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# Spin Equilibrium and Quaternary Structure Change in Hemoglobin A. Experiments on a Quantitative Probe of the Stereochemical Mechanism of Hemoglobin Cooperativity†

Kar Cheong Cho‡ and J. J. Hopfield*

ABSTRACT: The molecular mechanism of hemoglobin cooperativity was studied kinetically by flash photolysis on mixed-state hemoglobins which consist of three ferrous carboxy subunits and one hybrid ferric subunit including fluoromet, azidomet, cyanatomet, and thiocyanatomet. The effects of conformational transitions on the hybrid subunit were detected by kinetic absorption spectroscopy after the CO was fully photodissociated from the binding sites by a large pulse of light from a tunable dye laser. The hemoglobin conformational transition rate was observed to depend on its state of ligation. At 22 °C, pH 7, and 0.1 M phosphate, the deoxy R → T conformational change rate is $4 \times 10^4$ s$^{-1}$. The rate decreases to $1.4 \times 10^4$ s$^{-1}$ for singly ligated hemoglobin. The R → T conformation change alters the energy separation between the high- and low-spin states for azidomet, cyanatomet, and thiocyanatomet subunits by about 700, 300, and 300 cal/mol, respectively. There are two possible implications of this result: (1) the iron atom spin state is not the only major factor in the determination of its position with respect to the heme plane or (2) the change with conformation of the protein force exerted by the proximal histidine on the iron atom (for an iron to heme-plane displacement of less than 0.3 Å) is less than 50% of that expected from simple models in which this motion is responsible for cooperativity.

The essence of the cooperative binding of oxygen by mammalian hemoglobin lies in the several 100-fold difference in the oxygen affinity between the two quaternary structures of the hemoglobin molecule. While there are measurable structural changes within one particular subunit when a second subunit is ligated, the energy of binding at a given subunit depends chiefly on the quaternary structure (and pH, temperature, and other aspects of solution conditions), not on the state of ligation per se of the other subunits (Shulman et al., 1975; Perutz, 1976). One thus seeks a quantitative structural understanding of the way in which the binding energy of oxygen (or other ligands) can affect and be affected by the quaternary structure of the protein. The problem is made difficult by the fact that many of the observed structural changes do not have a significance for the oxygen affinity, by the fact that only a small amount of the affinity change is localized as an energy at the heme (Ogawa & Shulman, 1972; Susser et al., 1974), and by the myriad of small structural changes observed.

The most conspicuous heme structural change occurring upon oxygenation is the movement of the iron atom from 0.6 Å out of the mean porphyrin plane into coplanarity with the heme (Perutz, 1970; Fermi, 1975). Simultaneously, the iron changes from a high-spin to low-spin state. The conventional explanation for such a 0.6-Å geometrical difference, according to Hoard (1971), is that the high-spin deoxy iron atom has too large an effective ionic radius to be fitted into the porphyrin core. However, the out-of-plane nature may also arise from steric repulsions between the axial ligands and the porphyrin nitrogen orbitals (Olafson & Goddard, 1977; Warshel, 1977). Hoard (1971) and Perutz (1970) have both proposed that the iron–proximal histidine coordinate is central to the cooperativity and that the $\delta_L$ movement of 0.6 Å acts as a trigger for the concerted movement of the protein framework. The more recent model compound studies (Kastner et al., 1978) combined with EXAFS studies (Eisenberger et al., 1979) put into question the simple connection between spin, ionic radius, and

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geometry of heme compounds and raise quantitative questions about the ambiguous "out of mean heme plane", but the general idea of a domed deoxyheme and a planar oxyheme seems verified. Following up on his trigger mechanism, Perutz (1972) further proposed that the reduced affinity in the T state can be explained by the globin exerting a tension on the heme along the iron–histidine direction which "raises the spin state of the iron and diminishes the oxygen affinity by opposing the change to low-spin state". The quantitative behavior of this heme tension or protein force (if it indeed exists) as a function of the out-of-plane displacement $\delta_L$ of the Fe atom should quantitatively describe the modification of the binding energy of the heme by the action of *this particular coordinate*. (Other heme coordinate changes on ligation may also be important.) By assuming that the cooperative free energy is not localized in a few bonds, and assuming with Perutz that the heme doming to planar geometry change was the only significant geometry change, Hopfield (1973) predicted that in the T structure there should exist a constant protein force of about 6 kcal/Å along $\delta_L$.

To support his heme tension theory, Perutz and co-workers (Perutz et al., 1974a–c, 1978; Messana et al., 1978) have advanced spectroscopic results on various Hb derivatives upon addition of IHP, a chemical which for some ligands can presumably switch the equilibrium from R to T by locking the Hb tetramer in the T structure. The IHP-induced spectral changes are interpreted as demonstrating extra heme tension associated with the T state. Such conclusions have been questioned by Henseley et al. (1975) and Edelstein & Gibson (1975), who suggested an additional intrinsic effect of IHP in various Hb derivatives. Although it has been verified by X-ray studies that IHP can indeed switch specifically fluoromet-Hb to the T structure (Fermi & Perutz, 1977), the possible side effects of IHP remain unsettled. The spectral changes could not, in any event, be given a significance in terms of energy.

We report here a kinetic experiment designed to test the heme tension proposal quantitatively without the use of IHP. The basis of the proposal is the use of the high-spin to low-spin equilibrium of a mixed-spin ferric hemoglobin (Beetlestone & George, 1964; Iizuka & Kotani, 1969) as a "strain gauge" measuring the force on the heme. With such a strain gauge, comparison of the force in the two quaternary structures can be made. The concept of the experiment is static, but for technical reasons (and to avoid using mutant or modified hemoglobins) the method of study is kinetic. To interpret a force from our experiments which measure energies, we used the "known" change in geometry between high- and low-spin states. This change in Fe out-of-plane distance may not be the relevant coordinate, but we have not been able to devise a general strain gauge. Heme tilt may be important (Gelin & Karplus, 1976) and, to the extent that it is caused by the doming → planar geometry change, is included in the present study.

