Distal and Proximal Control of Ligand Reactivity: A Transient Raman Comparison of COHbA and COHb (Zürich)

Thomas W. Scott,[†‡] Joel M. Friedman,*[‡∥] and Victor W. Macdonald[§]

Contribution from AT&T Bell Laboratories, Murray Hill, New Jersey 07974, and Johns Hopkins Medical Institutions, Department of Medicine, Baltimore, Maryland 21205.
Received March 5, 1984

Abstract: When Raman scattering is used, it is shown that for a given solution condition, both the yield of picosecond geminate recombination and the quantum yield of photolysis averaged over a 10-ns laser excitation pulse are consistently lower for COHb (Zürich) than for COHbA. In going from pH 8.2 to pH 6.2 + 3 mM IHP, both hemoglobins exhibit the same proportional increase in the photolysis yield. The transient Raman spectrum of these photolyzed carboxyhemoglobins indicates that the behavior and geometry of the proximal side of the heme is very nearly the same in both proteins. The persistent difference in ligand-binding properties between these two proteins is therefore ascribable to a static variation in another structural degree of freedom, one that is likely to involve the distal side of the heme. The findings suggest that both distal and proximal variations in the heme environment account for base-line differences in ligand-binding properties between different hemoglobins, but within a given protein cooperativity are under proximal control.

X-ray crystallographic studies[1] have revealed two well-defined quaternary structures associated with hemoglobin. A ligation-dependent equilibrium between the low-affinity T-state and high-affinity R-state quaternary structures can account for many of the gross features of cooperative ligand binding in hemoglobin.[1,2] It is still uncertain how, on a structural level, the change in quaternary structure alters the localized ligand binding site. High-resolution X-ray crystallographic studies reveal changes in the tertiary structure about the heme when quaternary structure and state of ligation are simultaneously changed.[3] These studies reveal specific structural features about the heme that are of potential importance in the regulation of affinity. These include features on both the proximal and the distal side of the heme. The nature of X-ray crystallography is such that it is not feasible to systematically compare many different hemoglobins under different conditions in order to determine which structural features correlate with changes in ligand-binding properties. There have been numerous studies showing that ligand off and on rates are sensitive to protein structure. However, these processes take place over time scales that typically exceed the relaxation time of the tertiary structure. Geminate recombination occurs over a time scale ranging from picoseconds to ~200 ns.[4-9] The yield and rate appear to depend upon the ligand and the protein.[6-9] Because the recombination times are typically faster than structural relaxation times,[10-13] the determinants of reactivity are associated with the structure of the parent ligated hemoglobin. Consequently, a comparison of the fast rebinding subsequent to photolysis as a function of the tertiary structure of the ligated species would be of value in assessing the nature of the structural determinants of reactivity. The difficulties with this approach are finding the systems that exhibit a range of known tertiary structures.

Hemoglobin Zürich (HbZ) is a mutant human hemoglobin in which the distal histidine (β^{63} E7 His) of the β subunits is replaced by an arginine. X-ray studies indicate that this substitution results in modifications of the heme pocket.[14,15] Most noticeable is the increase in free volume on the distal or ligand binding side of the heme. Ligand-binding studies show that there is an increase in CO-binding affinity on these modified β subunits.[16,17] Recently[18] the rate of geminate recombination of CO in isolated β_z chains has been studied over an extensive range of temperatures (10–300 K). From this study it appears that the potential energy barrier controlling CO rebinding subsequent to photolysis is lower in β_z relative to both Mb and the unmodified β chains. Although the most noticeable perturbation associated with the β_z substitution is the altered distal side of the heme, the potential for structural coupling of even remote segments of the protein means that attributing the decreased barrier height purely to a distal effect is premature.

The resonance Raman spectrum of Hb contains many vibrational frequencies that can be related directly to specific degrees of freedom of the heme. Of these, the iron–proximal histidine stretching mode (Fe–His) has shown the most variation with protein structure.[19-27] The frequency of this mode (ν_{Fe-His}) has

[†] Present address: Corporate Research Science Laboratories, Exxon Research and Engineering Co., Annandale, NJ 08801.
[‡] AT&T Bell Laboratories.
[§] Johns Hopkins Medical Institutions.
[∥] This work was supported in part by Grant HL02799 from The National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD.

