Resonance Raman and Electron Paramagnetic Resonance Studies of the Quaternary Structure Change in Carp Hemoglobin. Sensitivity of These Spectroscopic Probes to Heme Strain

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Abstract: It is possible to induce a change between T and R quaternary conformations of carp (*Cyprinus carpio*) hemoglobin merely by changing the pH and inositol hexaphosphate concentration. This provides a unique opportunity to examine the properties of liganded and of unliganded hemes in *both* low- and high-affinity conformations by resonance Raman (RR) and electron paramagnetic resonance (EPR) spectroscopies. The change in quaternary structure causes *no* shifts in the heme RR frequencies of the unliganded Hb, or of either high- or low-spin liganded derivatives. However, the induced structure change does alter the high-spin/low-spin equilibrium of azidomet-Hb from 10/90% to 45/55% and changes do occur in the EPR parameters of the low-spin liganded derivatives. These results have led us to examine the relative sensitivities of the two spectroscopic probes to small deformations (strains) in the heme geometry. For a low-spin six-coordinate heme, RR is predicted to be insensitive to small out of plane motions of the metal ion, while EPR is quite sensitive to coordination-geometry deformations. RR might be sensitive to deformations of a high-spin heme; EPR is not.

Introduction

Unliganded mammalian hemoglobins adopt the low-affinity "T state" and the cooperative binding of O_2 or other ligands is in general accompanied by an obligatory transition to the high-affinity "R state".[1] At neutral pH, such fish hemoglobins as that of the carp (*Cyprinus carpio*) also show cooperative effects characteristic of a T $\rightleftarrows$ R switch upon ligation. However, at low pH, particularly in the presence of organic phosphates, both unliganded and liganded forms remain in the T state, whereas at high pH both forms adopt the R conformation.[2-5] Therefore, it is possible to induce a change between T and R merely by changing the pH and effector concentration, and this provides a unique opportunity to examine the properties of liganded and of unliganded hemes in *both* low- and high-affinity conformations.

Resonance Raman spectroscopy provides one probe of heme properties. A number of vibrational bands have been found to exhibit shifts of up to 35 cm^{-1} upon changes in heme spin and oxidation state.[6] The underlying cause of observed variations in vibrational frequency is perturbations in the porphyrin structure, most probably related to "core expansion",[7] or changes in the diameter of the porphyrin "central hole".[8] These "structure-sensitive" Raman bands provide a means of investigating whether changes in protein conformation can indeed induce strains in the heme prosthetic group. As a complementary probe of heme properties, EPR spectroscopy is well established as a monitor of the primary coordination sphere of the ferric iron in various met-Hb forms.[9]

We discuss here our investigation of the effects of the carp hemoglobin (Hb) T $\rightleftarrows$ R conformational conversion upon heme structure through the joint use of these two spectroscopic tools. The change in quaternary structure causes no shifts in the heme RR frequencies of the unliganded Hb, or of either high- or low-spin liganded derivatives. However, the induced structure change does alter the high-spin/low-spin equilibrium of azidomet-Hb, and changes do occur in the EPR parameters of the low-spin liganded derivatives. These results have led us to examine the relative sensitivities of the two spectroscopic probes to small deformations (strains) in the heme geometry. We have formulated a semiempirical relationship between heme strains and RR band shifts and discuss EPR changes, where observed, through the use of other semiempirical theories. For a low-spin six-coordinate heme, RR is predicted to be insensitive to small out of plane motions of the metal ion, while EPR is quite sensitive to coordination-geometry deformations. RR might be sensitive to deformations of a high-spin heme; EPR is not. The observed change in the spin-state equilibrium of azidomet-Hb is discussed in the light of these results.

Experimental Section

Carp blood collected in ACD (acid citrate–dextrose) anticoagulent was processed as in the literature.[4] All EPR and RR measurements were performed on between two and four preparations of carp Hb. Human hemoglobin was prepared as described elsewhere.[10] Both carp and human met-Hb were prepared by the addition of 1.5 molar equiv of $K_3Fe(CN)_6$ for 20 min at 0 °C and immediately desalted on a Rexyn-I-300 (Fisher) column. The met-Hb were judged unperturbed by the criteria of Cameron.[11] An ϵ_{405} 161 mM^{-1} [12] was used for analysis of both human and carp aquomet-Hb.

Optical spectra were recorded on Beckman ACTA III or IV spectrophotometers, while the IHP-induced carp azidomet-Hb optical difference spectrum was recorded on a Cary 17-D instrument. Conditions for the difference spectra were [heme] = 61 μM, [N_3^-] = 0.1 in 0.05 M cacodylate buffer, pH 6.0, with 1 mM IHP, pH 6.0, added to the sample cell.

All carp and human hemoglobin samples for EPR and RR spectroscopic measurements were ~1.0 mM/heme in a medium of 0.05 phosphate–citrate, pH 5.7–6.2 (low pH), or of 0.05 M Tris, pH 8.0–9.0 (high pH). Azido- and cyanomet-Hb samples contained respectively 0.1 M N_3^- and CN^- at both low and high pH in order to assure complete ferriheme ligation at room temperature. Fluoromet-Hb cannot be fully formed at room temperature because of the low F^- association constant.[13] Fluoromet-Hb samples at pH 6 contained 0.3 M NaF and at room temperature had ≥95% fluoride-bound ferrihemes; samples at pH 8.1 contained 0.4 M NaF and were estimated to be 90% fluoride bound. Deoxy Hb samples were prepared from met-HbCN by addition of minimal dithionite. Since the T state of both deoxy- and liganded carp-Hb is stabilized by low pH and the presence of IHP, low-pH samples of deoxy- and cyanomet-Hb were examined with [IHP] = 4, 10, and 20 mM in order to investigate the effects of concentration; no differences were observed. All other low-pH samples contained 10 mM IHP. The pH of a sample was measured, and then half was placed in a 4-mm quartz tube for examination by EPR and frozen in liquid nitrogen; the other half was placed in a 5-mm glass tube for collection of RR spectra and refrigerated.