## Materials and Methods

Human hemoglobin was prepared from whole blood as described elsewhere (Treu & Hopfield, 1975) except that the sample was further purified by a carboxymethylcellulose column and eluted with a pH gradient. The purified sample was concentrated to about 10 mM by pressure dialysis and was converted to carboxyhemoglobin for storage. Samples were used within 2.5 months. Mixed-state hemoglobins were prepared as follows. A few cubic centimeters of pH 7, 0.1 M phosphate buffer were mixed with the required amount of 50 mM solutions of potassium ferricyanide and concentrated solutions of ligand complexes (sodium azide, sodium fluoride, potassium cyanate, and potassium thiocyanate). The resultant solution was sealed with a rubber septum and was bubbled with a Matheson 10% CO–90% $N_2$ gas mixture under slight excess pressure for 30 min. A 10 mM concentrated solution of carboxyhemoglobin was then introduced into the solution, and the 10% CO gas mixture continued to blow over the sample for 30 min. The final heme concentration was about 450 $\mu$M and the level of oxidation was either 15 or 50% as desired. Absorption spectra were recorded with a McPherson Eu-701 double-beam spectrophotometer. The exact concentration of ferric heme was determined by a least-squares fitting of the absorption spectrum to a mixture of the two derivative spectra. For the four ferric heme derivatives, fluoromet, azidomet, cyanatomet, and thiocyanatomet, the ratios of the ligand concentration to the ferric heme were 1000:1, 100:1, 500:1 and 1000:1, respectively. The distribution of the ferric hemes among the tetramer is given by a binomial distribution, since the oxidation of carboxyhemoglobin is governed by the CO "off" rate which is the same for both subunits (S. Ogawa, personal communication; Q. Gibson, personal communications). At 15% oxidation, tetramers with no more than one oxidized heme dominate.

An R → T transition was initiated in the mixed-state Hb by a flash of light which removed the CO from the ferrous subunits. The photolysis source used was a Synergetics flashlamp-pumped dye laser. The dye used was $10^{-4}$ M Rhodamine 6G in spectral-grade methanol. The laser pulse has a width of 0.5 $\mu$s. The pulse energy of about 150 mJ was focused to a diffuse spot 6 mm in diameter at the sample. The monitoring beam was a 450-W xenon lamp, prefiltered before the sample by Corning 3-69 and Oriel G 776-7100 filters to eliminate wavelengths shorter than 530 nm and longer than 800 nm. A Jarrell Ash f/6.3 2 nm/mm dispersion spectrograph was used to analyze the transmitted light. Small photo detectors in the image plane provided simultaneous observability of several wavelengths. The photo detectors were discrete channels of EG&G UV 100B photodiodes coupled to Harris HA 2625 op-Amps. The signals from channels at different monitoring wavelengths could be individually monitored or scaled and added or subtracted to each other to null certain signatures. The recorder used was a Biomation 802 transient recorder and the output device was an XY chart recorder. Under typical experimental conditions, the system has a time constant of 2.5 $\mu$s and a signal to noise ratio of 1000:1 in 1 $\mu$s. However, because of the presence of electrical pickup associated with the firing of the flashlamp and laser light scattering into the detector, there were false signal spikes which took 8 $\mu$s to decay to the normal noise level. Therefore, the first 10 $\mu$s of information was always discarded.

In concept, we study the dependence of the high-spin ferric heme concentration on quaternary structure by monitoring the 630-nm (high-spin) absorption band at an appropriate time. This experimental method cannot contain the IHP artifacts (i.e., explicit IHP effects not related to quaternary structures per se) which are suspected when IHP is used to change the quaternary structure. We are measuring parameters which are in principle obtainable from equilibrium binding studies. However, in equilibrium studies the relevant species would be present in very small concentrations and subtraction problems would prevent meaningful studies. The time resolution and nonequilibrium species populations available in kinetic studies are essential to obtaining the desired results.

## Results

Studies were made on carboxyhemoglobin and hybrid

hemoglobin consisting of carboxy derivatives mixed with azidomet, cyanatomet, thiocyanatomet, and fluoromet subunits, respectively. The level of CO photolysis attained for all of the samples was about 96% as determined by using an extinction coefficient difference of $\epsilon_R - \epsilon_{RCO} = 4.7\ mM^{-1}$ at 537 nm. The pH and the free CO concentrations were chosen to optimize the resolution of the R → T transition from the CO recombination signal. Transient optical changes were monitored at isosbestics as follows: at 537 nm, $\epsilon_R = \epsilon_T$; at 547 nm, $\epsilon_T = \epsilon_{RCO}$; at 625 nm ↔ 640 nm, $\epsilon_R = \epsilon_T$; at 670 nm, $\epsilon_R = \epsilon_T$, $\epsilon_{RX} = \epsilon_{TX}$. Subscripts R and T in the extinction coefficient $\epsilon$ denote the deoxy subunits in the R and T state and subscripts RX and TX refer to the ferric subunits.

In the Monod–Wyman–Changeaux allosteric model of hemoglobin with equivalent subunits (Monod et al., 1965), for any state of ligation there are two possible quaternary structures, R and T. The kinetic processes of greatest importance at short times in the mixed-state experiments [in which one heme is oxidized and has a ligand which is not removed by photolysis (D denotes the dimers present)] are

$$Hb^R \xrightarrow{K_0} Hb^T$$

$$Hb^R{\cdot}x \xrightarrow{K_1} Hb^T{\cdot}x$$

$$Hb^R{\cdot}x^i + CO \xrightarrow{(4-i)l^*(CO)} Hb^R{\cdot}x^i{\cdot}CO \qquad i \leq 3$$

$$Hb^T{\cdot}x^i + CO \xrightarrow{(4-i)l(CO)} Hb^T{\cdot}x^i{\cdot}CO$$

$$D + CO \xrightarrow{2l^*} DCO$$

Later in time, further reactions in which partially ligated Hb reverts to the R structure are also important. It is difficult to get the information desired from the data at later times, so the analysis will be restricted to short times.

