes will necessarily be smaller. It takes the same amount of work (500 cal/mol) to move the low-spin fraction from 0.7 to 0.5 as it does to move it from 0.955 to 0.90, yet the change in α in the former case is almost 4 times that in the latter. A real example is given by nitrito and azido human MetHb. Nitrito MetHb is approximately 60% low spin at 20 °C,^{17,21} and azido MetHb is around 90–95%.^{16,22} If 240 cal/mol of "strain energy" acts on both systems, then the nitrito will change to 50% low spin and the human will change to 86% or 92.5%, respectively. Thus the $\Delta\alpha$ in the nitrito will be 2.5 times greater (or 4 times greater) than that in the azido which was originally at 90% (or 95%). Noble et al. observed a 5-fold-greater visible absorption change in nitrito than in azido upon the addition of IHP.¹⁷ Thus small changes in the observable

Table I. Literature Values of the Low-Spin Fraction, α , of Azido Methemoglobin from a Variety of Techniques^a

workers	technique	α
George et al. ²²	magnetic susceptibility	0.955
Iizuka and Kotani ²⁵	magnetic susceptibility	0.87
Alben and Fager ²⁷	IR	0.95
Perutz et al. ¹²	IR	0.92
Neya et al. ¹⁵	visible spectra	0.92
Anusiem and Kelleher ²⁸	visible (Soret)	0.963
Philo and Dreyer ¹⁶	magnetic susceptibility	0.88
Neya and Funasaki ²⁹	NMR	0.88

^a All measurements were made at 20 °C.

spin parameters in the azido system can have the same thermodynamic significance as the large changes in the nitrito system. This implies that in order to make good quantitative estimates of $\Delta(\Delta G)$ it is absolutely crucial to have accurate values of α .

Determination of the Best Value for the Magnetic Moment of Pure Low-Spin Azido Human Methemoglobin and Refinement of α

The literature values of the low-spin fraction, α , of human MetHbN₃⁻ vary from 0.87 to 0.96 (Table I). From the viewpoint of α this is only a 10% difference, but from the viewpoint of Gibbs energy it is a difference of about 750 cal/mol. In light of eq 6 a more refined value is necessary in order to make accurate calculations of $\Delta(\Delta G)$. Many experimental techniques have been used to determine the thermodynamic values of the spin equilibrium; the most direct technique, however, is that of magnetic susceptibility. Equation 3 shows that α can be calculated from the measured effective magnetic moment and the magnetic moment of the pure high-spin and low-spin states. For iron(III) complexes nearly all workers agree that the spin-only value of 5.92 μ_B is a good assumption for the pure high-spin magnetic moment, but there is little agreement as to the value to be taken for the low-spin magnetic moment due to the possibility of extensive orbital contribution.¹⁰ For the pure low-spin magnetic moment, workers have used values ranging from 1.73 to 2.5.^{9,16,17,23} Each group of workers presented a rationale for their choice, but the following experimental facts will enable a better value to be chosen. The ESR spectra at liquid nitrogen temperatures (77 K) show that human MetHbN₃⁻ is in the pure low-spin state.²⁴ Iizuka and Kotani²⁵ have measured the effective magnetic moment of MetHbN₃⁻ from room temperature down to 77 K. It decreases with temperature until about 150 K, where it levels off to a constant value of 2.31. The cyanide complex exhibits a temperature-independent value of 2.23. Thus the pure low-spin azide μ not only is comparable to that of the cyanide but also is slightly greater. Finally, the magnetic moment of K₃Fe(CN)₆, a pure low-spin complex, increases slightly from 1.90 at 80 K to 2.25 at 300 K. This slight increase with temperature is explained by the van Vleck equation.¹⁰

Putting these three results together leads to an estimate of 2.66 and 2.58 for the low-spin magnetic moments of the azido and cyano complexes at room