EPR spectra were obtained from a Varian Associates E-4 spectrometer as described elsewhere.[14] The field linearity was verified with a F. W. Bell 660 gaussmeter, and, in addition, strong pitch (g =

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Table I. Frequencies of Structure-Sensitive RR Bands for Carp Deoxy-Hb, Cyanomet-Hb, and Fluoromet-Hb

deoxy-Hb		cyanomet-Hb		fluoromet-Hb			intensity[b]		
T state[c]	R state[d]	T state[c]	R state[d]	T state[c]	R state[e]	P[a]	*r*	*s*	*t*
1354	1355	1373	1372	1371	1370	p	vs	s	s
1471	1469	1504	1505	1481	1479	p	m	w	w
1545	1543	1560	1562	*f*	*f*	dp	vw	m	s
1557[g]	1559[g]	1589	1588	1556	1557	ap		*h*	m
1564	1565	1583	1585	1563	1561	p	s	m	
1604	1603	1641	1640	1607	1605	dp	m	m	s

[a] P = polarization; p = polarized; dp = depolarized; ap = anomalously polarized. [b] *r* = 4579 Å excitation; *S* = 4965 Å excitation; *t* = 5145 Å excitation; vs = very strong; s = strong; m = medium, w = weak; vw = very weak. [c] ~1 mM in heme, 0.1 M phosphate-citrate pH 5.8 plus 10 mM IHP. [d] ~1 mM in heme, 0.1 M Tris pH 8.8. [e] ~1 mM in heme, 0.1 M Tris pH 8.1. [f] This peak cannot be resolved owing to the proximity of the 1557-cm^{-1} (ap) peak. [g] This peak clearly resolved from the adjacent (1545 cm^{-1}) peak in successive spectra taken with the parallel and perpendicular orientation of the polarized analyzer. [h] This peak is not clearly resolved from its neighboring polarized peak.

2.0027) was used as a field marker. Met-HbN_3 and met-HbF spectra were taken in liquid nitrogen (77 K) while met-HbCN samples were run at 4–15 K using an Air Products LTD-3 apparatus.

Resonance Raman spectra were obtained using a Spectra Physics 164 Ar^+-ion laser for excitation. Spinning glass cells were used in a 180 °C backscattering geometry; spectra were obtained with a 0.85-m Spex 1401 double monochromator with photon counting detection.[15] Polarization data were obtained by placement of a Polaroid analyzer in the backscattered beam. Peak positions are reproducible to ±1 cm^{-1} for all strong bands and to ±2 cm^{-1} for broader and/or smaller bands. Three excitation lines were employed to observe all RR vibrations.

(1) 5145 Å. This line excites into the α-β region and selectively enhances non-totally-symmetric vibrations.[6] The vibrational frequencies and polarizations of all the RR bands between 50 and 1700 cm^{-1} were monitored for deoxy-Hb and the liganded, low-spin met-Hb derivatives. For liganded, high-spin met-Hb, however, this excitation falls at a minimum in the electronic spectrum and give a low resonance enhancement and relatively poor spectra. Thus, only the 1100–1700-cm^{-1} region was monitored with this excitation.

(2) 4965 Å. This laser line is useful for studying the liganded, high-spin met-Hb derivatives, since it is near the visible electronic absorption band maxima for these derivatives. Again, non-totally-symmetric vibrations are selectively enhanced[6] and all bands in the region 50–1700 cm^{-1} were monitored. This excitation was also used to monitor the "structure-sensitive" bands for deoxy Hb and the liganded, low-spin met-Hb derivatives; these bands fall between 1300 and 1700 cm^{-1}.

(3) 4579 Å. In all derivatives this excitation is near the Soret band and enhances the totally symmetric vibrational modes.[6] In all cases, spectra were recorded at 50–1700 cm^{-1}.

Spectra were generally obtained at room temperature (23 °C), with the exception of some carp met-HbF and initial carp deoxy-Hb experiments. These were performed at 4 °C with cooling provided by a stream of cold N_2. The lower temperature promotes binding of the fluoride ligand (especially at pH 8) and, for the deoxy-Hb, helps prevent sample deterioration.

A quantitative study of the effects of spin state on the RR spectra of met-Hb employed high- and low-spin reference RR spectra taken with identical instrument conditions and protein concentration. First, a spectrum of high-spin human fluoromet-Hb (~96% fluoride bound, 99% high spin) was taken (1.0 mM met-Hb in 0.3 M F^- and 0.05 M P_i buffer, pH 6.9). Then this sample was converted to the low-spin, cyanomet-Hb derivative by adding 0.5 M NaCN (pH 6.9) to a final concentration of 0.01 M and its spectrum was recorded. Comparison to a spectrum from another sample containing 0.1 M CN^- showed complete low-spin formation. Such reference spectra were generated with all three excitations. The spectrum of an experimental sample exhibiting a spin-state equilibrium was modeled by computer summing appropriate percentages of the reference spectra. The high/low spin percentages obtained from a best visual fit with a synthetic spectrum have experimental uncertainties of ~ ±5% imposed by the noise of the RR spectra. However, this technique monitors the heme directly and is wholly unaffected by the presence of free azide in solution. As such it presents a decided advantage over the use of infrared spectroscopy. With this latter technique, it is impossible to add excess azide, needed to ensure 100% binding by the hemes, since the IR-active vibrations of azide free in solution have the same frequency as that of the high-spin azidomet-hemoglobin.[16]

Results

Carp hemoglobin can be forced by suitable choice of solution conditions to adopt either the T or R quaternary conformations, independent of the heme ligation state.[2-5] Resonance Raman spectra of a number of carp Hb derivatives in both T and R conformations have been obtained. In all cases, spectra of the analogous human Hb forms have been measured under comparable conditions. Since corresponding spectra of the two proteins are in general similar or even indistinguishable, results for the human Hb are only mentioned as needed. In addition, EPR spectra of the several paramagnetic met-Hb forms have been obtained for carp Hb in the R and T states, and for human Hb under comparable conditions.

A. Deoxy-Hb. Table I lists the frequencies, relative intensities, and polarizations of the more significant high-frequency RR bands for the high-spin, five-coordinate heme of carp deoxy-Hb in both the T state (pH 5.8 plus 10 mM IHP) and the R state (pH 8.8). Results with all three excitations are included. Figure 1 presents the T- and R-state spectra with 4579 Å excitation. Within experimental error, the transition between T and R states induces *no* changes in the frequencies, intensities, or polarizations of any porphyrin vibrational band.

B. Low-Spin Liganded Heme. RR. Previous studies have shown that carp HbCO, a low-spin liganded form of the protein, exhibits a pH-induced R ↔ T conformation change. However, HbCO is photosensitive and difficult to observe in RR.[17] Therefore, we have studied the structural analogue, cyanomet-Hb. This also exhibits the R ↔ T conformational change, as evidenced by a large decrease in CN^- binding constant upon the addition of IHP to solution of the protein at low pH (pH ~6).[18]

The RR spectra of the low-spin liganded heme of cyanomet-Hb in both R (pH 8.8) and T (pH 5.8, 10 mM IHP) states have been obtained using all three exciting wavelengths. Figure 2 presents the spectra in both T and R states with 5145 Å excitation. Again, the T → R transition causes no shift in vibrational peak position or change in band intensity or polarization for *any* band.