The data presented below are proportional to signal voltages, which are related to absorbance changes by $\Delta V/V_0 = 10^{-\Delta OD}$. The data were first digitized by hand, converted to absorbance units, and fitted to the correct functional dependence with a nonlinear least-squares fitting program kindly provided by E. Groth of Princeton University.

The effects on the hybrid subunits induced by conformation change are monitored by studying the high-spin band around 630 nm. However, at that wavelength there is also a sizable signal due to recombination with CO. A transient at 670 nm due solely to recombination was therefore directly subtracted from the transient monitored at 630 nm before recording. The undesired large ligation-induced signal is thus eliminated, yielding good sensitivity in the detection of the weaker conformational induced spectral change. Since the actual measured quantity is a voltage change instead of absorbance change, we have implicitly assumed in this subtraction scheme that the voltage change scales linearly with absorbance. The assumption is valid in our case at the early times being analyzed. In the results shown below from the 625- ↔ 640-nm region, a large fraction of the CO deoxy signal has already been removed by subtraction with transients at 670 nm.

*Carboxy-Hb.* Figure 1 shows the first 400 μs of carboxyhemoglobin transient following laser flash at 547, 537, 630, and 670 nm, respectively, in voltage units. The level of photolysis attained is 96%. Assuming that the CO concentration remains constant, and neglecting the small dimer correction, the first 50 μs of absorption change at 547 nm can be approximated by

$$A = A_0 e^{-Kt} \qquad (1)$$

where $K = K_0 + 4l^*(CO)$. From the fit, we obtained $A_0 = (-5.6 \pm 0.15) \times 10^{-3}$ and $K = (5.3 \pm 0.2) \times 10^4\ s^{-1}$. By use

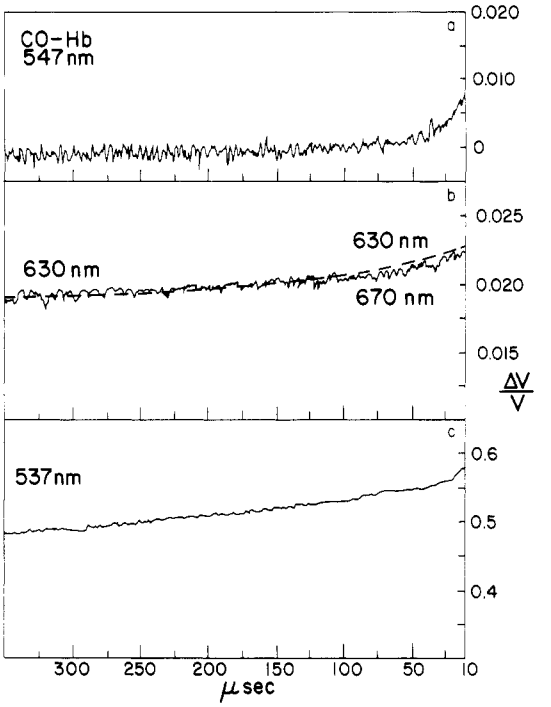


FIGURE 1: Transient relaxation of carboxyhemoglobin at (a) 547, (b) 630 and 670 (dashed line), and (c) 537 nm. Heme concentration is 440 μM. Other parameters are free CO concentration (150 μM), pH 7, 0.1 M phosphate, 22.5 °C, and 1-mm path length.

of a value of 6 $\mu m^{-1}$ for $l^*$ (Sawicki & Gibson, 1976), the deoxy R to T transition rate $K_0$ at pH 7, 22.5 °C, is found to be $K_0 = 4 \times 10^4\ s^{-1}$. The number is consistent with that obtained by Sawicki and Gibson under similar conditions. Such fast relaxation is not observed by us with whale myoglobin, further confirming our assignment of this 20-μs phase to R → T. In addition to the observed fast phase, Sawicki and Gibson have observed another anomalous fast component which lasts several hundred microseconds at 425 nm ($\epsilon_R = \epsilon_T$) for pH values below 8. We did not see this anomalous component at 547 nm ($\epsilon_R = \epsilon_T$) at pH 7.

Relaxation at 537 nm was analyzed from 150 to 350 μs. In this time scale, any conformationally induced relaxations should have been essentially completed. During this time the concentration of CO can also be assumed constant. The transient therefore can be represented by

$$A = A_0[(1-f)e^{-l(CO)t} + fe^{-l^*(CO)t}] \qquad (2)$$

The data within this short period of time are not sufficient to determine the three parameters $l$, $l^*$, and $f$ separately. The main parameter of interest to us is $1-f$, the fraction of tetramers found in the T state following photolysis. By use of $l$ and $l^*$ numbers of 0.1 and 6 μm/s (Sawicki & Gibson, 1976), a best fit to the data yields $f$ (the fraction recombining rapidly) equals 10.4 ± 0.4%. Seven percent of this 10% is expected from (a) the branching ratio of the conformational transition to the CO recombination rate as obtained by this experiment and (b) a 2 μM tetramer to dimer dissociation constant (Antonini & Brunori, 1971). The remaining 3% is attributed to slightly incomplete and nonuniform photolysis. We considered the agreement between experiment and expectation adequate.