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0002-7863/85/1507-3702$01.50/0

been shown to be sensitive to the quaternary state of the protein, increasing from ~215 to ~222 cm^- in going from the equilibrium deoxy T to the equilibrium deoxy R structures.[19-23,28] Furthermore Raman studies of transients[24-27,29,30] have revealed that for each quaternary state, ligand binding induces additional changes in the tertiary structure about the heme. As a result of the ligand-binding-induced changes, ν_{Fe-His} is, relative to the corresponding deoxy species, higher for the transient deoxy species occurring within several nanoseconds subsequent to photoinduced ligand dissociation. However, even for a given quaternary structure, ν_{Fe-His} in the deoxy transient (at 10 ns) has been shown to vary both with the animal source and the solution conditions.[12,13,24-27,29] In comparing these frequencies, it is observed that there is a general trend for this frequency to scale with ligand affinity. High-affinity hemoglobins exhibit the higher frequencies. Conditions that decrease the affinity also tend to decrease this frequency. A more rigorous correlation is observed between this frequency and the yield of geminate recombination.[12,13,24] In any comparison of closely related Hb's, the protein with the lower frequency has the lower yield of geminate recombination. Similarly this frequency appears to be related to the rate of spontaneous ligand dissociation—the lower the frequency the higher the rate. Given this relationship between the iron–proximal histidine linkage and ligand-binding related parameters, it is of interest to determine to what extent the differences between HbA and HbZ might be related differences in the proximal heme–protein linkage that might be induced by the distal environment.

In this study, we compare COHbA and COHbZ with respect to the following: the rate and yield of the subnanosecond geminate recombination, the yield of photolysis averaged over a 10-ns pulse that only partially photolyzes the sample, and the low-frequency Raman spectrum of the transient deoxy photoproduct occurring within 10 ns of photolysis. Both of the nanosecond comparisons were done at pH 8.2 (Tris) and at pH 6.2 + IHP (0.3 and 3.0 mM) whereas the picosecond study was carried out only at pH 8.2. Because the actual subpicosecond photodissociative process appears to be independent of the protein environment about the heme,[9] differences in the time -averaged yield of photolysis are ascribable to differences in those processes which contribute to the yield of geminate recombination. Previous low-resoltuion studies of the recombination of CO with HbA revealed an absence of a subnanosecond geminate process. Improving the pulse-to-pulse stability by using an acitve–passive mode-locked Nd:YAG laser has recently enabled a more detailed measurement of this process. It is observed that there is weak but discernible geminate rebinding of CO in HbA occurring over seveal hundred picoseconds and extending into the nanosecond regime (to be published). In the present work, we compare this process for HbA and HbZ. Because of this inherently low probability for geminate rebinding and the possibility of further reductions in the probability with a decrease in pH (+IHP), direct monitoring of the small changes in the picosecond process did not appear to be the most appropriate method of measuring the effect of solution conditions upon this process. Instead we used the Raman spectra to compare the yield of partial photolysis averaged over a 10-ns pulse. The high contrast ratio between the intense ligated and deoxy Raman peaks at 1375 and 1355 cm^{-1} s, respectively, allows for a very accurate comparison of different samples. Measurement of the relative quantum yield of photolysis under intensity conditions of partial photolysis is a method of amplifying the small differences observed in a direct study of the subnanosecond geminate recombination. In the quantum yield study, one is in first approximation, repeating and averaging the picosecond experiment over the 10-ns pulse. In earlier studies it was shown that in going from high pH to low pH in the presence of millimolar concentrations of IHP, the 10-ns time-averaged photolysis yield for oxy-HbA is increased and ν_{Fe-His} of the 10-ns deoxy photoproduct from either oxy- or carboxy-HbA decreased by a few cm^{-1} s.[12] Recent picosecond geminate recombination studies directly demonstrate that this change in the average yield for O_2 arises from a change in recombination that is occurring primarily at 200–400 ps subsequent to photolysis (to be published). These changes in the geminate rebinding appear to arise from an IHP-induced modification of the β subunits.[12,13,16] The present study also aims at establishing the extent to which the proximal portion of the altered heme pocket of the β subnit in COHbZ behaves as in COHbA.