EPR. Cyanomet-Hb gives an unresolved EPR spectrum at 77 K because of rapid spin-lattice relaxation. However, lowering the temperature below ~20 K permits observation of the highly anisotropic low-spin cyanoferriheme EPR spectrum for both carp and human proteins. The largest *g* value, g_1, could be measured with reasonable accuracy (±0.02) from ordinary absorption-derivative spectra obtained at low (unsaturating) microwave power. The intermediate *g* value, g_2, was best determined in the absorption-shaped spectrum obtained under adiabatic rapid passage conditions;[19] because of this, however, uncertainty in g_2 is considerably increased (±0.05). The lowest *g* value, g_3, was not accurately obtained using either condition and is not reported.

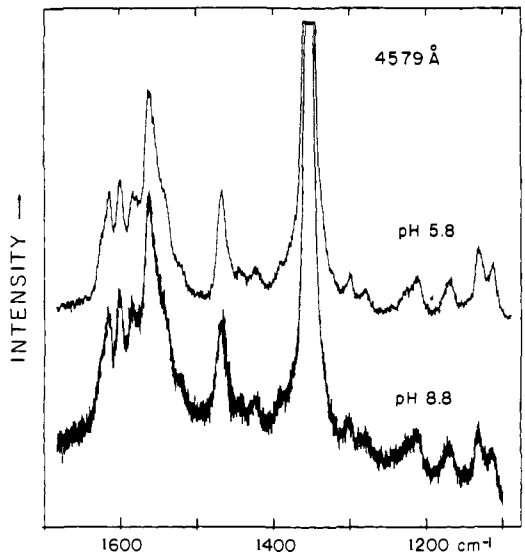

Figure 1. High-frequency resonance Raman spectra of carp deoxy-Hb obtained with 4579-Å (near-Soret) excitation: upper spectrum, T state, 0.05 M phosphate-citrate buffer, pH 5.8 plus 10 mM IHP; lower spectrum, R state, 0.05 M Tris, pH 8.8. Samples are from two different preparations of carp blood and contain minimal amounts of dithionite. Experimental conditions: 40 mW power, 4.5 cm^{-1} slits, T-state spectrum scan rate 10 cm^{-1}/min, R-state spectrum scan rate 20 cm^{-1}/min.

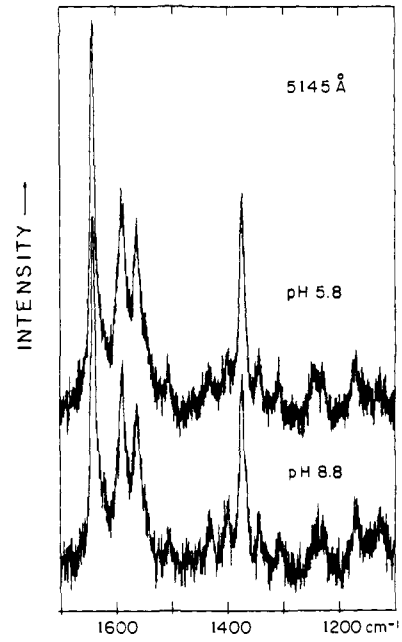

Figure 2. High-frequency RR spectra of carp cyanomet-Hb with 5145-Å (near-visible) excitation: upper spectrum, T-state, ~1 mM in heme, 0.1 M CN^-, 0.05 M phosphate-citrate buffer, pH 5.8 plus 10 mM IMP; lower spectrum, R state, ~1 mM in heme, 0.1 M CN^-, 0.05 M Tris pH 8.8. Experimental conditions: ~100 mW power, 3.7 cm^{-1} slits, 20 cm^{-1}/min scan.

Table II. *g* Values for Carp and Human Azido- and Cyanomet-Hb

		carp		human	
		T state (pH 5.8; 10 mM IHP)	R state (pH 8.8)	R state (pH 5.8; 10 mM IHP)	(pH 8.8)
N_3^-	g_1	2.897	2.816	2.823	2.818
	g_2	2.189	2.207	2.215	2.202
	g_3	1.624	1.695	1.689	1.696
CN^-	g_1	3.48	3.42	3.43	3.44
	g_2	1.92	1.97	1.95	1.95
	g_3[a]				

[a] Not measured.

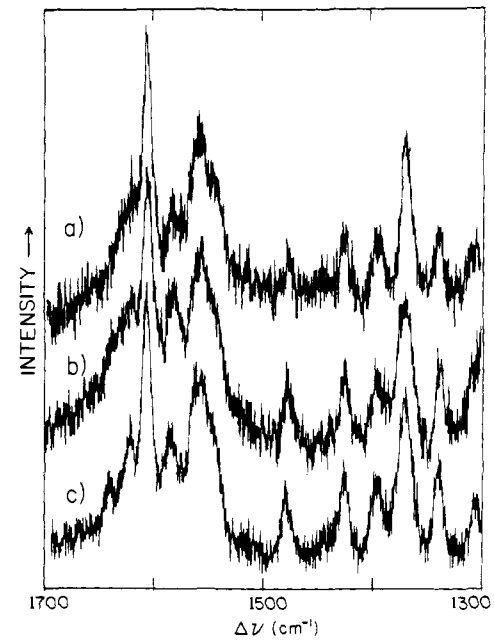

Figure 3. High-frequency RR bands of carp fluoromet-Hb obtained with 4965-Å (visible) excitation: (a) T state, 0.05 M phosphate-citrate buffer, pH 5.7, 0.3 M NaF, 10 mM IHP; (b) R state, 0.05 M Tris, pH 8.1, 0.4 M NaF; (c) synthetically generated spectra obtained by the sum of 95% high-spin plus 5% low-spin state reference spectra. Experimental conditions for (a) and (b): 100 mW power, 4.0 cm^{-1} slits, 10 cm^{-1}/min scan.

The observed *g* values for human and carp cyanomet-Hb are listed in Table II. For the carp protein, the value of g_1 undergoes a small, but significant, increase upon conversion from the R to the T state; g_2 may also change, but the larger limits of error make this uncertain. In contrast, human met-Hb (CN^-) does not change quaternary state upon the same change in solution conditions and also does not show a significant change in *g* values. Thus, the EPR spectra indicate a difference in the properties of the cyanoferriheme of T- and R-state met-Hb (CN^-), even though no such difference is manifested in the RR spectra. Discussion of the *g*-value variations is given below.