It is not known from previous experiments if there exists any R → T spectral change of the deoxy subunits near 630 nm, which could be confused with that arising from the hybrid subunits. The 630 transient obtained was therefore compared to the 670-nm relaxation which has been normalized to match

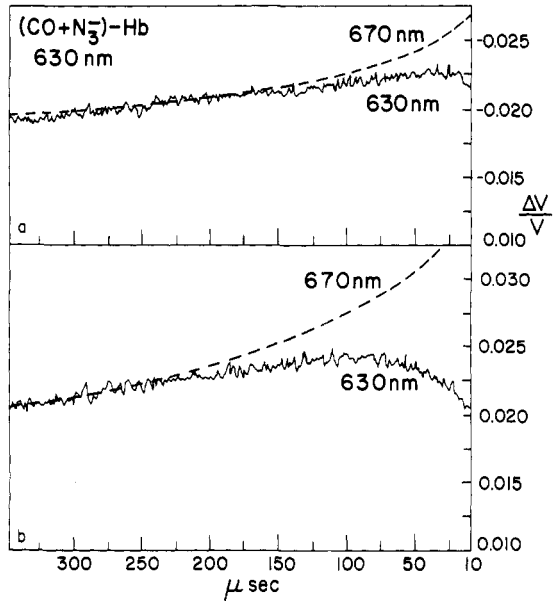


FIGURE 2: Transient relaxation of mixtures of carboxy- and azidomethemoglobin at 630 and 670 nm (dashed line). Heme concentrations are as follows: (a) HbCO = 368 ± 5.5 μM, $Hb^+ \cdot N_3$ = 68.5 ± 8 μM; (b) HbCO = 267 ± 5 μM, $Hb^+ \cdot N_3^-$ = 260 ± 5 μM. Other experimental conditions are the same as those specified in Figure 1.

the 630-nm result near 400 μs. In this way, any mismatch of the two curves at early times (~50 μs) should reflect the presence of an R → T induced signal at 630 nm. It is evident from Figure 1 that there is no observable R–T spectral difference at 630 nm coming from the deoxy subunits.

*HbCO Plus $Hb^+ \cdot N_3^-$ Hybrid.* The carboxy and azidomet hybrid system was examined with 15 and 50% of azidomet subunits. Only the 15% results were analyzed quantitatively. Transient signals at 537 nm from 150–350 μs were fitted to eq 2. The fraction of fast recombining species obtained under the conditions specified in the caption of Figure 2 was 21.4 ± 0.6%. This extra 11% fast component as compared to the pure CO result can be understood as a simple and expected consequence of the presence of oxidized subunits. Assuming that the hybrid subunits are distributed among the tetramers according to a binomial distribution, a 7% contribution to the fast component will arise from single oxidized $Hb^R \cdot N_3^-$ by using the branching ratio $K_1/3l^*(CO)$ as obtained (see below). The other 4% can be accounted for if 80% of the doubly oxidized hybrid tetramers $Hb^R \cdot (N_3^-)^2$ do not switch to T. Following such arguments, we calculate the concentration of azidomet hemes in T conformation following photolysis to be 25 μm among a total of 68 μM.

Figure 2 shows the transient monitored at 630 and 670 nm for 15 and 50% azidomethemoglobin. The difference between these two curves corresponds to a conformational-induced signal at the azide subunit because no transients of this nature are observed with studies on pure carboxy-Hb. Control experiments with azidomet-Hb and pure carboxy-Hb with free azide in the solution confirm that the transients observed are not temperature- or azide-artifacts. The difference curve from 10 to 100 μs for a 15% azidomet sample was fitted to

$$A(t) = A_0\left[e^{-Kt} + \left[\frac{F(t)}{F(t_m)} - 1\right]\right] \qquad (3)$$

where $K = K_1 + 3l^*(CO)$ and $F(t)/F(t_m) - 1$ is approximately a 15% correction term which arises from matching the 630- and 670-nm curves at $t_m$ of 400 μs instead of at $t = 0$. $F(t)$ was obtained by fitting the first 100 μs of transient at 670 nm

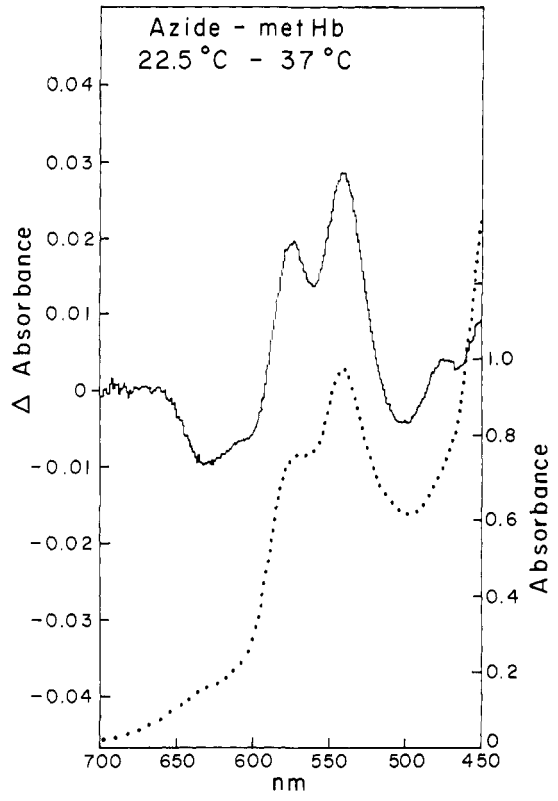


FIGURE 3: Absorption spectrum (dotted line) at 22.5 °C and temperature difference spectrum between 22.5 and 37 °C of approximately 87 μM azidomethemoglobin. Experimental conditions are total azide concentration (50 mM), pH 7, 0.1 M phosphate, and 1-cm path length.

to an empirical function. We obtained $A_0 = (1.52 \pm 0.4) \times 10^{-3}$ and $K = (2.6 \pm 2) \times 10^4$ s$^{-1}$ or $K_1 = 1.7 \times 10^4$ s$^{-1}$ using an $l^*$ value of 6 μm$^{-1}$ s$^{-1}$.

Figure 3 shows a temperature difference spectrum of azidomethemoglobin between 22.5 and 37 °C. The spectrum resembles closely that of azidometmyoglobin reported by Beetlestone & George (1964). They have interpreted this kind of spectrum as arising from temperature-induced change in the high-spin to low-spin equilibrium. Following their interpretation, using a high-spin population of 10% at 20 °C and a spin enthalpy difference of about 4 kcal/mol (Beetlestone & George, 1964; Iizuka & Kotani, 1969), we obtained from the magnitude of our observed temperature spectrum a high-spin to low-spin extinction coefficient ($\epsilon_h - \epsilon_l$) of 4 mM$^{-1}$ cm$^{-1}$ at 630 nm for azidomethemoglobin. A comparable number of 3 mM$^{-1}$ cm$^{-1}$ was obtained by measurements of absorption and paramagnetic susceptibilities by Messana et al. (1978) for carp hemoglobin.