Experimental Section

Geminate recombination was studied by using single 30-ps pulses (10 Hz) at the second harmonic (532 nm) of an active–passive mode-locked Nd:YAG laser (Quantel YG 471C) to photolyze a carboxyhemoglobin sample. The recombination was monitored by probing the transmission of blue pulses (436 nm) of ~25-ps duration which were generated by passing the second harmonic through a hydrogen cell (300 psi). The blue pulse results from the first anti-Stokes Raman transition. The change as a function of delay in the transmission through a 1 mm path length cell was achieved by using a programmable optical delay line (Klinger), providing delays of up to 2.4 ns. Because of the high pulse-to-pulse stability (≤5%), it was possible to generate the rebinding curve by slowly scanning the delay line. The reproducibility was sufficiently good to allow for signal averaging (repetitive scans) when necessary. The transmission was monitored by a photodiode screened with appropriate optical filters. The output of this photodiode and another which monitored the incident pulse energies were averaged in a boxcar integrator, and the ratio was taken to compensate for pulse-to-pulse variations. The output of the boxcar averager was digitized and stored in a computer. Computer-controlled shutters provided a base-line value for the transmission of the sample in the absence of the photolysis pulse. The curves were converted to absorbence and displayed as relative concentration of deoxyheme vs. delay.

The relative time-averaged yield of partial photolysis was measured by using a single 10-ns pulse to both partially photolyze the sample and probe the degree of photolysis. The relative degree of photolysis was determined by using the ratio of the 1355-cm^{-1} and the 1375-cm^{-1} Raman bands. These two well-separated and intense peaks are characteristic of deoxy- and ligated heme, respectively. Each spectrum was generated in identical fashion from a solution of identical heme concentration (0.09 mM) by using fixed pulse energies (~0.1 mJ/pulse) at 10 Hz. The excitation frequency is 4400 Å (excimer laser pumped dye laser, Stilbene III). The hemoglobin samples were isolated and purified from fresh CO-equilibrated hemolysate by the techniques of Huisman and Dozy.[36] The low-frequency Raman spectra of the photolyzed carboxyhemoglobins were generated and collected as previously described.[12,13,24-27]

Results and Discussion

A comparison between the subnanosecond geminate recombination of COHbA and COHbZ at pH 8.2 is shown in Figure 1. It can be seen that in contrast to oxy-HbA and HbZ, where a substantial fraction of the photodissociated ligand rebinds within 400 ps (to be published), only a relatively small fraction of the CO recombines even within a few nanoseconds. This rebinding proceeds to approximately 200 ns with a total geminate yield of about 50%.[4-7] Despite the low yield of geminate recombination over this 2-ns interval, it can be seen that HbZ exhibits a greater yield relative to HbA. This result was observed in all of several repetitions of the experiment. The actual difference is greater than what is seen in the figure since the α subunits are the same

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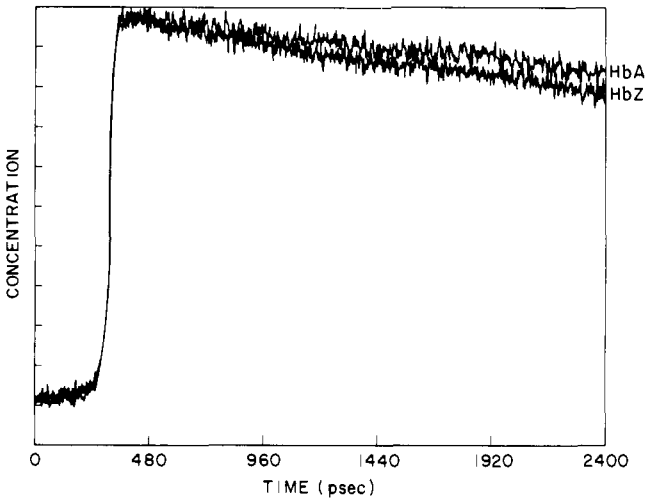

Figure 1. Comparison of the geminate recombination of carboxy-HbZ and carboxy-HbA at pH 8.2. Concentration refers to relative concentration of deoxyheme computer generated from the initial time-dependent transmission curves. Geminate recombination was studied by using single 30-ps pulses at the second harmonic (532 nm) of an active–passive mode-locked ND:YAG laser (Quantel YG 471C) operating at 10 Hz to photolyze a ligated hemoglobin s ample in a 1 mm path length cell at ~10 °C. The hemoglobin concentration was sufficiently low (~2–3 × 10^{-4} M) that absorption at the photolyzing wavelength was negligible, so that uniform concentration of excited species through the cell path could be assumed. The recombination was monitored by probing the transmission of attenuated blue pulses (436 nm) of ~25-ps duration which were generated by passing the second harmonic through a hydrogen cell (300 psi). The time between pump and probe pulses was varied with a programmable optical delay line. The result seen here were obtained in all the several repetitions (different days) of this experiment. The base line was checked and found to be flat compared to the observed decay seen in the figure.