C. High-Spin Liganded Heme. RR. Optical spectra[2,20] indicate[21] that high-spin liganded derivatives of carp Hb are R state at high pH and adopt the T-state structure at low pH (+IHP). Aquomet-Hb, of course, cannot be examined under both conditions and so we studied the high-spin species, fluoromet-Hb in the R (pH 8.1) and T (pH 5.7 plus 10 mM IHP) states, at room temperature (~23 °C) and also at 4 °C to maximize fluoride binding. No significant effects of temperature were observed and only room temperature results are discussed. Figure 3 displays the high-frequency portion of the spectra obtained with 4965 Å for R- and T-state carp fluoromet-Hb. The high-frequency bands for 5145-, 4965-, and 4579-Å excitation of both quaternary states are listed in Table I. No RR band shows *any* change in position or polarization upon the R-T transition.

The peaks in the spectrum at pH 5.8 (Figure 3a) do show slight intensity differences if compared to those in the spectrum at pH 8.1 (Figure 3b). These differences can be attributed to

Table III. Frequencies of Structure-Sensitive RR Bands of Carp Azidomet-Hb

	T state			R state			
P[a]	4579 Å	4965 Å	5145 Å	4579 Å	4965 Å	5145 Å	spin[b]
p	1374 (vs)	1373 (s)	1375 (s)	1374 (vs)	1372 (s)	1374 (s)	hs, ls
p	1478 (w)						hs
	1505 (w)	1504 (w)	1505 (vw)	1505 (w)	1502 (w)	1503 (vw)	ls
p[c]	1562 (m)	1558 (m)					hs
	1582 (s)	1582 (m)		1584 (s)	1583 (m)		ls
ap			1559 (m)				hs
			1587 (m)			1586 (m)	ls
dp	1603 (w)	1603 (m)	1605 (m)				hs
	1640 (m)	1639 (m)	1641 (s)	1640 (s)	1639 (m)	1639 (s)	ls

[a] See Table I for abbreviations used. [b] All bands listed are characteristic of the ferriheme; hs and ls indicate the spin state exhibited by each band. [c] Interference from nearby bands makes the position and polarization of these bands the least reliable.

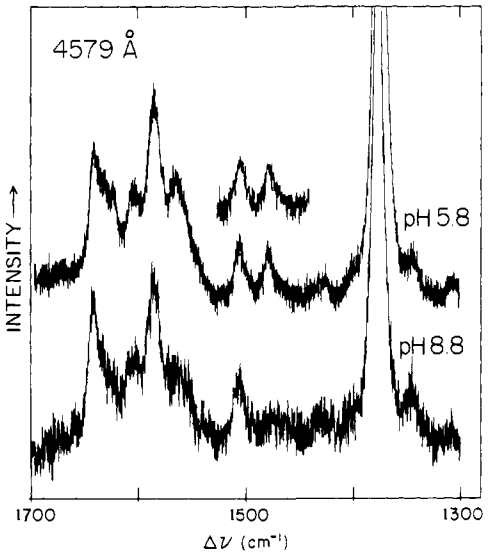

Figure 4. High-frequency RR spectra of T- and R-state carp azidomet-Hb using 4579-Å excitation: upper, T state, 0.1 M NaN_3, 0.05 M phosphate-citrate buffer, pH 5.8, plus 10 mM IHP; lower, R state, 0.1 M NaN_3, 0.05 Tris, pH 8.8. Experimental conditions: ~79 mW power, 4.5 cm^{-1} slits, T-state spectrum 10 cm^{-1}/min scan, R-state spectrum 20 cm^{-1}/min scan. Inset: a computer-summed spectrum consisting of 55% low-spin state plus 45% high-spin state reference spectra.

the presence of a minor amount of low-spin met-Hb at pH 8.1, which is present because the residual hydroxymet-Hb is in a spin-state equilibrium. Figure 3c is a spectrum synthesized (see Experimental Section) by adding 95% of a pure high-spin spectrum (human met-HbF) and 5% of a pure low-spin spectrum (human met-Hb(CN^-)). The effects of adding a low-spin component to the high-spin reference spectrum (Figure 3a) are clearly similar to the effect seen upon raising the pH. Thus, there are *no* changes in the RR spectrum of a high-spin liganded heme upon R ↔ T interconversion.

EPR. The EPR spectrum of human fluoromet-Hb is typical of a high-spin ferriheme, with $g_\perp = 6$, $g_\parallel = 2$.[9] At 77 K a ^{19}F hyperfine splitting from the ligand is observed at $g_\parallel$, but the perpendicular splitting is resolved only at lower temperatures. EPR measurements at 77 and at 10 K show no measurable difference between the R- and T-state protein. Thus, neither the RR nor EPR spectra show an effect of R ↔ T interconversion on a high-spin ferriheme.

D. Spin Equilibrium Liganded Heme. Carp azidomet-Hb can also be forced to adopt either R or T conformation, depending on conditions. First, as with the CN^- ligand, we find addition of IHP to solution of carp met-Hb to cause a significant reduction in the affinity for azide as a ligand (~threefold at pH 6). Second, at low pH in the presence of a saturating azide concentration (0.1 M), large differences in optical spectra are observed upon addition of IHP to azidomet-Hb. The UV region of the IHP-induced difference spectrum exhibits maxima at 279 and 287 nm and a minimum at 293 nm. (Such a difference spectrum has been interpreted as indicating an R ↔ T conversion.[21]) The visible region of the carp azidomet-Hb IHP-induced difference spectrum exhibits maxima at 505 and 631 nm and minima at 543 and 572 nm,[20] consistent with the change from a largely low-spin heme in the R form (−IHP) to an equilibrium mixture of high- and low-spin hemes in the T (+IHP) form.

RR. There is *no* change in the positions of the low-spin ferriheme structure-sensitive bands upon change in the carp azidomet-Hb quaternary structure (Table III). However, the R-T conversion causes changes in the relative peak intensities and the appearance of new bands (Figure 4 and ref 10). Azidoferrihemes are well-known to exhibit spin equilibria,[22,23] and, in light of the IHP-induced change in the optical spectrum, these observations are such as would be expected if R-state carp met-Hb(N_3^-) is largely, but not totally, low spin and suffers an increase in the fraction of high-spin ferriheme upon R → T conversion.

