PICOSECOND ABSORPTION STUDIES ON THE PHOTODISSOCIATION OF α- AND β-NITROSYL HEMOGLOBIN MONOMERS

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ABSTRACT Transient absorption studies of the pump-probe type were performed on the NO forms of the α- and β-monomers of hemoglobin using a Nd^{3+} phosphate-glass laser. A second harmonic 531-nm, 8-ps fwhm pulse pumped the Q-band while a delayed continuum generated pulse was used to monitor $\pi\pi^*$ Soret absorption changes in the 410–453-nm region. Photodissociation of nitrosyl α- and β-monomers was found to differ markedly from the tetramer in what we believe to be the formation of a five-coordinate HbNO (with proximal imidazole detached) photoproduct within the first 50 ps after photon absorption.

INTRODUCTION

In an earlier communication (1) we discussed the results of a picosecond transient absorption study on the α- and β-Hb monomers. Our analysis was based on a fit of the data to a nonadiabatic mechanism invoking excited octahedral states of the complex, both predissociative six-coordinate and postdissociative deoxy five-coordinate photointermediates observed during the first 50 ps after photon absorption. We found that the most notable differences between these monomers were the rate and extent of geminate recombination, results (*a*) showing that the β-chain geminately recombined faster than the α-chain (0.25 vs. 0.015 ps^{-1} for O_2 and 0.13 vs. 0.015 ps^{-1} for CO, respectively), and (*b*) consistent with a final state indicating an iron fully relaxed out of the porphyrin plane in the α-monomer and one that has not completely relaxed in the β-monomer indicating a mixture of deoxy $^3T_1$ (with the iron partially relaxed) and deoxy $^5T_2$ (with the iron fully relaxed). Where noise is not limiting, we have found the geminate rate is dependent on the spin states of the reacting partners. However there are conditions reported under which CO and O_2 recombine with deoxy-Hb with nearly equal rates in the distal heme pocket (contrary to predictions based on spinorbit considerations) in support of an adiabatic mechanism (2–4). In either case the tertiary heme geometry, distal and proximal, must be carefully considered in interpreting results. Our transient absorption work shows that variations in the proximal histidine heme linkage and the distal pocket hole size play important if not dominant roles in governing the rate of geminate recombination and photodissociation mechanism. For example, work on synthetic hemes (5) showed evidence of the transient formation of a pseudo four-coordinate for compounds having strained imidazole linkages that was absent in the nonstrained complexes. In the work reported here we find evidence for the formation of five-coordinate HbNO complex (with the proximal imidazole detached) in the α- and β-monomers, but not in the tetramer.

EXPERIMENTAL AND ANALYSIS

Preparation and purification of the α- and β-monomers from the tetramer Hb(A) are discussed in reference 1. Preparation of the NO complexes from the CO forms was done in complete absence of oxygen to avoid higher oxides of nitrogen (6, 7) which would denature Hb. NO (Union Carbide Corp., Lindy Div., New York, NY) was purified by bubbling through 20 cm of saturated KOH solution followed by 40 cm of KOH pellets. The sample was kept under flowing NO for ~1 h before being transferred to the experimental 1-mm optical pathlength cell. All sample preparations and experiments were performed at 5°C to minimize protein degradation. Ground state absorption spectra were taken before and after each set of experiments and compared with reference spectra to assure sample quality. Sample concentrations were adjusted with 0.025 M bisTris-Tris buffer, pH 7.40, to obtain absorbances of ~1.0 at the Soret maximum, ~50 μM. These low concentrations are necessary to maintain monomers for both the α and β forms (8). With a pump spot size of 1 mm we estimate that the sample receives a pulse of not more than 350 μJ/mm, conditions that should minimize nonlinear artifacts.