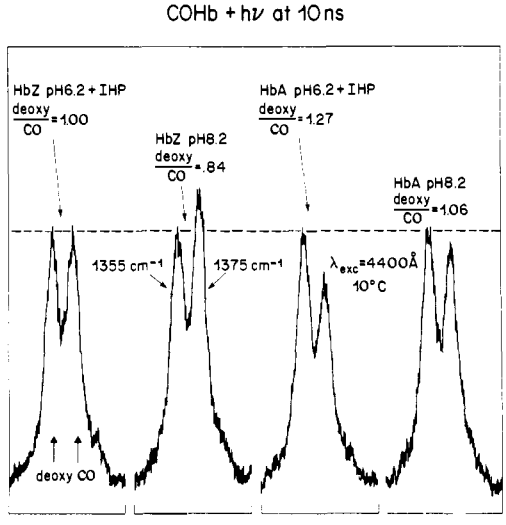

Figure 2. Comparison over 10 ns of the degree of partial photolysis in COHbA and COHbZ as a function of solution conditions. Each doublet corresponds to the deoxy (~1355 cm^{-1}) and carboxy (~1375 cm^{-1}) component of the ν_4 mode. Each of the four spectra spans 100 cm^{-1} s (1320–1420). The spectra were all normalized to the deoxy component. Above each spectrum is the ratio of the deoxy-to-carboxy peak height. The ratios were obtained from 4× expanded versions of these spectra.

in both cases and the difference originates only from the rebinding in the β subunits. A larger difference would be observed if the contribution of the α subunits could be subtracted out. A similar difference is observed in the fast rebinding seen in the oxy derivatives (to be published).

Figure 2 shows a comparison of the 10-ns time-averaged yields of photolysis for COHbA and COHbZ at pH 8.2 and pH 6.2 + IHP (3 mM). This region of the Raman spectrum contains the deoxy (~1355 cm^{-1}) and ligated (~1375 cm^{-1}) components of

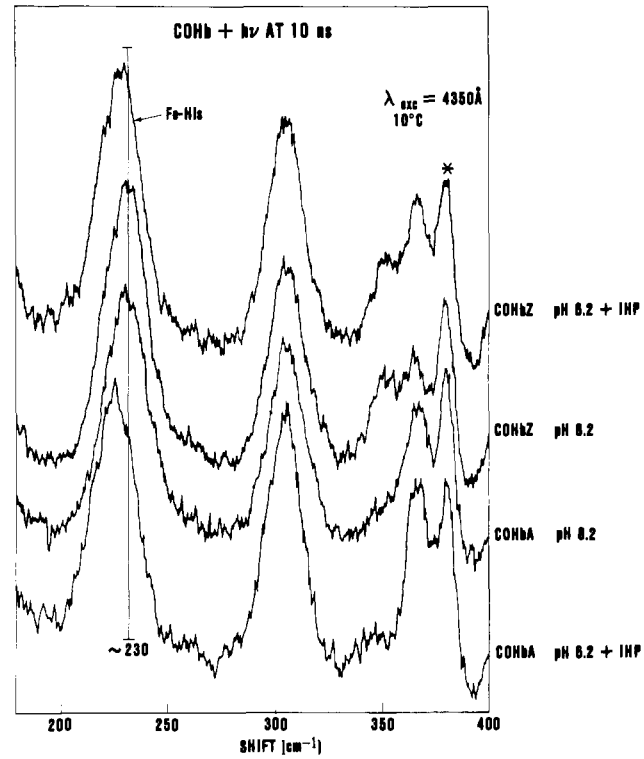

Figure 3. Low-frequency Raman spectra of the deoxy photoproduct occurring within 10 ns of photolyzing COHbA and COHbZ. The starred peak is from the sapphire front window of the sample cell. A front scattering geometry was employed. The pulse energies were ~1 mJ/pulse at 10 Hz.