Consider first R-state (pH 8.8) carp azidomet-Hb. With 4579-Å (Figure 4, lower) and 5145-Å excitation (not shown), close inspection shows subtle intensity differences from the low-spin reference sample in the relative intensities within a spectrum. With 4965-Å excitation, there are noticeable differences in the relative band intensities between the spectrum of carp met-Hb(N_3^-) and that of a low-spin reference.[10] Comparison with computer-generated spectra shows the R-state azidomet-Hb to be 10 ± 5% high spin. A similar analysis of human azidomet-Hb spectra at high pH yields ~10% high spin, in satisfactory agreement with results from other techniques.[22-24]

Upon conversion of carp azidomet-Hb to the T state (pH 5.8, 10 mM IHP), there are *no* changes in the positions of any RR band. However, the feature in the high-frequency RR spectra associated with high-spin ferrihemes become quite pronounced. With excitation at 5145 Å, the T-state spectrum shows the appearance of two "high-spin" bands at 1605 and 1559 cm^{-1} (Table III); the latter is anomalously polarized and particularly distinct in polarization spectra. With excitation of the T-state protein at 4579 Å (Figure 4), the 1478-cm^{-1} high-spin band is clearly seen and the 1505- and 1640-cm^{-1} low-spin bands decrease in intensity relative to the 1372-cm^{-1} band. Finally, with 4965-Å excitation, the T-state spectrum shows a marked decrease in the intensity of the 1640-cm^{-1} (dp) band and a definitive increase in the 1603-cm^{-1} (dp) band.

The readiest measure of the high-low-spin equilibrium ratio in the T state is afforded by the relative intensities of the "low-spin" band at 1505 cm^{-1} and the "high-spin" band at 1478 cm^{-1}. Computer-generated spectra obtained by adding

Table IV. Crystal Field Parameters for Azidomet-Hb[a]

		$\tilde{V}$	$\tilde{\Delta}$
	Human[b]		
R state	(pH 8.8)	2.33	4.73
	(pH 5.8 + 10 mM IHP)	2.29	4.56
	Carp[b]		
R state	(pH 8.8)	2.31	4.68
T state	(pH 5.8 + 10 mM IHP)	2.04	4.43

[a] $\tilde{V}$ and $\tilde{\Delta}$ are given in units of the iron spin-orbit coupling constant (λ),[28] and are calculated from the data in Table II as described in ref 28. [b] For complete solution conditions, see text.

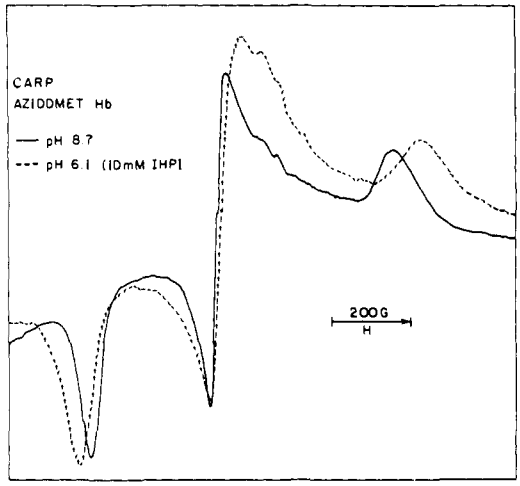

Figure 5. EPR spectra of carp azidomet-Hb in the T state (1 mM in heme, 0.1 M N_3, 0.05 M phosphate-citrate, pH 6.1 plus 10 mM IHP) and in the R state (1 mM in heme, 0.1 M N_3^-, 0.05 M Tris, pH 8.7) at 77 K. Instrumental conditions: modulation amplitude, 16 G; power, 10 dB; time constant, 0.1 s; scan time, 8 min for 2000 G.

45 ± 5% high-spin and 55 ± 5% low-spin reference spectra match the relative intensities quite well (see inset, Figure 4). The relative intensities in the 1640- and 1603-cm^{-1} bands, obtained with 4965-Å excitation, are also best matched using these percentages, although the reference spectrum exhibits better resolution of the 1603-cm^{-1} band than is observed experimentally. Thus, it appears that at room temperature, R-state carp azidomet-Hb is ~10% high spin, 90% low spin, and becomes ~45% high and 55% low spin in the T state. In contrast, human azidomet-Hb remains in the R form at pH 5.8, 10 mM IHP, and the percentage of high-spin hemes remains unchanged.

EPR. Azidomet-Hb gives a well-resolved EPR spectrum at 77 K, typical of a low-spin ferriheme with a rhombic **g** tensor (Figure 5). The three g values obtained for human met-Hb(N_3^-) at pH 8.8 are in good agreement with those previously reported,[25] and the g values for R-state carp azidomet-Hb (pH 8.8) are comparable. At low pH (pH 5.8, 10 mM IHP) the human protein, which remains locked in the R state, exhibits small changes in the g values. However, conversion of the carp protein to the T state with the low-pH medium causes appreciable changes in the spectra and calculated g values. Thus, the EPR results indicate that the properties of a low-spin liganded ferriheme are influenced by the change from R to T conformations. In addition, only low-spin EPR signals are observed for R-state carp met-Hb(N_3^-), but a small percentage of high-spin signal ($g \sim 6$) is seen in the T state. This suggests that the spin states may be in equilibrium even at 77 K, but the amount of this high-spin signal varied somewhat depending on the manner of freezing the sample and quantitation of the signals was not attempted.

The changes in EPR spectra of human and carp azidomet-Hb can be interpreted in terms of the usual model of the ferric ion of a low-spin ferriheme, due to Griffith[26] and Kotani,[27] and described by Weissbluth.[28a] The model assumes that the porphyrin and axial ligands subject the metal to noncubic ligand fields, with an axial component, $\tilde{\Delta}$, along the heme-normal (z axis), and a smaller rhombic component, $\tilde{V}$, proportional to the difference in ligand field strength along x and y axes. The values of $\tilde{\Delta}$ and $\tilde{V}$ in units of the iron spin-orbit coupling constant are readily obtainable from measured g values.[28] The results for azidomet-Hb with $\tilde{\Delta} > 0$ correspond in crystal-field terminology to an axial "compression", with axial ligand-field strengths larger than the in-plane ligand field.

Table IV contains the calculated values of ($\tilde{\Delta}$, $\tilde{V}$) for human and carp azidomet-Hb at high pH and at low pH in the presence of IHP. Previous work reported comparable g values, but had calculated the crystal field values incorrectly.[29] The values for the two proteins at high pH are quite comparable. Since human azidomet-Hb is locked in the R state, the changes which occur upon going from high- to low-pH medium must be tertiary in nature. They correspond to a small decrease in $\tilde{V}$, $\delta\tilde{V} \sim -0.05 \pm 0.01$, and a noticeably larger decrease in $\tilde{\Delta}$, $\delta\tilde{\Delta} \sim -0.19 \pm 0.01$ (Table IV). The latter indicates that the axial and in-plane bonding become more nearly equal. The former means that the rhombic distortion of the iron is lessened. In contrast, the carp protein, which undergoes a quaternary structure change, exhibits large decreases in both $\tilde{V}$ and $\tilde{\Delta}$ (Table IV).