Details of the experimental setup and kinetic modeling procedure may be found in references 5 and 9. Modeling is necessary to obtain estimates of lifetimes and rate con-

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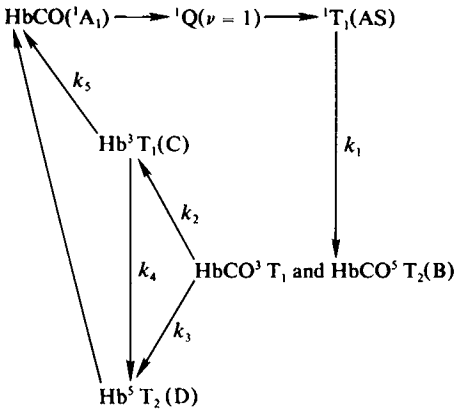
stants associated with the evolution of intermediates observed experimentally during the dissociation. Analysis of the early ΔA difference spectra, where the time delay between the probe and pump pulses is ≤10 ps, is complicated by the fact that each pulse has a 8-ps fwhm width. In effect the pump pulse excites different molecules at different times over a range of >8 ps depending on its temporal shape, which we assume to be gaussian. As the probe pulse passes through the sample, it "interrogates" molecules at different stages of excitation and delay while overlapped with pump pulse, extending the range of an experiment over 16 ps. This complication is resolved by analyzing the experimental absorbance change ΔA as a function of the delay between the probe and pump pulses using a numerical integration (Runge-Kutte-Nystrom) method which convolutes the pump and probe pulses together with rate expressions used to kinetically model the photodissociation. Experimental parameters used in the modeling procedure include the λ_{max}, the ϵ_{max}, and the fwhm for the ground state and intermediates which we are able to isolate at certain delay times by addition of the ground state spectrum to the ΔA spectrum: $A(\lambda,\Delta t) = \Delta A(\lambda,\Delta t) + A(\lambda,\text{ground})$. These parameters are not adjusted during the fitting procedure. However, initial guesses for the rate constants are needed. We obtain these from estimates of the intermediate lifetimes after workup of transient ΔA data. The rate constants are the only parameters adjusted so as to obtain the best computer fit to the experimental difference spectra for the entire set of pump-probe delays. Clearly, an important factor affecting the accuracy of this methodology is the degree of experimental noise present. This is particularly problematic in the blue region of difference spectra where the light available from the continuum generation for the absorption ($\log I_O/I$) analysis is comparatively weaker than that found in the red region. In our study of HbCO and HbO_2 (9) the noise in the blue region was ≤10% of the maximum ΔA excursion. Based on the isolation of four intermediates (AS, B, C, and D) and the calculated fit invoking radiationless transitions between octahedral 'bottleneck' states (10, 11), we proposed the following mechanism for the photodissociation of HbCO:



In our work on synthetic hemoglobins (5) and also on the photodissociation of the CO and O_2 forms of the α- and β-chains (1), noise in the blue spectral region was greater, ≤15% of the maximum ΔA, permitting us to experimentally isolate and kinetically model only intermediates B, C, and D (time delays starting at −20 ps are necessary to capture A. Results were consistent with the mechanism presented above, with the exception of synthetic CO complexes having strained proximal imidazole groups. These complexes showed evidence of a 'pseudo' four-coordinate intermediate, with both axial ligands dissociated, that preceded formation of B. In the case of HbNO discussed in the next section, the noise was comparable with these studies. The dissociation was consistent with the mechanism described above starting with intermediate B. Experiments on the NO forms of the α- and β-Hb chains are quite noisy, ≤20% of the maximum ΔA, precluding the detection and kinetic analysis of intermediates preceding C. However, we can model the recovery of the ground state HbNO (through analysis of k_5 and k_4), after experimentally isolating and characterizing intermediate C and D (which appears to be a five-coordinate HbNO complex with the proximal imidazole detached) in specific time delay frames as discussed in the next section. The effects of early intermediates found in the general mechanism are eliminated from the analysis by assigning large values to rate constants k_1, k_2, and k_3. We estimate, from differences found between the calculated and experimental $\Delta A(\lambda,\Delta t)$ curves for various choices of rate constants, that inaccuracies in the rates and lifetimes reported in the next section (using our methodology and proposed mechanism) to be no greater than ±10%.