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Table II. Room Temperature Magnetic Susceptibility Data for the Calculation of α_1 of Azido MetHb

workers	species	temp (°C)	$\chi_{N_3^-}$	$\mu_{N_3^-}$	χ_{CN^-}	μ_{CN^-}	χ_{F^-}	μ_{F^-}	$\alpha_1^{N_3}$
Coryell et al. ³⁰	cow	24			2610	2.50	14 610	5.92	
Coryell and Stitt ³¹	cow	25	3360	2.84					0.937
Blanck and Scheler ³²	human	20	3500	2.84	2800	2.57	14 800	5.91	0.941
Noble et al. ⁹	human	18		2.67		2.45			0.961
Philo and Dreyer ¹⁶	human	20	3327	2.804	2505	2.43	14 300	5.81	0.933

Table III. Literature Values of $\Delta(\Delta G)$ on the Spin-State Equilibrium of Azido Human MetHb Induced by the Addition of IHP^a

workers	technique	temp (°C)	α_1	α_{IHP}	$\Delta(\Delta G)$ (cal/mol)
Perutz et al. ¹²	IR	20 (?)	0.921	0.888	224
Neya et al. ¹⁵	UV-vis	20	0.919	0.890	197
Noble et al. ⁹	magnetic	18	0.898	0.866	180
			$\mu_1 = 2.00$		
recalculated Philo and Dreyer ¹⁶	with $\mu_1 = \mu_{CN^-}$ magnetic	20	0.961	0.927	383
			0.878	0.865	68
			$\mu_1 = 2.00$		
recalculated Perutz et al. ³	with $\mu_1 = \mu_{CN^-}$ NMR		0.935	0.922	114
				no data given but confirm small effect of ref 16	

$$^a \Delta(\Delta G) = -RT \ln \left[\frac{\alpha/(1-\alpha)}{(1-\alpha_{IHP})/\alpha_{IHP}} \right]$$

temperature. Table II gives a collection of room temperature values for the magnetic moments of the azido, fluoro, and cyano complexes of MetHb. The average value for cyanide is 2.49. This is quite close to the estimate above. Note that the azide values in the table are higher because at room temperature they have some high-spin component. The important point is that the experimentally measured magnetic moment of the cyanide complex is not only a good estimate of the pure low-spin value of the azide complex but also a lower estimate. Using the cyanide and the fluoride magnetic moments as the pure low-spin and high-spin values for the azide complex, the fraction of low spin, α , is calculated from numerous results in Table II. The average value of α is 0.943. If 0.08 is added to the cyanide values, then a new average of 0.958 results. Thus a new refined estimate of the low-spin fraction of human MetHbN₃⁻ at 20 °C is 0.95 ± 0.01.

Harmonizing the $\Delta(\Delta G)$ Values

Table III contains values of the IHP-induced $\Delta(\Delta G)$ on the spin equilibrium of human MetHbN₃⁻ calculated from data in the literature. The values of Noble et al. and Philo and Dreyer were calculated for two different values of μ in order to see the effect on α and $\Delta(\Delta G)$. In their analysis Philo and Dreyer used 2.00.¹⁶ In both cases an increase in the value of μ_1 causes a significant increase in α and a doubling of the value of $\Delta(\Delta G)$. The recalculated values yield an average $\Delta(\Delta G)$ of 250 cal/mol. Taking into account the other values in Table III, a good estimate of the IHP-induced effect on the spin equilibrium of human MetHbN₃⁻ is 200–300 cal/mol. If IHP brings about only a 25% R–T transition, then a complete transition should bring about a change of 800–1200 cal/mol in the spin equilibrium. This harmonizes with that found for carp.¹³

The Problem of Precision

There is one final aspect that sheds even more light on why the human MetHbN₃⁻ controversy arose, and that is the problem of precision. Smith and Williams have pointed out (i) that when the observed paramagnetic susceptibility is close to the low-spin limit, it is

difficult to decide whether the data are consistent with a 100% low-spin compound, and (ii) that the presence of only a few percent of one form could be crucial in mechanistic arguments.²⁶ The human MetHbN₃⁻ is such a system.