The relatively incomplete data for the cyanomet-Hb makes a quantitative treatment unreliable. However, the nature of the g-value changes for the carp protein is consistent with a similar kind of change in crystal-field parameters upon the R → T conversion. Recall that changing the medium caused no change in the EPR of R-state human cyanomet-Hb.

Discussion

The T ⇄ R conversion does not cause observable change in the RR band positions for any carp hemoglobin derivative. However, the conversion does cause changes in the EPR spectra of low-spin met-Hb forms. We first discuss the respective sensitivities to heme strain of the two techniques. We then consider the effect of quaternary structure on the spin equilibrium of azidomet-Hb in the light of these observations.

Sensitivity to Heme Strain. Attempts to relate heme geometry to RR frequencies by detailed calculations are currently underway.[30] However, at present we believe a semiempirical approach to be particularly useful for understanding the effects of *small* perturbations. Consider an unconstrained heme in a spin and ligation state labeled i, with an RR band at frequency ν_i. The frequency, ν, which is observed after an applied stress causes an infinitesimal change in a particular heme coordinate, r, can in principle be expressed by a Taylor's series.

$$\nu = \nu_i + \left(\frac{\partial \nu}{\partial r}\right)_{r_i} \delta r + \tfrac{1}{2}\left(\frac{\partial^2 \nu}{\partial r^2}\right)_{r_i} (\delta r)^2 + \ldots \qquad (1)$$

Frequently eq 1 will be reexpressed in terms of $\delta\nu = \nu - \nu_i$, the shift in vibrational frequency upon deformation. Parametrizing eq 1 will allow us to place limits of δr. The observation that no RR band shifts by ±1 cm^{-1} means that $|\delta\nu| < 2$ cm^{-1} upon T ⇄ R conversion. This approach is only valid for small perturbations around a defined reference state and will not *explain* the relationship between ν and r, but will provide a *correlation* between small changes in each.

How can eq 1 be parametrized? Spaulding et al.[8] noticed that for a wide range of metalloporphyrins the frequency of

the anomalously polarized band at ~1590 cm^{-1} is not obviously correlated with either the distance of the metal atom from the porphyrin plane (Δ) or with the metal-N(pyrrole) distance (R), but is smoothly related to the size of the porphyrin "central hole", or d. The relationship is in fact very close to linear as is shown[10b,c] by a plot of the data from Table II[31] of Spaulding et al.[8] Several other bands have also been shown to vary linearly with d.[32] For simplicity, these discussions will focus on the *ap* band; the others may be treated equivalently. The linear variation means that to good approximation one may associate $r \rightleftarrows d$ in eq 1, while retaining only the linear term

$$\delta\nu = \nu - \nu_i = \left(\frac{\partial\nu}{\partial d}\right)_{d_i} \delta d \tag{2a}$$

From a linear least-squares fit to the data of Spaulding et al.[8] one obtains $(\partial\nu/\partial d)_{d_i} = -500\ cm^{-1}/Å$:

$$\delta\nu = -(500\ cm^{-1}/Å)\delta d \tag{2b}$$

It will also prove useful to invert the linear relationship (eq 2b) to express d in terms of ν:

$$\delta d = d - d_i = \left(\frac{\partial d}{\partial \nu}\right)_{\nu_i} \delta\nu \tag{3a}$$

$$\delta d \approx -(0.002\ Å/cm^{-1})\delta\nu \tag{3b}$$

Using eq 3, the invariance of the "1590" band to $\pm 1\ cm^{-1}$ shows only that the R-T conversion changes the diameter of the central hole by less than 0.004 Å. However, taking these equations to be the fundamental relationship between structure and vibrational frequency, it is also possible to obtain secondary equations which relate RR changes in frequencies to changes in other distances in the coordination geometry, and, in particular, to changes in Δ. We write (d_i, R_i, Δ_i) as the appropriate uncontrained distances for an i-state heme, with the value of the associated vibrational frequency, ν_i, related to d_i by eq 2. Imposition of a stress will produce small deviations from these values, δR, $\delta\Delta$, δd, and also the associated $\delta\nu$ given by eq 2. The well-known flexibility of the porphinato macrocycle[33] suggests that, in a low-spin porphyrin, the small stress which causes a slight heme deformation would alter the porphyrin conformation through changes in Δ_i and d_i, *without* an energetically prohibitive equivalent change in the covalent bond length, R_i. The Fe-N_c bond is thought to be much weaker in high-spin heme,[30] but crystal structures of a variety of heme models[34] suggest that changes in Δ might nevertheless correlate with changes in d. We thus examine a model in which the deformed geometry of an i-state heme is related to its perturbed RR frequency simply by using the Pythagorean theorem

$$R^2 = R_i^2 = (d_i + \delta d)^2 + (\Delta_i + \delta\Delta)^2 \tag{4}$$

and relating δd and $\delta\nu$ through eq 2 and 3.

In expanding eq 4 it is instructive to simplify further. We ignore terms in $(\delta d)^2$. For those states i where $\Delta_i \neq 0$, $(\delta\Delta)^2$ is also ignored. Low-spin hemes, with $\Delta_i = 0$, are a separate case; the term linear in $\delta\Delta$ vanishes, and the quadratic term is required. This is a general symmetry requirement of considerable importance and will be returned to. Utilizing eq 3 and these approximations, the two cases become

$$\text{low-spin liganded heme: } \delta\nu = \frac{-1}{2d_i}\left(\frac{\partial\nu}{\partial d}\right)_{d_i} (\delta\Delta)^2 \tag{5a}$$

$$\approx +(125\ cm^{-1}/Å^2)(\delta\Delta)^2 \tag{5b}$$

$$\text{other states, } i\text{: } \delta\nu = -\left(\frac{\Delta_i}{d_i}\right)\left(\frac{\partial\nu}{\partial d}\right)_{d_i} (\delta\Delta) \tag{6a}$$

$$\approx +(250\Delta_i\ cm^{-1}/Å)(\delta\Delta) \tag{6b}$$

We have also taken $d_i = 2.0$ independent of i.

From eq 5, we see that this "geometric" model predicts the RR of low-spin hemes to be rather insensitive to the position of the metal atom: the experimental result that $\delta\nu < \pm 2\ cm^{-1}$ means that Δ between R and T states could be $\lesssim 0.1$ Å, yet go undetected by RR spectroscopy!