RESULTS AND DISCUSSION

Experimental ΔA vs λ spectra at pump-probe delays ranging between −12 and 50 ps for Hb(α)NO are shown in Fig. 1. Because of the relatively high degree of noise in the blue region, the spectra have been redrawn using a 31-point smoothing algorithm on the right side. This also facilitates comparison of the experimental to the kinetically modeled spectra found in Fig. 2. Absorption spectra of photointermediates C and D are shown in Fig. 3. Results for Hb(β)NO are similar. For comparison, we ran a parallel set of experiments on the tetramer Hb(A)NO finding no major differences with the previously reported work (12), but revealing significant differences between the spectroscopic characteristics of D found in the tetramer, also shown in Fig. 3, in comparison to D seen in the α- and β-complexes in this as well as the previous study (1) on the CO and O_2 derivatives.

With reference to the photodissociation scheme discussed in the previous section, we wish to emphasize that the assignment of intermediates to specific octahedral $Fe^{2+}(d_6)$ states remains speculative at this point and that no new information is presented in this investigation to

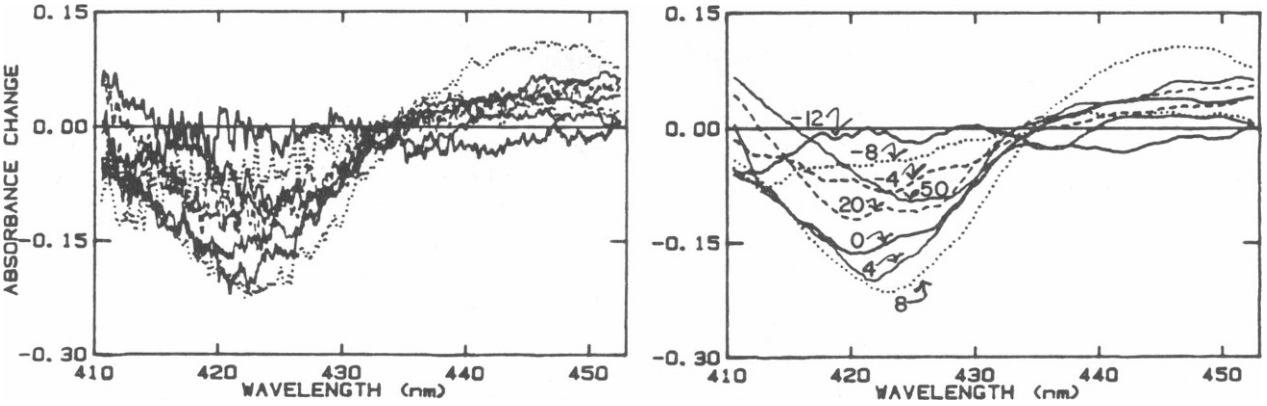


FIGURE 1 Experimental spectra for Hb(α)NO. Delays range from −12 to 50 ps. Spectra on the right have been redrawn using a 31-point smoothing algorithm to facilitate comparison of the kinetically modeled spectra found in Fig. 2.

strengthen these assignments. In the CO complexes, for example, we have linked intermediate AS to $^1T_1$ found directly below the porphyrin $\pi\pi^*$ Q level where the 531-nm pump pulse initiates the dissociation and B to a mixture of $^3T_1$ and $^5T_2$, also in the predissociative six-coordinate manifold. Noise in the α- and β-NO experiments was too severe to isolate intermediates corresponding to AS or B. In the six-coordinate NO manifold these states are expected to be quite different in character than those found in the CO and O_2 manifolds. However, in the tetramer we do find evidence of B in the form of a broad, low extinction absorption characterized by early bleaching in the Soret region ~419 nm and appearance of photoproduct in the red portion of spectrum for early negative delay experiments. The origin of this intermediate in the NO complex is not clear; it might be due to an electronically excited predissociative state(s) or in part a consequence of the reported high population of low-lying charge-transfer states (13) assessed radiationlessly that are linked to the cause of the low quantum yield of photodissociation through stabilization of the R structure of HbNO (14).