the ν_4 (oxidation-state marker) mode. It can be seen from Figure 2 that at both high and low pH, the relative yield of deoxy heme is lower for COHbZ. However, for both hemoglobins, the pH 6.2 + 3 mM IHP solution conditions induce proportionately the same increase in the yield of deoxyheme relative to the sample at pH 8.2. At pH 6.2 + 0.3 mM IHP, there is no change in the deoxy-ligated ratio compared to pH 8.2. For the reasons discussed in the introduction, these differences in the average yields of photolysis are attributed to the differences in the geminate rebinding seen in Figure 1.

Figure 3 contains the low-frequency Raman spectra of the deoxy photoproduct occurring within 10 ns of the photolysis of the CO derivatives of HbA and HbZ under the two conditions utilized for Figure 2. In contrast to the conditions of partial photolysis employed in generating the spectra in Figure 2, these spectra were generated with pulses having both a higher intensity and a more resonant frequency. The last sample that was run (COHbZ, pH 6.2 + IHP) exhibited near but not complete photolysis which was due in part to a drop in pulse energy. At pH 8, ν_{Fe-His} is the same to within an inverse centimeter for HbA and HbZ. In both cases, the frequency decreases a few inverse centimeters in going from pH 8 to pH 6 + 3.0 mM IHP. That this is an IHP effect is supported by the absence of similar shifts for an IHP concentration of 0.3 mM at pH 6.2. In contrast to HbA, HbZ exhibits a discernible peak at ~345 cm^{-1}. This peak generally does not correlate with Hb reactivity but does appear to be related to protein changes induced by ligation regardless of the quaternary structure.[27,29,30] the results in Figure 3 indicate that with respect to the structural determinants of *this* Raman band, ligated HbZ has a more deoxy-like heme environment than ligated HbA in spite of both species having ν_{Fe-His} values that are characteristic of the ligated tertiary structure.

At pH 8.2, the 10-ns time-averaged yield of photolysis is higher for COHbA relative to COHbZ. The low-frequency Raman spectrum of the corresponding deoxy photoproducts indicates that the iron–proximal histidine linkage is the same in both proteins. In both cases, the addition of IHP at low pH both increases the relative yield of deoxyheme and produces a shift to lower frequency

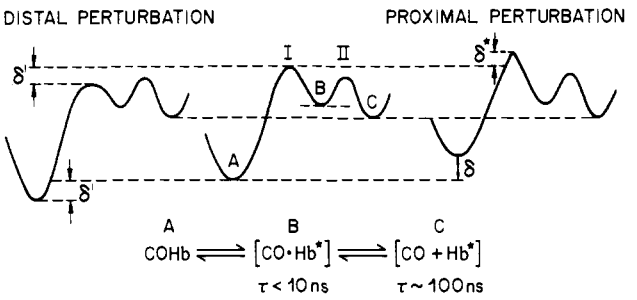

Figure 4. one-dimensional potential surfaces for ligand dynamics within the protein showing the effect of proximal and distal perturbations. Each surface corresponds to a fixed configuration of the protein. Well A corresponds to a ligated heme (COHb), well B to the initial adiabatically generated photoproduct consisting of a deoxyheme with the ligand in the immediate vicinity of the iron [CO·Hb*], and well C to the same deoxyheme but with the ligand in the bulk protein [CO + Hb*]. The deoxyheme is designated Hb* because its properties are modified by a nonequilibrium, i.e., ligated configuration of the protein. The lifetimes of the B and C associated species shown in the figure are based on geminate recombination studies. A detailed explanation of these diagrams can be found in ref 12. The middle configuration corresponds to COHbA at high pH. Relaxation of the distal constraints as might occur in COHbZ gives rise to the changes seen in the left-side configuration. Lowering the pH and adding IHP produces the modifications shown on the right.

of the Fe–His mode. From studies[12,13,26] on hybrid hemoglobins, it is clear that this IHP-induced shift in frequency and change in ligand reactivity originates primarily if not entirely in the β subunit. The difference in the magnitude of the frequency shift between HbA and HbZ (Figure 3) may be explainable in terms of both the laser-power-related decrease in the yield of photolysis of the HbZ sample at pH 6.2 and preferential rebinding to the β_z chains.

