Spin Equilibrium in Human Methemoglobin: Effects of Inositol Hexaphosphate and Bezafibrate As Measured by Resonance Raman Spectroscopy

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ABSTRACT: The spin equilibria of several derivatives of human methemoglobin were probed by resonance Raman scattering. The intensity of lines in the Raman spectrum gives a measure of the high-spin ($S = {}^5/_2$) to low-spin ($S = {}^1/_2$) ratio which agrees well with the spin equilibria determined from direct magnetic susceptibility measurements. The addition of bezafibrate (BZF) to methemoglobin in the absence of organic phosphate, IHP, has very little effect on the spin equilibrium, whereas in the presence of IHP it augments the change in spin significantly. When both IHP and BZF are added to the mixed-spin derivatives (H_2O, SCN^-, OCN^-, and NO_2^-) of human methemoglobin, the spin equilibrium is shifted toward higher spin by about 700 cal/mol, similar to the spin change detected in derivatives of carp methemoglobin upon addition of IHP alone. These data support a general mechanism for the allosteric transition in which a constant fraction of the cooperative energy (≈20%) is detected at the heme of the ferric ligand-bound forms.

The spin equilibrium in methemoglobins has been studied extensively to monitor the influence of protein structural changes on the heme group (Perutz et al., 1978; Noble et al., 1983; Henry et al., 1985). The addition of organic phosphates to the protein has been shown to alter this equilibrium and in some cases to bring about a change in the quaternary structure of the protein. In several studies it has been shown that the addition of organic phosphates, such as inositol hexaphosphate (IHP), to certain proteins such as carp hemoglobin induces a larger change in the spin equilibrium than in other hemoglobins such as human hemoglobin (HbA) (Henry et al., 1985; Philo & Dreyer, 1985). Whether this difference in behavior is a result of a lack of generality of the effect of IHP on the electronic configuration of the iron atom or due to an incomplete R to T transition in the human protein has not been determined. Recently, Noble et al. (1987) reported a preliminary set of experiments which indicated that the combination of IHP and bezafibrate [2-[4-[2-(*p*-chlorobenzamido)ethyl]phenoxy]-2-methylpropionic acid (BZF)] produced a larger change in the spin-state equilibrium of the nitrite derivative of human met HbA than did IHP alone. In this paper we use resonance Raman scattering to explore the effect of BZF on ferric HbA in greater detail. In an accompanying paper (Noble et al., 1989) the results of the magnetic susceptibility and visible optical absorption spectra are presented.

Resonance Raman scattering has been used in the past to study spin equilibria in methemoglobins with great success. The frequencies and the intensities of heme vibrational modes are sensitive to the porphyrin macrocycle core size, the amount of doming, and the degree of π back donation. As these properties are dependent on the electronic configuration of the iron atom, some lines in the Raman spectrum have been found to be reliable indicators of the spin state (Spiro & Strekas, 1974; Spiro & Burke, 1976; Spiro et al., 1979; Choi et al., 1982; Dasgupta et al., 1989). Furthermore, owing to the rapidity with which the measurements can be carried out, questions concerning the stability of various derivatives can be explored directly. In the past, comparisons of the spin equilibrium determined from the Raman scattering data have agreed reasonably well with the spin content determined from susceptibility measurements. In an extensive study, Henry et al. (1985) used the Raman technique to determine the effect of IHP on the spin states of both carp and human methemoglobins. In that study, it was found that the change in energy at the iron atom was about 700 cal/mol in carp hemoglobin but only 300 cal/mol in human hemoglobin for those ligands in which IHP could bring about a change in the quaternary structure. Moreover, these results are in general agreement with reported susceptibility measurements (Philo & Dreyer, 1985). This leads to the question of whether the linkage between the heme and the subunit interface is fundamentally different for these two proteins or whether the IHP-induced allosteric translation in the human ferric protein is simply incomplete.