*HbCO Plus $Hb^+ \cdot OCN^-$ Hybrid.* Figure 4 shows the transient of two different mixtures of carboxy- and cyanatomet-Hb at 625 and 670 nm, respectively. The difference between these two curves for the low cyanatomet concentration samples was again fitted according to eq 3. We obtained $A_0 = (-8.3 \pm 0.04) \times 10^{-4}$ and $K = (1.9 \pm 0.3) \times 10^4$ s$^{-1}$ which implies $K_1 = 1 \times 10^4$ s$^{-1}$ for $l^* = 6$ μm$^{-1}$ s$^{-1}$. The most important feature to be noticed is that the conformational-induced spectral change *is opposite in sign* to that obtained in the azidomet. From the 537-nm relaxation, we calculate in a fashion similar to the azidomet case that 24 μm out of 61 μm of cyanatomet-Hb switches to the T structure following photolysis.

Figure 5 shows a temperature difference spectrum of cyanatomethemoglobin. The 625-nm high-spin band indicates a much higher population of high spin. The sign of the temperature spectrum at 625 nm also shows that the spin enthalpy

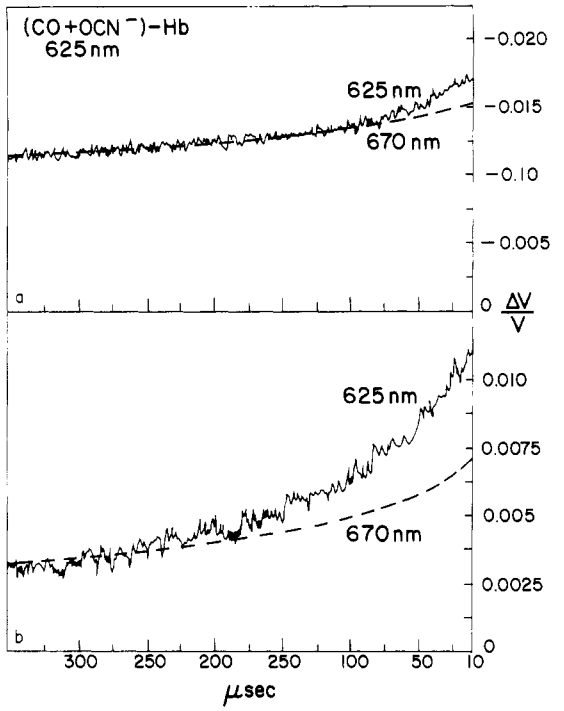


FIGURE 4: Transients of mixtures of carboxy- and cyanatomethemoglobin at 625 and 670 nm (dashed line). Heme concentrations are as follows: (a) HbCO = 358 ± 2.5 μM, $Hb^+ \cdot OCN^-$ = 61 ± 6.5 μM; (b) HbCO = 214 ± 3.5 μM, $Hb^+ \cdot OCN^-$ = 213 ± 2.5 μM. Other conditions are the same as those specified in Figure 1.

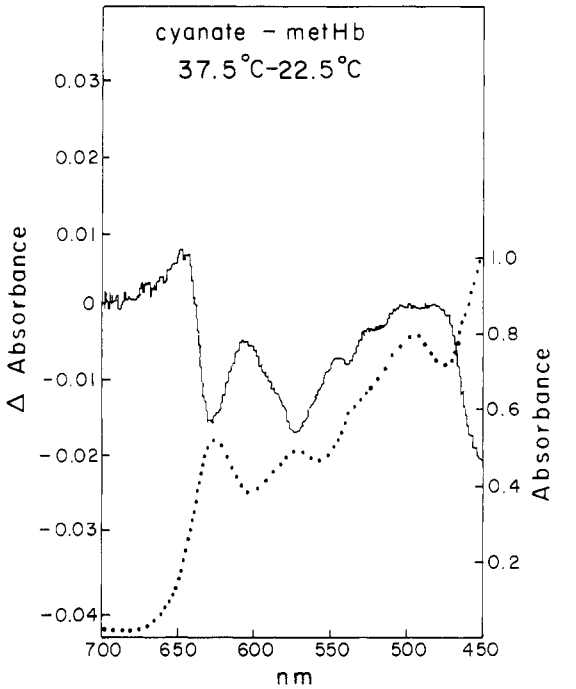


FIGURE 5: Absorption spectrum (dotted line) at 22.5 °C and temperature difference spectrum between 37.5 and 22.5 °C of approximately 110 μM cyanatomethemoglobin. Experimental conditions are total potassium cyanate concentration (~50 mM), pH 7, 0.1 M phosphate, and 1-cm path length.

difference $\Delta H_s(l - h)$ is positive. From resonance Raman studies on human cyanatomethemoglobin (Cho et al., unpublished experiments), it was inferred that this derivative is predominantly high spin (80%) at room temperature but does switch to low spin at lower temperatures. These results are in contrast to those reported by Iizuka & Kotani (1969) for horse azidomet-Hb, where the high-spin fraction at 20 °C is

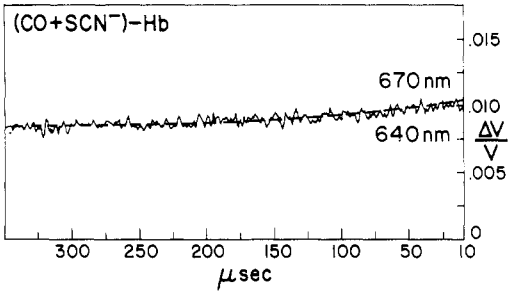


FIGURE 6: Transients of a mixture of carboxy- and thiocyanatomethemoglobin at 640 and 670 nm (dashed line). Heme concentrations are HbCO = 390 ± 3 μM and $Hb^+ \cdot SCN^-$ = 54 ± 5.5 μM. Other conditions are the same as those specified in Figure 1.