It would be useful to make some quantitative calculations relating the expected changes in the magnetic susceptibility to a given change in α . According to Beeston and George,²³ the low-spin fraction, α , and the magnetic susceptibility at a given temperature are related by the equation

$$\chi^T = \alpha \chi_l^T + (1-\alpha) \chi_h^T \quad (7)$$

Rearranging and solving for α yields

$$\alpha = \frac{\chi_h^T}{\chi_h^T - \chi_l^T} - \frac{\chi^T}{\chi_h^T - \chi_l^T} \quad (8)$$

Since the first term on the right-hand side is a constant at a given temperature,

$$\Delta \chi^T = -(\chi_h^T - \chi_l^T) \Delta \alpha \quad (9)$$

Table III shows that many workers were measuring $\Delta(\Delta G)$ values of around 200 cal/mol. Equation 6 indicates that the magnitude of $\Delta \alpha$ for a given amount of work will depend on the initial value of α , and thus from eq 9 the same will hold true for $\Delta \chi^T$. If MetHbN₃⁻ is initially 95% low spin, then the anticipated change in χ when 200 cal/mol of work is applied is 234×10^{-6} emu/mol. The estimated error of Philo and Dreyer is 200×10^{-6} ,¹⁶ and thus the actual change is completely masked by the error of the experiment. In carp Hb the changes are much larger, and thus magnetic susceptibility can yield reasonable results on that system.

A Spectroscopic Resolution of the Problem

The extent of the induced R–T transition in human MetHbN₃⁻ depends on the type of allosteric effector,

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with the order of magnitude being IHP < IHP plus bezafibrate < L345. Magnetic susceptibility data have been published only for the IHP effect (Table III). However, the effect of these allosteric effectors on the visible absorption bands has been published.^{6,17,18} The low-spin fraction can be calculated from the spectral data according to eq 4. The 20 °C value of the millimolar absorptivity of the azide MetHb at 542 nm is 11.4.⁶ Good estimates of ϵ_l and ϵ_h are 11.7 and 5.7, respectively. The values of $\Delta\epsilon$ (mM) for IHP, IHP plus bezafibrate, and L345 are 0.18, 0.306, and 1.0, respectively. From these data the values of $\Delta(\Delta G)$ can be calculated according to the equation in footnote *a* of Table III. The values are respectively 290, 440, and 970 cal/mol of heme. The value of 290 is in excellent agreement with the range previously found for IHP. The value of 970 implies that L345 brings about a fairly complete R-T transition according to the range 800–1200 previously calculated.

Conclusion

Although IHP induces small changes in the spin-state parameters of human MetHbN₃⁻, small does not imply thermodynamic insignificance because the spin equilibrium is positioned at an extremum. IHP induces a fractional (25%) shift in the R-T equilibrium which quantitatively can account for the change in the spin equilibrium without attributing the change to merely tertiary structural alterations. The degree of the R-T shift increases with the addition of more powerful allosteric effectors, which is reflected in the greater changes in the spin-state parameters. The magnitude

of the changes in the spin-state parameters ($\Delta\mu^2$, $\Delta\epsilon$) alone does not indicate directly the $\Delta(\Delta G)$ (viz., eq 6). The same amount of work may bring about a larger or smaller change in spin parameters, depending on the initial position of the equilibrium, α . The human MetHbN₃⁻ enigma or controversy arose because (i) IHP induces only partial shifts in the R-T equilibrium which in turn induce very small shifts in the spin equilibrium because it is initially near an extremum; (ii) these consequent small shifts could not be measured with the desired precision using magnetic susceptibility due to relatively large errors in the determination of the concentration of the concentrated MetHb solutions;^{16,17} (iii) there was a large variation in the $\Delta(\Delta G)$ values calculated from the data due to the variation in the choice of reference parameters such as the μ_l of the azide complex. The recalculated $\Delta(\Delta G)$ values for a *complete* R-T transition of human MetHbN₃⁻ are in the range of 1000 cal/mol from both the magnetic and spectral analyses, implying that the mechanism for heme-heme interaction is not only qualitatively but quantitatively similar to that of carp. Thus the quaternary state of the globin is *thermodynamically* linked to the spin state of the heme in human hemoglobin. Although the precise nature of this linkage remains to be clarified (whether due to bond tensions, steric restraints, or changes in bond strengths due to heme tilt, etc.), the Perutz model is still a good reference point.

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