Experimental Procedures

Ferric human adult hemoglobin (HbA) was prepared as described by DiIorio (1981) using nitrite as the oxidant. Washed red blood cells were lysed with 2 volumes of 1 mM Tris, pH 8.0. The hemolysate was made 0.1 M in NaCl and centrifuged at 50000*g* for 30 min to remove the stroma. An excess of sodium nitrite (3 mol/mol of heme) was added and allowed to incubate at 4 °C for 30 min. This mixture was then passed through a Sephadex G-25 column equilibrated with 1 mM Tris, pH 8, and finally through a Dintzis column to remove all ions including organic phosphates (Garlick et al., 1979).

The buffer used in all measurements was Bis-Tris-HCl, pH 6.5, adjusted to yield a final chloride ion concentration of 0.1 M. All ligands were added to give a final concentration of 0.1 M. Inositol hexaphosphate was prepared as a pH-adjusted

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stock solution without other anions by titrating with the acid form of Amberlite IR-120 resin as previously described (Noble et al., 1987). Bezafibrate [2-[4-[2-(*p*-chlorobenzamido)-ethyl]phenoxy]-2-methylpropionic acid] was kindly supplied to us by Boehringer-Mannheim Corp.

The resonance Raman data were obtained on previously described Raman difference instrumentation (Rousseau, 1981). By use of the difference technique, an accurate assessment could be made in those cases where only a small change in the frequency or the intensity of a line was induced by the additive. The spectra were obtained at 5-cm^{-1} resolution with 413.1-nm excitation. To analyze the resonance Raman data, a curve-fitting routine was utilized. In this procedure the spectra were deconvoluted on a computer by using a nonlinear least-squares fitting procedure based on Marquardt's algorithm (Marquardt, 1963; Nash, 1979). It was found that the best fits could be obtained when a 1:1 Gaussian/Lorentzian combination was used in the fitting procedure. In these analyses, the positions and the intensities of the Raman lines were permitted to differ for each derivative while the bandwidths were held constant for all fittings. Once the optimal line frequencies were obtained for any particular derivative, these were then fixed for the subsequent deconvolution of data obtained in the presence of IHP and/or BZF. The intensities of the marker lines derived from the fits were used in the calculation of the spin equilibrium.

Results

The region of the resonance Raman spectrum of ferric heme proteins between 1400 and 1600 cm^{-1} is sensitive to the spin equilibrium. In particular, lines at about 1510 and 1585 cm^{-1} are associated with a low-spin iron configuration, whereas lines at 1480 and 1565 cm^{-1} are associated with a high-spin six-coordinate configuration. In several studies (Scholler & Hoffman, 1979; Cho et al., 1981; Henry et al., 1985) the relative intensity of these lines has been used to determine the spin equilibrium of methemoglobins, although it has been recognized that these lines cannot be used as an absolute measure of the high- and low-spin content because in the limit of a pure high-spin (or low-spin) derivative the low-spin (or high-spin) marker lines do not go to zero. Furthermore, the Raman scattering cross sections for the high- and low-spin states need not be the same. Therefore, it is necessary to have reference points to calibrate the correlation between the line intensities and the spin configuration content.

To extract the spin equilibrium information from the Raman data, we have tried various methods of analysis. For example, if the high-spin marker lines at 1480 and 1565 cm^{-1} [which we label A(h) and B(h), respectively] and the low-spin marker lines at 1509 and 1585 cm^{-1} [which we label A(l) and B(l), respectively] gave a linear measure of the spin content, the ratios

$$R(A) = A(\mathrm{h})/A(\mathrm{l}) \quad \text{and} \quad R(B) = B(\mathrm{h})/B(\mathrm{l}) \qquad (1)$$

would be expected to yield measures of ratios of the high- to low-spin iron configuration, i.e., the spin-state equilibria. However, these ratios are not always mutually consistent and become very sensitive to the underlying lines at the extremes of high spin and low spin. A function that is more stable and less sensitive to variations in unresolved lines is the average fraction of the sum of the intensities of the high-spin line pairs:

$$F(A,B) = \left[\frac{A(\mathrm{h})}{A(\mathrm{h}) + A(\mathrm{l})} + \frac{B(\mathrm{h})}{B(\mathrm{h}) + B(\mathrm{l})}\right] \Big/ 2 \qquad (2)$$

We have used this function to analyze the data obtained in the present study.