Photointermediate C was identified with the postdissociative short lived $^3T_1$ state in past work (1). Results and the kinetic modeling workup in this study also support this conclusion. It is seen in all derivatives (CO, O_2, and NO) of both isolated chains, the tetramer (9), as well as in the CO and O_2 synthetic heme complexes that we have studied (5). In Hb(α)NO it is characterized through a broad flat shoulder in the red spectral region of the 0- and 4-ps ΔA spectra shown in Fig. 1, (the 4- and 8-ps spectra in the β-monomer) leading to a transient absorption spectrum having a maximum at 445 nm (Fig. 3).

Intermediate C ($^3T_1$) has the potential to geminately recombine with NO in a spin allowed process to form the parent doublet ground state complex HbNO or radiationlessly relax to the lower $^5T_2$ state (intermediate D) in the deoxy manifold. As in past work we see clear experimental evidence for the arrival of D in the tetramer in competition with the relaxation of C; noticeable sharpening of the 20- and 50-ps ΔA spectra in the red spectral region concomitant with >50% recovery of the ground state by 50 ps. From the kinetic modeling procedure we find that geminate relaxation to the ground state dominates the competition for C,

$$\mathrm{Hb}(^3T_1) \xrightarrow{0.01\ \mathrm{ps}^{-1}} \mathrm{Hb}(^5T_2) \text{ vs. } \mathrm{Hb}(^3T_1) + \mathrm{NO} \xrightarrow{0.3\ \mathrm{ps}^{-1}} \mathrm{HbNO},$$

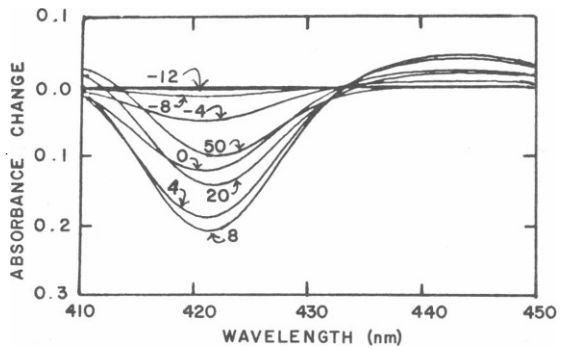


FIGURE 2 Modeled spectra for Hb(α)NO at the delays indicated. The rate constants that gave the best fit to the experimental spectra are discussed in the text with regard to the proposed photodissociation-geminate recombination mechanism.

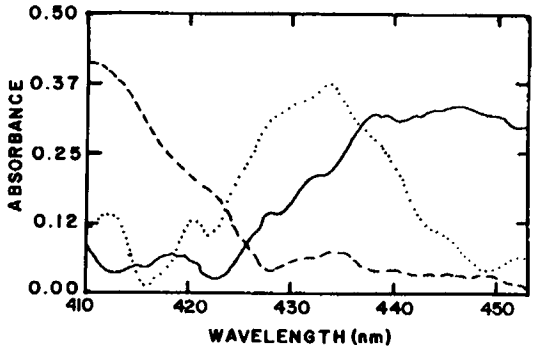


FIGURE 3 Smoothed absorption spectra (31-point algorithm) of the C (*solid*) and D (*dashed*) intermediates found in Hb(α)NO. The intermediate D (*dotted*) found in Hb(A)NO tetramer is included for comparison.

resulting in a short ~3-ps lifetime for C. The 100-ps transient relaxation is identical to the result reported in the work by Cornelius and co-workers (9) for the weak component of the biphasic relaxation using a 353-nm pump pulse. They attribute a faster 17-ps decay to geminate recombination. Our kinetic modeling–pulse deconvolution analysis indicates that this rate is somewhat faster, 0.3 vs 0.06 ps^{-1}. The redshifted λ_{max} of C (~449 nm) in comparison with the λ_{max} of D (~432 nm) indicates that the iron in C is not fully relaxed out of the plane of the porphyrin yielding a high-affinity shortlived "R-like" species that can rapidly recombine with NO. The λ_{max} of D is close to the steady-state deoxy value of 430 nm (15), suggesting that the iron in this state is almost fully relaxed out of plane where the heme would be closer to being described as a low-affinity "T-like" structure. In such a structure, geminate recombination through D ($^5T_2$) would be expected to be slower due to tertiary structural restrictions as well as spin orbit considerations. Results found in this and our earlier studies are not a proof of the nonadiabatic mechanism, but they are consistent with it.