These findings indicate that at high pH, the observed difference in the yield of photolysis is not due to a difference in the iron–proximal histidine linkage. Furthermore, because of the shifts in the ν_{Fe-His}, the IHP-induced increases in the photolysis yield for COHbA and COHbZ both appear related to a change in the proximal linkage. Thus, whereas changes in the proximal linkage appear to be responsible for the IHP effect, the persistent IHP independent difference between COHbA and COHbZ must be explained in terms of other structural features such as the distal-side environmnet of the heme.

Infrared[31] and Raman studies[32] indicate that the Fe–CO linkage is modified in COHbZ vis-a-vis COHbA. The data is consistent with a less tilted or bent geometry for this linage in HbZ.[32] Although the changes in the associated bond strengths do not account for differences in reactivity between the two proteins, the spectral differences indicate a more open distal pocket for HbZ. Because of this difference, the structural constraints or changes in the distal pocket that are allegedly necessary for CO binding with HbA are reduced in HbZ. This difference should affect equally both the transition state for ligation and the potential energy well of the ligated heme as previously suggested.[33] Figure 4 depicts this distal-effect-induced alteration of the reaction coordinate diagram for geminate rebinding in hemoglobins. With model compounds in which geminate recombination is typcially not observed, this type of distal perturbation is anticipated and is observed to influence the association but not the dissocation rate.[33] It is evident from Figure 4 that in systems undergoing geminate recombination, the distal perturbation can affect both the macroscopic on and off rates. The figure also indicates that the effect should originate entirely from the change in the recovery of A from B (or C) because the instantaneous dissociation rate (A - B) should be unaltered ($\Delta A = \delta'$, $\Delta I = \delta'$). Evidence that barrier I is the primary variable in this distal effect includes transient absorption studies[18] and transient Raman studies at cryogenic temperatures[34] which show that barrier I is lower for the HbZ-derived subunits in the isolated β chains and in the intact tetramers, respectively.

The presented results show that proximal effects can be superimposed upon those derived from the relatively pH-insensitive distal perturbations discussed above. Insight into the basis for the proximal effect is afforded through the proposed relationship between ν_{Fe-His} and the tilt of the proximal histidine (in its own plane).[12,25,27] In this model based on X-ray crystallographic data,[3] Raman spectra, and theoretical studies,[35] the protein conformation, in particular the α_1–β_2 interface, determines the "tension" on the proximal histidine. For both equilibrium and transient deoxy species, it is claimed that the greater this tension, the more tilted is the histidine. Increased tilting increases the nonbonded repulsive interaction between the nearest pyrrole nitrogen and the same-side imidazole carbon. For comparable displacements of the iron out of the heme plane, the more tilted geometry is associated with the weaker iron–histidine bond due to the enhanced repulsive force between the imidazole and the pyrrole nitrogen. This weakening of the bond is reflected in the decrease in ν_{Fe-His}. Based on Raman studies of ν_{Fe-His} from both equilibrium and transient deoxy-Hb's derived from a large variety of mammalian, reptilian, and fish sources, it is observed that in general, this tension increases in going from T-state deoxy to T-state transient deoxy to R-state deoxy to transient R-state deoxy. For HbA and HbZ, low pH + IHP increases this tension for the R-state transient deoxy species as seen in this work. This increase in tension, as reflected in the decrease in ν_{Fe-His} is qualitatively similar to but smaller than the change observed in those COHb's that can be switched to the T state in the presence of IHP at low pH. The correlation between ν_{Fe-His} and reactivity stems from the increase in energy necessary to move the iron in plane upon ligand binding for the more tilted geometry.[12,35] This increase in energy can be viewed as an added frictional contribution associated with the movement of the iron into the heme plane. The effective result is to both increase the barrier height for binding at the heme (I) and destabilize the potential surface of the ligated heme (A) as shown in Figure 3. The extent to which I changes with respect to A is determined in part by the ligand-dependent nature of the transition state.[37]

Conclusion

The results of this and previous related work indicate that whereas the affinity in different hemoglobins is affected by both distal and proximal differences at the heme, cooperativity within a given hemoglobin appears to be largely under proximal control.

Registry No. HbA, 9034-51-9; HbZ, 9035-33-0.

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