Resonance Raman data were recorded from the H_2O, SCN^-, OCN^-, NO_2^-, and CN^- derivatives of human methemoglobin. For the cases of the mixed-spin derivatives (all but CN^-, which is fully low spin), spectra were taken of the hemoglobin derivative without additives, of the derivative in the presence of either IHP or BZF, and of the derivative in the presence of both additives (IHP plus BZF). We find that when only BZF is added to a derivative, the change in spin equilibrium is very small ($\ll$5%). However, larger changes were observed when IHP was added, and these changes were significantly augmented by the addition of BZF. In all cases, as was found with the addition of allosteric effectors in the past (Henry et al., 1985), the equilibrium was shifted toward higher spin; i.e., the low-spin state was destabilized relative to the high-spin state upon the addition of IHP or IHP plus BZF, although which state is actually affected cannot be determined from our data alone.

For a given change in the free energy of a process, the largest change in spin equilibria will occur for that reaction whose reactant and product are of most nearly equal concentrations at equilibrium. With human ferric hemoglobin it is the nitrite derivative that best fulfills this condition. In the absence of effectors such as organic phosphates the square of its magnetic moment, μ^2, is approximately 14 (Bohr magnetons)^2, which corresponds to $\sim$34% high spin. The effects of IHP and the combinationof the IHP and BZF on the resonance Raman spectrum from 1450 to 1650 cm^{-1} of this derivative are shown in Figure 1. In the left-hand panels are presented the spectral data points along with the fitting results from the deconvolution of the spectra into distinct bands. The separate bands obtained from the deconvolution procedure appear in the right-hand panels. From top to bottom are presented the spectral data for human HbA at pH 6.5 in Bis-Tris buffer, human HbA in the same buffer containing 1 mM IHP, and the human HbA in the pH 6.5 buffer containing 1 mM IHP plus 5 mM BZF.

Striking differences between these spectra are apparent. In the absence of effectors (spectrum A), the two low-spin marker lines at 1509 and 1585 cm^{-1} are more intense than their associated high-spin marker lines at 1480 and 1565 cm^{-1}. With the addition of IHP (spectrum B), the 1480- and 1509-cm^{-1} lines become of roughly equal intensity as do the 1565- and 1585-cm^{-1} lines. In the presence of IHP and BZF (spectrum C), the intensities of the high-spin marker lines are further increased relative to the intensities of the low-spin lines, consistent with an additional effect of BZF on the spin-state equilibrium in the presence of IHP. Similar sets of spectra were obtained for the cyanate, thiocyanate, and aquo derivatives of ferric human HbA. In addition, the spectrum of the cyanide derivative in the absence of both IHP and BZF was obtained as a low-spin reference state. The spectra of all of these derivatives were subjected to the same band deconvolution analysis as described above for the nitrite derivative.

In carrying out these experiments we were concerned about the stability of the samples being examined. Instability could result from the intrinsic properties of a derivative, the reactivity of the ligand, or the effects of exposure to intense laser radiation. To control for this possibility the Raman spectrum of each sample was initially collected in a single scan (5 min) that was stored separately. Then a spectrum was collected by averaging multiple scans (typically 5). This spectrum was then compared to that obtained initially. Significant differences were not found.