Considering the crudeness of these estimations, it is important to realize that the results embodied in eq 5 are required by general considerations, independent of the details of a model. As noted by Hopfield,[35] a low-spin six-coordinate heme, with $\Delta \sim 0$, has an approximate reflection plane lying through the heme. By symmetry, then, in this case, $(\partial\nu/\partial\Delta)_{\Delta=0} = 0$, and the linear term in eq 1 must vanish if $r \Leftrightarrow \Delta$. Thus, any means of parametrizing eq 1 must give a result of the form of eq 5a,in which $\delta\nu \propto (\delta\Delta)^2$, with ν insensitive to small changes in Δ.

What about the EPR of a strained low-spin ferriheme? Again taking the simplest semiempirical approach, the observed changes in EPR spectra can be readily interpreted through use of the angular overlap model (AOM)[36] for bonding to transition-metal ions. In the AOM it is assumed that splitting of d orbitals occurs because they undergo differential antibonding interactions with the filled ligand. The contributions of a given ligand are written $\epsilon_\lambda{}^q = (\epsilon_\lambda{}^q)_0 S_{\lambda,q}{}^2$, where λ represents the symmetry of the interaction, (σ, ϵ, δ), q indexes the ligand, the $S_{\lambda,q}$ are the metal-ligand overlap integrals, and the $(\epsilon_\lambda{}^q)_0$ are the proportionality constants to be obtained from experiment.

Using the AOM and the observation that the Fe-N_3 and imidazole planes very nearly coincide,[37] then $\tilde{V}$ is the sum of the energies of interaction of d_{y^2} with the two axial ligands and $\tilde{\Delta} = \tilde{\Delta}_p + \tilde{V}/2$, where $\tilde{\Delta}_p$ is the contribution to $\tilde{\Delta}$ from interaction with the porphyrin macrocycle. Thus, a protein-induced heme strain which influences only the metal-porphyrin interaction would change only $\tilde{\Delta}$ through $\tilde{\Delta}_p$, leaving $\tilde{V}$ unaffected. Conversely, a strain influencing only the strength of the axial bonds should change $\tilde{V}$ and $\tilde{\Delta}$ roughly in a ratio $\delta\tilde{\Delta}\tilde{V}/\delta\tilde{\Delta} \sim 2$.

The tertiary changes which occur in human azidomet-Hb upon going from high to low pH correspond to a small decrease in $\tilde{V}$, $\delta\tilde{V} \sim -0.05 \pm 0.01$, and a noticeably larger decrease in $\tilde{\Delta}$: $\delta\tilde{\Delta} \sim -0.13 \pm 0.01$ (Table III). Since any change in axial ligation should change $\tilde{V}$ by almost twice as much as $\tilde{\Delta}$, this shows that the tertiary changes primarily influence the heme by changes in the metal-porphyrin interaction, $\tilde{\Delta}_p$.

On the other hand, the effects of the quaternary structure change in carp azidomet-Hb give large, comparable changes in both $\tilde{V}$ and $\tilde{\Delta}$. If these may be decomposed further into tertiary contribution plus an additional, purely quaternary effect, that is if $\delta\tilde{V} = \delta\tilde{V}$ (tert) + $\delta\tilde{V}$ (quat) and similarly for $\delta\tilde{\Delta}$ with the tertiary changes equal to those of the human protein, then one obtains $\delta\tilde{V}$ (quat)/$\delta\tilde{\Delta}$ (quat) $\sim (-0.23 \pm 0.02)/(-0.10 \pm 0.02)$. This is close to the approximate factor of 2 expected if the R $\rightarrow$ T conversion affects only the axial ligation through decreases in bond strength of imidazole and/or azide.

These qualitative pictures of the effects of tertiary and quaternary changes on the heme can be given a geometric interpretation for comparison with the model for frequency shifts in RR. From the ($\tilde{V}$, $\tilde{\Delta}$) entries in Table III, values of the $\epsilon_\lambda{}^q$

can be obtained and, by calculating overlap integrals, the proportionality constants can be parametrized. With this done, then the observation of a $\delta\tilde{V}$ (quat) and $\delta\tilde{\Delta}$ (quat) upon R $\rightleftarrows$ T switch can be used to obtain the $\delta\epsilon_\lambda{}^q$ which are proportional to $\delta(S_{\lambda,q}{}^2)$, the change in the squared overlap caused by, say, a bond elongation. Using any one of several methods of calculating overlap integrals, we find that the change in EPR parameters can be accounted for by small deformations. For example, assuming again that R_i remains fixed, but that one of the axial bonds elongates upon R $\rightleftarrows$ T switch in carp azidomet-Hb, then the change in g values corresponds to a bond elongation of $< +0.06$ Å. Other types of deformation and indeed other mechanisms[39] might contribute in small or large measure to the observed (δV, $\delta\Delta$), and this calculation and the RR models do not directly compare the same coordinate within the Fe coordination sphere. We nonetheless see that the semiempirical models are in harmony in that, even assuming all changes in EPR parameters to result solely from a single heme deformation, this deformation would be below the predicted limit of observability in RR.

The case of a high-spin heme is different and less clear. Equation 6 predicts a far greater sensitivity of the high-spin hemes to a small metal motion, $\delta\Delta$, with the sensitivity increasing with Δ_i. Correspondingly, the general symmetry considerations show that when $\Delta_i > 0$, as in the high-spin hemes, then the leading term in eq 1 will be linear in Δ, as is true in eq 6. Using the values of Δ_i for high-spin met-Hb[40] and deoxy-Hb[41] and the fact that we observe *no* frequency shifts in any band, but in particular the (ap) band, upon R $\rightleftarrows$ T conversion of fluoromet-Hb or of deoxy-Hb, eq 6 suggests that for these Hb forms any differential protein-induced strain of the heme coordination geometry is well under several hundredths of an ångstrom. Similarly, the model applies to the five-coordinate, low-spin cobaltous porphyrin of coboglobin, and validates the conclusion that no "heme strain" occurs in this hemoglobin analgoue.[42] However, if the Fe-N_p bond is indeed subject to stretching, the geometric parametrization breaks down; correspondingly, the leading term in Δ, eq 1, may be linear, but its magnitude might well be very small.

Thus, in general EPR is quite sensitive to small deformations of a low-spin heme, while RR is not. We show elsewhere[43] that the NO ferrohemes provide an interesting special case, in which RR provides a useful criterion for discussing the heme-iron coordination state, whereas EPR does not. In contrast, RR might be a good monitor for strains of a high-spin heme (or five-coordinate low-spin metalloporphyrin) but the question is open. EPR should not be sensitive to small deformations of a high-spin heme: changes in the out of plane distance of the Fe-axial ligand distance should alter the zero field splitting, D, but, because D is much larger than the Larmor frequency, the high-spin heme EPR spectrum is virtually independent of this parameter.[28] In short, the two spectroscopies appear to have at best complementary applications to hemoproteins.