Unlike the Hb(A)NO results or our study on the CO and O_2 isolated chains, intermediate D seen in the α- and β-NO complexes has a strikingly different λ_{max} located <410 nm. It shows up clearly in this region for the α-monomer and is less apparent in the β-subunit, but it is nevertheless present in each chain. In the 20- and 50-ps difference curves, Fig. 1, geminate recovery of the ground state is obvious at 422 nm, but a substantial amount of photoproduct remains without a typical domed ΔA at 440 nm that is characteristic of the "normal" arrival of D. Absorbances are essentially flat during these delay periods in this spectral region. In addition, the spectral characteristics of the bleach have changed to show a slight red shift in these time frames, indicating that an absorption is growing in.

In contrast to the O_2 and CO heme derivatives, the NO species has a shorter ligand-Fe bond (16, 17) and an odd electron which weakens or lengthens the transaxial Fe-imidazole bond (16, 18, 19) through a transfer of this electron to the Fe d_Z2 orbital (19) giving rise to the possibility of breaking the Fe-Im bond under added heme stress. For this reason the study of nitrosyl heme complexes with the addition of inositol hexaphosphate (IHP) has been popular (16–22). IHP produces a T six-coordinate configuration where the Fe-Im bond ruptures after photon absorption in Q or Soret bands. As heme conformation is governed by the *N*(proximal imidazole)-Fe bond length (18, 23, 24), the longer Fe-Im bond distance found in HbNO imposes less restraint on the equilibrium oxy structure allowing the IHP to switch the iron out of the plane of the porphyrin to the deoxy T conformation. The shorter Fe-Im bond of CO and O_2 Hb is believed to restrict formation of six-coordinate T hemoglobin (18).

Without the quaternary constraint of the tetramer, it is possible that the isolated subunits do not require added strain on the heme through addition of IHP for the "weak" Fe-Im bond to break. This supposition is supported through the similar absorbance of the late delay ΔA curves (Fig. 3) and the reported absorbance of the five-coordinate HbNO complexes (25–27) that possess a Fe-NO bond without a Fe-Im bond. These species typically show maximum absorbances at ~400 nm.

There are two possible ways in which D, the five-coordinate HbNO, may be formed in terms of intermediates introduced above: directly from B to D, or indirectly through B to C to D. The model proposed in this work requires (for both paths) that the iron be in or near the plane of the porphyrin, intermediates B or C, before the formation of D. If the iron being fully or almost fully relaxed out of the plane was a prerequisite to the formation of D, then an out-of-plane intermediate characterized by a λ_{max} at ~430 nm should have been observed in the α- and β-HbNO experiments and this was not the case.

Experimentally, the formation of five-coordinate Hb(α)NO is more prominent than that of the β-monomer as seen through the increased positive ΔA at 410 nm and by the degree of red shift in the bleach at 421 nm. IHP studies on the Fe-Im bond cleavage (16–22) have shown that in the tetramer β-chains exist mainly in six-coordinate hemes while α-chains were predominately five-coordinate. Arnone and Perutz (28) found that the β-chain does undergo a tertiary structure change upon addition of IHP, however there must be insufficient heme tension to produce a five-coordinate Hb(β)NO species without IHP. This chain nonequivalence is also pointed out in our recent work on the photodissociation of O_2 and CO from the isolated subunits (1) where the strain on the heme was evident through differing rates of geminate recombination; the larger amount of tension in the α-monomer was associated with a faster relaxation of the iron out of the plane of the porphyrin concomitant with a lower ligand affinity resulting in a slower geminate rate compared with that of the β-monomer. The faster geminate recombination of the β-subunit was attributed to several possibilities. The first involved steric hindrances imposed by the distal residues of the protein. Perutz and co-workers (29) have shown through x-ray analysis that the distal heme pocket of the α-subunit is larger than that of the β-subunit. Additionally in changing from six- to five-coordinate deoxy-β, val-Ell moves ~1 Å toward the heme plane. On this basis one could argue that the dissociated axial ligand would be held in close proximity to the iron due to the steric restrictions of val-Ell, thereby increasing the probability of geminate recombination. However, this differs significantly from the α-monomer where the size of distal pocket remains about the same in changing from the ligated to the unligated complex. In addition the steric hindrance of distal his-E7, which is believed to lower the binding affinity of CO compared with O_2 (30–33), is magnified in the smaller β-pocket. The 2:1 geminate rate ratio we see for O_2:CO in the β-monomer is an indication of the importance