In one experiment the spectra of the nitrite derivative were collected after exposure to the beam for 1 h and then after

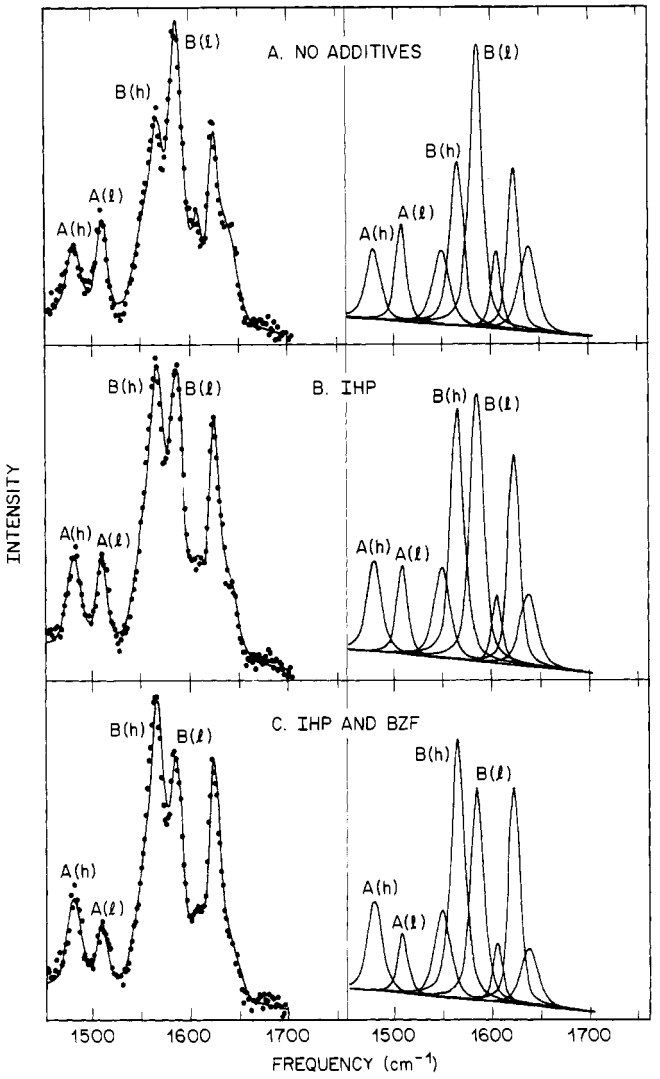

FIGURE 1: Resonance Raman spectra of the nitrite derivative of human methemoglobin in the absence and presence of additives. The unsmoothed raw data are given by the data points in the panels on the left side. The solid line is an eight-line fit to the data. The deconvoluted lines used to generate the fit are shown in the panels on the right side. The high-spin marker lines at 1480 and 1565 cm^{-1} are labeled A(h) and B(h), respectively, and the low-spin marker lines at 1509 and 1585 cm^{-1} are labeled A(l) and B(l), respectively. (A) The nitrite derivative in the absence of additives. (B) The nitrite derivative in the presence of inositol hexaphosphate (IHP). (C) The nitrite derivative in the presence of inositol hexaphosphate (IHP) and bezafibrate (BZF).

exposure for 2 h. Such long-term exposure to the beam produced small but progressive changes in the spectra with an increase in the high-spin marker lines. However, even after such treatment, the difference spectra resulting from the addition of IHP and BZF were unchanged.

The results of all of the analyses of the resonance Raman data are listed in Table I. We list the intensities of each of the spin marker lines and the function $F(A,B)$ (eq 2) from which we calculated the spin equilibrium of each sample. For most derivatives these results are the average of several data sets. The spin equilibria were determined by using the cyanide derivative as a zero high-spin reference point and aquo met HbA in the absense of additives as an 89% high-spin reference point, drawing from the magnetic susceptibility measurements. The percentage of high spin (α) and the square of the magnetic moment, μ^2, related by