Several additional comments are in order. First, assuming the validity of eq 6, it still cannot be used to relate two hemes in different states, say to discuss the small differences in ν between aquomet-Hb and fluoromet-Hb or the absence of differences between the α and β chain hemes of aquomet-Hb.[7] The model employs the reference geometry adopted by a particular state i. This is primarily determined by R_i and must be "parametrized" for each individual state, as witnessed by the appearance of Δ_i in eq 6. In fact, the relatively small difference in frequencies between deoxy- and fluoromet-Hb, when analyzed with eq 2, simply requires that the difference in R_i between these two states is accommodated more by a change in Δ than by a change in d. Thus, there need be no contradiction between the present conclusions and those of Spiro et al.[7] regarding the RR of high-spin hemes.

Second, the limitations we place on a metal motion in a sense complement those placed by EXAFS.[44] That technique places upper limits on δR, but is wholly insensitive to deformations which do not change bond lengths in the coordination sphere. We assume $\delta R = 0$, and then place upper limits on other types of deformations.

Spin Equilibrium in Hb(N_3^-). Both carp and human azidomet-Hb exhibit a high-spin (hs)-low-spin (ls) equilibrium at ambient temperatures. Changing from a high-pH medium to low pH with IHP present does not influence the quaternary structure of the human protein[12] and likewise causes no change in the spin equilibrium. However, the R $\rightarrow$ T conversion of the carp protein produced by the change in medium does cause an appreciable change in the equilibrium between high- and low-spin forms. If all hemes of the tetramer are equivalent, the equilibrium constant, K_s, describing the ratio of high-spin (hs) and low-spin (ls) azidoferrihemes for a given quaternary conformation is

$$K_s = \frac{[\mathrm{hs}]}{[\mathrm{ls}]} = \frac{g_{hs}}{g_{ls}} e^{-\Delta G_s/RT} \qquad (7)$$

where $g_i = 2S_i + 1$ and $S_{hs} = 5/2$, $S_{ls} = 1/2$. From the analysis of the RR spectra (Figure 4) the equilibrium constant for the R state is $K_s{}^R = 0.11$, corresponding to the hs state being higher in free energy than the ls state with $\Delta G_s{}^R = 1.9$ kcal/mol, while in the T state $K_s{}^T = 0.8$, corresponding to $\Delta G_s{}^T = 0.8$ kcal/mol. Thus, assuming equivalent hemes, the switch from R $\rightarrow$ T decreases the free-energy difference between high- and low-spin azidohemes by $\delta\Delta G_s \approx 1.1$ kcal/mol. A contrary assumption would be that carp Hb has the equivalent of α and β chains and that only one chain is affected upon R $\rightarrow$ T conversion, changing from ~5% hs to ~90% hs with a $\delta\Delta G_s = 2.6$ kcal/mol. This seems unlikely for a variety of reasons. First, the EPR of T-state azidomet Hb does not appear to be a superposition of an R-state spectrum with a perturbed spectrum (Figure 5), as would be the case if half the hemes were unaffected. In addition, studies[2-5] of ligand binding to carp Hb locked in the T state do not suggest appreciable chain inequivalence. Thus, we tentatively discount any appreciable contribution from chain nonequivalence.

The lessened free-energy difference between hs and ls azidoferrihemes in state carp met-Hb(N_3^-) could, in the extreme, arise either from a relative destabilization of the ls form or from a relative stabilization of the hs form. Since the above analysis of frequency shifts suggests that the hs form is not appreciably perturbed upon the R $\rightarrow$ T switch, while the EPR results indicate that the ls form is perturbed, it is natural to suggest that the effect of R $\rightarrow$ T is mainly experienced by the ls form. The effect of the R $\rightarrow$ T transformation is unlikely to be simply localized in a stretching of the Fe-N_ϵ (histidine) bond. The force constant for this bond should be ~100 kcal/Å^2.[35] To store $\delta\Delta G_s = 1.1$ kcal/mol by stretching such a bond would require an elongation of ~0.15 Å. The discussions above indicate that such a deformation should give a greater change in the EPR spectrum, and is expected to cause observable changes in the RR frequencies.

Finally, the addition of IHP to carp met-Hb (pH 6.0) causes a reduction in affinity for N_3^- by ~1/3, about twice the effect observed with human met-Hb. This added effect must be due to the R $\rightarrow$ T conversion in the fish Hb and any attempt to physically and mathematically describe it must incorporate the changes in the hs-ls equilibrium reported here. However, no final description is yet possible. The simplest interpretation of the azide affinity difference between the R and T states would involve only properties of the heme. However, incorporating the magnitude of the changes in both overall affinity and in the hs/ls ratio, one finds that the affinity of both hs and ls forms has been reduced. In short, both ls and hs forms of the azidoferriheme increase in free energy relative to the hs aquoferriheme and so the conformation change must also af-

fect the free energy of one or both of the hs heme derivatives involved. Other interpretations, however, might give a quite different picture of the influence of the T-R switch. For example, the affinity decrease in T can also be described by assuming a spin-state change and a modest decrease in IHP affinity upon conversion to T. In order to complete the description of the free-energy balance in carp Hb, a complete study of N_3^- and IHP binding to carp met-Hb would be required, including the difficult task of looking for cooperativity in N_3^- binding and/or studying the Hb/met-Hb(N_3) redox equilibrium.[45]

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Possible Interpretation of Long-Wavelength Spectral Shifts in Phytochrome P_r and P_{fr} Forms

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Abstract: Semiempirical calculations of the chiroptic properties qualitatively support the anionic form of phytochrome P_{fr} proposed by Rüdiger and co-workers, in which conjugation between rings A and B is interrupted. Among the infinity of conceivable skewed conformations of the chromophores of P_r and P_{fr} some are found which fit the observed chiroptic data in a consistent fashion. It is concluded that the loss of conjugation between rings A and B entails a change of conformation on going from native P_r to native P_{fr}.

I. Introduction

A remarkable fact in nature is the analogy between photochemically active pigments in plants and oxygen-binding pigments and their breakdown products in animals. We have previously[1,2] studied the long-wavelength chiroptic properties of gall pigments specifically bound to serum albumin. From a semiempirical quantum-chemical interpretation of the results we have drawn conclusions on possible conformations of bilirubin and biliverdin in their protein complexes, hoping to contribute to the understanding of the transport and transfer mechanisms of these molecules in vivo. In the present investigation we consider photochemically active phytochrome,[3] the structure of which is closely related to biliverdin and which, in the plant cell, is also specifically bound to a protein. Two

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