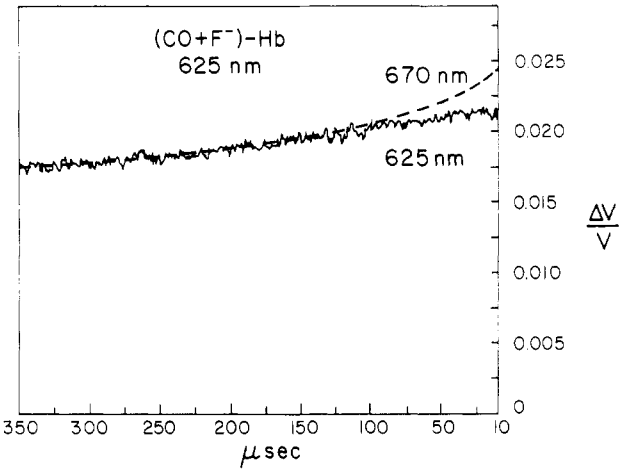


FIGURE 7: Transients of a mixture of carboxy- and fluoromethemoglobin at 625 and 670 nm (dashed line). Heme concentrations are HbCO = 401 ± 2 M and $Hb^+ \cdot F^-$ = 78 ± 4 μM. Other conditions are the same as those specified in Figure 1.

23% and $\Delta H_s(l - h)$ is negative. The resolution of this discrepancy is not obvious, for it is unlikely to be due to species difference. Assuming the Raman results, the temperature difference spectrum in Figure 5 can be understood if one assumes a significant spectral temperature dependence within a single spin state (see below).

*Hb·CO Plus $Hb^+ \cdot SCN^-$ Hybrid.* Figure 6 shows the transient of a mixture of 390 μm of carboxy and 54 μm of thiocyanatomet subunits at 640 and 670 nm, respectively. No observable conformational-induced spectral change associated with the ferric subunits could be seen. Similar results were obtained at 630 and 650 nm. The concentration of thiocyanatomet subunits in the T structure was calculated to be 22 μm from the 537-nm transient result as described before.

*Hb·CO Plus $Hb^+ \cdot F^-$ Hybrid.* The transient optical changes of the carboxy- and fluoromethemoglobin hybrid at 625 and 670 nm are shown in Figure 7. The fit to the difference curve yields $A_0 = (1.2 \pm 0.03) \times 10^{-3}$ and $K$ of $(2.4 \pm 0.2) \times 10^4$ $s^{-1}$ assuming $l^*$ to be 6 $\mu m^{-1}$ $s^{-1}$. The fraction of fluoromet subunits in the T conformation is equal to 28 μm.

As shown in Figure 8, fluoromethemoglobin also exhibits a temperature difference spectrum of magnitude comparable to that of the mixed-spin derivatives. This supports our previous assumption that there exists a sizable spectral temperature dependence within a single spin state.

## Discussion

*(A) Interpretation of the Conformation Change Induced Spectral Changes.* The conformational-induced spectral change associated with the ferric subunits is summarized in Table I. To interpret these results we must understand the

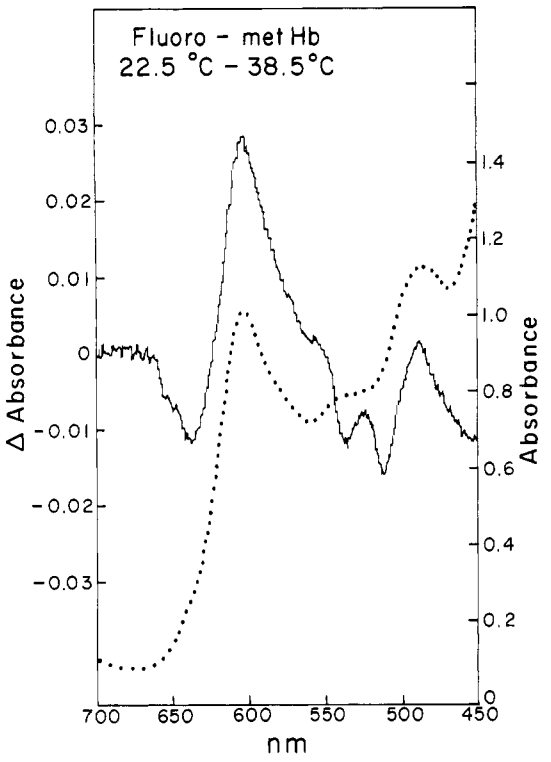


FIGURE 8: Absorption spectrum (dotted line) at 22.5 °C and temperature difference spectrum between 22.5 and 38.5 °C of fluoromethemoglobin. Experimental conditions are total fluoride concentration (100 μM), pH 7, 0.1 M phosphate, and 1-cm path length.

Table I: Summary of Conformationally Induced Spectral Changes of Various Ferric Subunits

| derivative | concn (μM) in T conformation | OD change | % high spin at 20 °C | $\epsilon_s(h-l)$* ($mM^{-1}$ $cm^{-1}$) |
|---|---|---|---|---|
| $Hb^+ \cdot N_3^-$ | 25 | $(1.52 \pm 0.04) \times 10^{-3}$ | 10[a] | 4 (630 nm) |
| $Hb^+ \cdot OCN^-$ | 24 | $(-8.3 \pm 0.04) \times 10^{-4}$ | ~80[b] | 6 (625 nm) |
| $Hb^+ \cdot SCN^-$ | 22 | ~0 | ~70[b] | 5.2 (640 nm) |
| $Hb^+ \cdot F^-$ | 28 | $(1.2 \pm 0.03) \times 10^{-3}$ | 100 | |

[a] Perutz et al. (1974c). [b] Cho et al. (unpublished experiments). The high-spin percentages have an error of ±20%. [c] $\epsilon_s$ of $Hb^+ \cdot N_3^-$ is deduced from the temperature difference spectrum as described. $\epsilon_s$ values for $Hb^+ \cdot OCN^-$ and $Hb^+ \cdot SCN^-$ are obtained from their absorption spectrum normalized by the assumed fraction of high spin.

origin of the observed signal. For the mixed-spin derivatives, does the conformational-induced absorption change reflect solely a change of high-spin population in the T conformation or does it contain effects of some R–T spectral difference of other origin? Since high- to low-spin equilibrium is much faster than $K_1$ (Beattie & West, 1974; Iizuka & Morishima, 1974; Perutz et al., 1978), the experiment cannot distinguish between the two possibilities on the basis of kinetics. From fluoromet- and cyanatomethemoglobin, we deduce that there are sizable spectral changes associated with the high-spin derivatives in the R and T conformations, the sign of which *at the peak* of the charge transfer band is opposite to that of an increase of high-spin population in the T state. Thus, we cannot hypothesize that spectral changes are solely due to spin changes. For the chiefly low-spin derivatives, absorption around the 630-nm region is rather weak, and it is therefore reasonable to assume for low-spin hemes there will be no significant conformational-induced spectral change near 630 nm.