$$\alpha = (\mu^2 - 3)/32 \qquad (3)$$

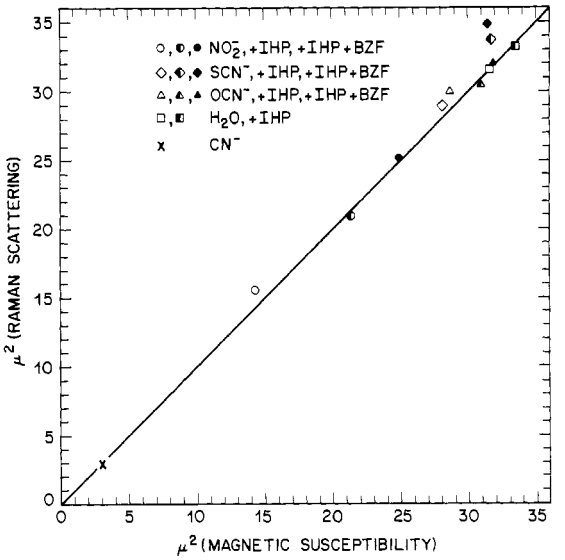

FIGURE 2: Comparison of the square of the magnetic moment (μ^2) determined from the resonance Raman data and from the direct magnetic susceptibility measurements. The Raman data were calibrated by setting the square of the magnetic moment of the cyanide derivative to 3 and that of the H_2O derivative to 31.6.

were determined for each derivative and are listed in Table I. In eq 3, we have used the spin-only values for μ of 1.73 μ_B for low spin and 5.9 μ_B for high spin.

To assess the accuracy of our procedures the values of μ^2 measured by magnetic susceptibility measurements are plotted against μ^2 determined from the Raman intensities in Figure 2. As is evident, the agreement between these two types of measurement is excellent. These results are also in good agreement with the spin changes detected in the visible absorption spectra of these derivatives reported in the accompanying paper (Noble et al., 1989). Only the SCN^- derivative in the presence of additives is not within the experimental error of the Raman and the magnetic susceptibility measurements. This discrepancy results largely from very unusual values of A(l) in the presence of IHP or IHP plus BZF, suggesting that the Raman spectrum of this derivative behaves differently from those of the other derivatives examined here. This is not the only pecularity of this derivative. As noted in the accompanying paper, BZF added to this derivative in the presence of IHP has little effect on its visible spectrum and causes no significant change in its spin equilibrium as judged by direct susceptibility measurements (Noble et al., 1989). Additional studies of this derivative appear warranted.

Discussion

Ferric hemoglobins may exist in high-spin ($S = {}^5/_2$) or low-spin ($S = {}^1/_2$) forms. For many derivatives there is an equilibrium between these forms, and the poise of the spin equilibrium depends on both the nature of the specific ligand and the protein. For example, the spin equilibrium is shifted toward higher spin in carp hemoglobin than in human hemoglobin in the absence of any allosteric effectors (Henry et al., 1985). This clearly indicates that there are significant structural differences between these proteins.

When IHP is added to carp methemoglobin, the spin equilibrium is shifted toward higher spin and, concomitantly, the quaternary structure is shifted from the R to the T state (Tan & Noble, 1973; Noble et al., 1987). Indeed, pH changes alone will also induce a change to higher spin and may partially shift the quaternary structure equilibrium to the T state in the carp protein. Since the change in spin-state equilibrium can

Table I: Resonance Raman Scattering Intensity Data, Spin Equilibria, and Free Energies Derived from the Spin Equilibrium[a]