(*a*) *Azidomet Derivative.* Azidomet is 90% low spin at room temperature (Perutz et al., 1974a–c). Thus, the spectral change due to the conformation effects on the high-spin spectrum will be small. We therefore interpret the large spectral change as arising chiefly from an increase of high-spin population in the T conformation. By use of $A_0$ of $1.5 \times 10^{-3}$ and $\epsilon_s$ of 4 $mM^{-1}$ $cm^{-1}$, the spectral change corresponds to an increase of 3.75 μm of the high-spin population in the T state among a total of 25 μm. This number can be translated to give an R–T conformational-induced free energy difference of 640 cal/mol between the two spin states; i.e.

$$(G_{HS}^R - G_{LS}^R) - (G_{HS}^T - G_{LS}^T) = 640 \text{ cal/mol of heme}$$

The energy differences increases to 790 cal/mol if $\epsilon_s$ is 3 $mM^{-1}$ $cm^{-1}$. This scale of energy difference is consistent with that obtained by Perutz and co-workers (Perutz et al., 1978; Messana et al., 1978) of ~700 cal/mol for hemes in Hb Iwate and HB Milwaukee.

(*b*) *Fluoromet Derivative.* The existence of a conformation change induced transient indicates that there is a sizable R–T spectral difference within the high-spin derivatives around 630 nm. The R–T extinction coefficient obtained for fluoromet at 625 nm is 0.43 $nm^{-1}$ $cm^{-1}$ which agrees quite well with the value of ~0.5 $mM^{-1}$ $cm^{-1}$ obtained by the fluoromet ± IHP measurements. Perutz et al. (1974a–c) have suggested that such spectral changes arise from an increase in the displacement of the iron atom from the mean porphyrin plane because of tension in the T conformation. There is no direct test of this suggestion available. Knowles et al. (1975) have reported that very similar difference spectra can be induced in many hemoglobin derivatives by perturbations such as temperature or pH even within the oxy quaternary structure. The similarity of such perturbation spectra suggests that the effects of perturbation are being propagated to the heme by a single general coordinate of importance, which is linked to many kinds of perturbation of the molecular conformation.

Fluoromethemoglobin shows a temperature difference spectrum comparable in magnitude to that of the mixed-spin derivatives in the small temperature of 4–40 °C. The presence of this temperature-induced spectral change within a single spin state limits the use of static temperature difference spectra as quantitative monitors of spin-state equilibrium.

(*c*) *Cyanatomet Derivative.* The spectral change in the cyanatomet case is difficult to interpret quantitatively because of the complication introduced by spectral changes within the high-spin fractions. In the case of the fluoromet ± IHP optical changes (Perutz et al., 1974a–c), the maximum scale of conformational-induced spectral changes associated with the high-spin state is about 0.7 $mM^{-1}$ $cm^{-1}$. Assuming such an extinction coefficient and an 80% high-spin fraction out of 24 μm, the R–T induced spectral change is $-1.33 \times 10^{-3}$. Compared to the observed change of $-0.83 \times 10^{-3}$, the spectral changes arising from an increase in high-spin fraction in the T state is given by $+0.50 \times 10^{-3}$. This corresponds to an increase of high spin of 0.82 μM or a conformational-induced energy difference of about 280 cal/mol. However, the dominating number and sign in this calculation are assumed from experiments on another ligand. The smallness of the effect is consistent with the absence of an effect of IHP on the high spin–low spin ratio for cyanate observed (Perutz et al., 1974a–c).

(*d*) *Thiocyanatomet Derivatives.* Since thiocyanatomet has a lower high-spin fraction than cyanatomet at room temperature, the transient spectral change should favor an increase in absorbance in the T conformation compared to that of cyanatomet. The small optical change observed is qualitatively

consistent with a cancellation resulting from a combination of two contributions opposite in sign. Assuming again an R–T spectral change of 0.7 $mM^{-1}$ $cm^{-1}$ and a 70% high-spin fraction among 22 $\mu$m, optical changes arising from high-spin fraction alone should be $-1.1 \times 10^{-3}$. By comparison with the small negligible transient observed in the experiment, this implies that the spectral change from spin population is about $+1.1 \times 10^{-3}$. The number corresponds to an energy difference of 280 cal/mol between the two spin states in the R and T structure. If the *assumed* intrinsic spectral change of the high-spin state were described as $0.7 \pm 0.7$ $mM^{-1}$ $cm^{-1}$, then the deduced energy is $280 \pm 280$ cal/mol. In IHP addition experiments (Perutz et al., 1978) an 11% increase in magnetic susceptibility was found for thiocyanate, while in our experiments changing quaternary structure without IHP corresponds to a $5 \pm 5$% change in paramagnetic susceptibility. A similar uncertainty can be placed on the cyanatomet example.