derivative	IHP	BZF	A(h)	A(l)	B(h)	B(l)	$F(A,B)$	%HS	μ^2	$\Delta G°$	$\Delta\Delta G_{IHP}$	$\Delta\Delta G_{tot}$	$\Delta\Delta G_{IHP\pm BZF}$
$HbNO_2$	−	−	0.54	0.76	1.4	2.3	0.39	39	15.5	−0.27			
	+	−	0.73	0.71	2.0	2.2	0.50	56	20.9	0.14	0.41	0.75	0.34
	+	+	0.81	0.54	2.4	2.0	0.58	69	25.2	0.48			
HbOCN	−	−	0.12	0.051	0.32	0.20	0.66	84	30.0	0.99			
	+	−	0.13	0.048	0.34	0.21	0.67	86	30.5	1.09	0.10	0.40	0.30
	+	+	0.14	0.044	0.35	0.21	0.70	91	32.1	1.39			
HbSCN	−	−	0.11	0.046	0.32	0.23	0.64	81	28.9	0.87			
	+	−	0.13	0.026	0.36	0.21	0.73	96	33.7	1.91	1.04	1.89	0.85
	+	+	0.14	0.021	0.38	0.22	0.75	99	34.8	2.76			
HbH_2O	−	−	0.19	0.085	0.58	0.25	0.69	89[b]	31.6[b]	1.25			
	+	−	0.18	0.073	0.55	0.21	0.72	94	33.2	1.65	0.40	0.66	0.26
	+	+	0.21	0.072	0.63	0.24	0.73	96	33.7	1.91			
HbCN	−	−	0.059	0.24	0.046	0.33	0.16	0[b]	3[b]	−	−	−	−

[a] A(h) and B(h) are the intensities of the high-spin marker lines at 1480 and 1565 cm^{-1}, respectively, and A(l) and B(l) are the intensities of the low-spin marker lines at 1509 and 1585 cm^{-1}, respectively. These intensities are relative within each spectrum only. $F(A,B)$ is the function of the intensities as described in the text from which the spin equilibria were calculated. The square of the magnetic moment, μ^2, and the percentages of high spin for each derivative were determined from $F(A,B)$ as described in the text. $\Delta G°$ is the standard free energy of the spin-state transition for each derivative. Stable high-spin derivatives have positive values for $\Delta G°$. $\Delta\Delta G_{IHP}$, $\Delta\Delta G_{tot}$, and $\Delta\Delta G_{IHP\pm BZF}$ are respectively the difference between $\Delta G°$ for the derivative in the absence of additives and in the presence of IHP, the difference between $\Delta G°$ for the derivative in the absence of additives and in the presence of both IHP and BZF, and the difference between $\Delta G°$ for the derivative in the presence of IHP and in the presence of both IHP and BZF. All values of energy are expressed in kilocalories per mole. [b] These values were used to calibrate the Raman intensity data. The value of 3 for the CN^- derivative is the spin-only value. In this derivative there are significant orbital contributions that raise the measured value of μ^2. However, it is generally agreed that this derivative is fully low spin.

be used to calculate directly the amount of energy transmitted to the heme when the protein quaternary structure is changed, it has been a useful probe of heme energetics. From such studies only about 300 cal/mol was found at the heme in those derivatives of human hemoglobins in which a quaternary structure change could be induced by the addition of IHP, but about 700 cal/mol was detected in the carp protein by IHP addition. The question then arises as to whether or not additional or different allosteric effectors could so destabilize the low-spin structure of human methemoglobin relative to the high-spin structure so as to induce large changes in the spin equilibrium, changes similar to those detected in the derivatives of the carp protein.

The energy associated with the spin-state equilibrium may be given by

$$[LS]/[HS] = e^{-\Delta G°/K_B T} \quad (4)$$

where [LS] and [HS] are the concentrations of the low- and high-spin species, respectively, and $\Delta G°$ is the standard free energy difference between the low-spin and high-spin states. To study the effect of allosteric effectors on the heme, $\Delta\Delta G$'s are defined as differences between the $\Delta G°$ values determined for derivatives with and without the additives.

The values for $\Delta G°$ obtained in this investigation are listed in Table I. These are in general agreement with the measurements reported in the past based on resonance Raman scattering (Henry et al., 1985) as well as from direct magnetic susceptibility measurements (Philo & Dreyer, 1985; Noble et al., 1987). We also list the $\Delta\Delta G$ values determined for each derivative with and without IHP ($\Delta\Delta G_{IHP}$), with and without both IHP and BZF ($\Delta\Delta G_{tot}$), and with IHP but with and without BZF ($\Delta\Delta G_{IHP\pm BZF}$). Some general observations on the $\Delta\Delta G$ values may be made. First, although the values for $\Delta\Delta G_{IHP}$ are in general agreement with those of other workers, they are somewhat larger than the values reported by Henry et al. (1985) based on resonance Raman measurements. We attribute this either to possible IHP concentration differences, pH differences, or data analysis differences. However, both sets of measurements yield values of ~300–400 cal/mol. (We do not include consideration of the SCN^- derivative because it appears to behave rather uniquely in our measurements and in both the magnetic susceptibility and visible spectral measurements, as discussed above.)

The most important results of this work are the values we obtain for $\Delta\Delta G_{tot}$ and $\Delta\Delta G_{IHP\pm BZF}$. The addition of BZF in the presence of IHP augments the IHP-induced energy change by approximately 300 cal/mol. In contrast, the addition of BZF to the same derivatives in the absence of IHP has no observable effect on the energetics. This indicates that the effect of BZF on the equilibria between the conformational states of hemoglobin is smaller than that of IHP. In the absence of either allosteric effector the conformational equilibrium may so greatly favor the R state that changes in this equilibrium brought about by BZF addition do not cause a measurable change in the concentration of the components of this equilibrium. However, the presence of IHP shifts the equilibrium to produce a more nearly equal balance between structural states, allowing the effect of BZF to be readily observed. These results are consistent with the reported effects of BZF on the ferrous form of the protein (Perutz & Poyart, 1983). It was observed that it binds to the T-structure form of the protein but not the R-structure form and binds in the central cavity of the tetramer (Perutz et al., 1986). It acts in concert with IHP rather than competitively because it does not bind at the same site.

The effect of the combination of IHP and BZF on HbA results in a $\Delta\Delta G_{tot}$ of ~700 cal/mol. Interestingly, this is about the same value that has been found for the effect of IHP alone on carp hemoglobin, indicating that the forces on the hemes are the same for the two proteins under these conditions (Henry et al., 1985). This raises the interesting question as to whether or not the NO_2^- derivative of met HbA may be switched to the T state by the addition of both IHP and BZF. It is known that it is not switched to the T state by IHP alone (Henry et al., 1985). Unfortunately, at present we are unable to determine if the combination of IHP and BZF can switch the NO_2^- derivative of HbA to the T state because the optical properties of BZF include a very strong absorbance in the UV region which precludes use of the UV and CD marker lines as determinants of quaternary structure.

In conclusion, the results reported here demonstrate that the protein structure of ferric human hemoglobin may be modified by the addition of IHP and BZF to exert similar forces on the heme as IHP alone does in ferric carp hemoglobin. A significant effect from BZF occurs only when IHP is present, possibly because the former has a smaller effect on

the free energy of the conformational transition of the protein than the latter. Although it is clear from our data that BZF influences the heme spin equilibrium, we do not have any data relating to how it influences the overall protein energetics. Thus, whether or not the R ↔ T equilibrium is also changed cannot be directly addressed by the data reported here. However, there is a growing body of evidence derived mainly from studies of the ferrous state that the energetics at the subunit interface are linked to the heme group via the proximal histidine (Rousseau & Friedman, 1988). If we infer that the shift in the spin equilibrium occurs due to conformational changes in the proximal histidine, then the changes we detect at the heme may be associated directly with changes in the R ↔ T equilibrium as well. The magnitude and the nature of these changes remain to be explored.

Within the limitations stated above, the results of this study are consistent with the hypothesis that although IHP does alter the average quaternary state of human HbA at pH 6.5, it does not cause a complete conversion to the T state. A more complete transition appears to be achieved by the combination of IHP and BZF, although the available data do not permit us to assess quantitatively the extent of this transition. Therefore, the observations of relatively small spin-state changes in human HbA in response to IHP may reflect an incomplete transition from the R to the T state rather than the lack of generality in the linkage between quaternary structure and spin-state equilibrium that has been observed in carp hemoglobin.

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