*(B) Relation between Conformational-Induced Energy Difference and Spin States.* We have measured the difference in energy between high- and low-spin states of a mixed-spin derivative in different protein conformations. In order to compare the observed scale of energy difference to predictions, the conformational energy term can be described as follows. If the high-spin fraction of a mixed-spin derivative sits preferentially out of the heme plane [as expected from Hoard's (1971) and Perutz' (1970) argument] toward the proximal side by a distance $\delta_s$, the existence of any tension or protein force $\mathbf{F}_p$ on the heme within one conformation would introduce an energy difference between the two spin states given by

$$G_{spin}^{protein} = \int_0^{\delta_s} F_p \, dx$$

The force acts in such a direction as to pull on the iron atom, and the high-spin state is energetically favored. Depending on the value of $F_p$, the protein couples a different free energy to the high- and low-spin states between the R conformation and T conformation. Consistent with the energetics of hemoglobin cooperativity, the force would act to raise the population of the high spin in the T conformation relative to that of the R state. The energy is given explicitly by

$$G_{spin}^{RT} = \int_0^{\delta_s} F_{RT} \, dx$$

where $F_{RT} = F_R - F_T$. In Hopfield's linearly distributed energy mode, $F_{RT}$ is constant in $\delta_s$ and is on the scale of 6 kcal/(mol Å). A high- and low-spin $\delta_s$ difference of 0.2 Å will produce a $G_{spin}^{RT}$ of 1.2 kcal/mol.

*(C) Application to Mechanism of Hemoglobin Cooperativity.* The azidomet derivative ($G_{spin}^{RT} \sim 700$ cal/mol) has a conformational-induced energy difference between the high-spin and low-spin states which is significantly lower than the 1.2 kcal expected above. The values for $Hb^+ \cdot OCN^-$ and $Hb^+ \cdot SCN^-$ are only about 300 cal/mol. (It is not impossible that the low numbers obtained for cyanate and thiocyanate derivatives are due to high-spin fractions of both derivatives which are larger than the numbers assumed in Table I. However, in the absence of any evidence that this is so, serious discussion based on such speculation seems unjustified.) The discrepancy can be accounted for by either a smaller $\delta_s$ or a smaller average protein force or a combination of both. We discuss the two possibilities separately. (1) According to Hoard (1971) and Perutz (1970), the high-spin iron atom stays preferentially out of the heme plane because it has effectively too large an ionic radius to be fitted into the porphyrin core. Recently, Warshel (1977) and Olafson & Goddard (1977) have proposed instead that the iron–heme geometry is governed primarily by the steric repulsion between the axial ligands and the porphyrin nitrogen orbitals. This proposal has received some support from Perutz' revised aquomet iron–mean heme distance (Perutz, 1976) and from recent X-ray results on certain high-spin model heme compounds (Kastner et al., 1978). It is difficult to distinguish clearly between the two out-of-plane mechanisms, and both effects may contribute. However, it remains a likely possibility, especially for sixfold coordinated complexes, that because of steric effects $\delta_s$ is smaller than 0.2 Å and is ligand dependent. The measured value of $G_{spin}^{RT}$ would be consistent with $\delta_s < 0.06$ Å for both cyanatomet and thiocyanatomet. In such a case, the lack of an increase in the high-spin population does not rule out the existence of a large protein force acting on this coordinate of the iron atom in the T conformation. (2) If the high- and low-spin iron of all the mixed-spin derivatives has a geometrical difference $\delta_s \sim 0.2$ Å, our results would imply that the protein force is $\lesssim 3$ kcal/Å averaged over the distance from 0.0 to 0.2 Å of the heme plane. Similarly, a combined analysis of the EXAFS (Eisenberger et al., 1979) and Raman studies (Kincaid et al., 1979) shows that in deoxy-Hb, where $\delta_L = 0.6$ Å, $F_{RT} < 2.7$ or $< 0.4$ kcal/Å, depending on the method of analysis. Thus, the forces observed at both ends of $\delta_L$ are $\lesssim 50$% of those expected. Either the iron–histidine coordinate is not "the" coordinate central to hemoglobin cooperativity (i.e., other coordinates make major contributions) or heme tension is confined mainly to a narrow range of displacement $\delta_L$ between 0.2 and 0.6 Å. This later alternative could most reasonably be true if the free energy is not distributed widely among the protein framework but is localized in and shared in fewer bonds or even is due to a few specific bonds.

*(D) Kinetics of $R \rightarrow T$ Structure Change.* The kinetic results demonstrate directly that the conformational transition rate is dependent on the state of ligation. At pH 7, 22.4 °C, and 0.1 M phosphate, the conformational transition rate decreased from $K_0$ of $4 \times 10^4$ $s^{-1}$ to an average of $K_1 = 1.4 \times 10^4$ $s^{-1}$ in singly liganded tetramers with one fluoride, azide, or cyanate ion bound. Among the derivatives examined, $K_1$ does not depend strongly on the nature of the ligand. The ratio $K_0/K_1$ of 2.9 agrees reasonably well with the number reported by Sawicki & Gibson (1976) for carboxyhemoglobin.

## Conclusion

The spin changes we observe for the three thermally mixed-spin derivatives correspond to energies 25–60% of the one anticipated (Hopfield, 1973) and are in addition ligand specific. (If the 0.4-Å motion inferred from the high-resolution studies of myoglobin (Takano, 1977) were used for the calculation, the observed energies would be only 15–40% of that anticipated.) One of the basic suppositions appears to be in error. The anticipated energy is based on the following suppositions: first, for all ferric ligands, the iron atom moves a full 0.2 Å on change of spin state; second, the iron atom motion is the dominant pathway for the change in affinity; third, the energy is widely delocalized in the protein. Which and whether the error is qualitative or only quantitative must come from experiments which give more information about structures and forces within a single state of spin and ligation. For new results in this direction, see Scholler & Hoffman (1980), Kincaid (1979), and Nagai (1979).

Our conclusions about energies are in quantitative agreement with those of Perutz et al. (1978) and Messana et al. (1978) in the case of human hemoglobin azide but are systematically lower for cyanate and thiocyanate. (Their experiments on carp hemoglobin yield somewhat higher numbers, possibly reflecting the greater cooperativity of that molecule.)

There are many experimental differences which may cause the divergence, including singly vs. fully oxidized tetramers, IHP vs. kinetics, and the myriad of detailed suppositions which must be made to obtain a quantitative analysis of the result.

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