of his-E7 in this process. The second possibility for the observed differences in geminate rate could be due to slight differences in the proximal his-F8 tilt between the α- and β-monomers; x-ray analysis by Baldwin and co-workers (34) shows that the tilt of the proximal imidazole in the β-subunit is less than in the α-subunit. On this basis, there is less "strain" on the porphyrin plane in the β-complex favoring a higher affinity which is also consistent with our results.

In this investigation we find that the greater tension on the heme in the α-monomer not only results in a difference in the geminate rate, 0.3 ps^{-1} for β vs. 0.09 ps^{-1} for α from the kinetic modeling, but is also evident as a higher concentration of five-coordinate Hb(α)NO by 50 ps. The difference seen between the α- and β-monomers in the lifetime of C, 3.5 ps for β vs. 10 ps for α, in the NO complexes is smaller than that seen for the O_2 and CO forms (for O_2, 3.7 vs. 25 ps and for CO, 7 vs. 31 ps for the β- and α-monomers, respectively). However, a clear distinction is not to be expected here due to the fact that NO geminately recombines very rapidly.

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REFERENCES

1. Guest, C. R., and L. J. Noe. 1987. Photodissociation of CO and O_2 from α and β hemoglobin chains studied by using picosecond absorption spectroscopy. *Biophys. J.* 52:885–889.
2. Jortner, J., and J. Ulstrup. 1979. Dynamics of nonadiabatic atom transfer in biological systems. Carbon monoxide binding to hemoglobin. *J. Am. Chem. Soc.* 101:3744–3754.
3. Redi, M. H., B. S. Gerstman, and J. J. Hopfield. 1981. Hemoglobin-carbon monoxide binding rate. *Biophys. J.* 35:471–484.
4. Frauenfelder, H., and P. G. Wolneys. 1986. Rate theories and puzzles of hemeprotein kinetics. *Science (Wash. DC).* 229:337–345.
5. Caldwell, K., L. J. Noe, L. D. Ciccone, and T. G. Traylor. 1986. Photodissociation of carbon monoxide and dioxygen forms of synthetic heme complexes studied using picosecond absorption spectroscopy. Evidence of a pseudo-four-coordinate intermediate. *J. Am. Chem. Soc.* 108:6150–6158.
6. Chevion, M., A. Stern, J. Peisach, W. E. Blumberg, and S. Simon. 1978. Analogous effect of protons and inositol hexaphosphate on the alternation of structure of nitrosyl fetal human hemoglobin. *Biochemistry.* 17:1745–1750.
7. Scholler, D. M., M. R. Wang, and B. M. Hoffman. 1979. Resonance Raman and EPR of nitrosyl human hemoglobin and chains, carp hemoglobin, and model compounds. *J. Biol. Chem.* 154:4072–4078.
8. Valdes, R., Jr., and G. K. Ackers. 1977. Thermodynamic studies and subunit assembly in human hemoglobin. *J. Biol. Chem.* 252:74–81.
9. Hutchinson, J. A., and L. J. Noe. 1984. A study of the photodissociation of the CO and O_2 forms of hemoglobin and myoglobin by picosecond absorption spectroscopy. *J. Quant. Electronics QE.* 20:1353–1362.
10. Eaton, W. A., L. K. Hanson, P. J. Stephens, J. C. Sutherland, and J. B. R. Dunn. 1978. Optical spectra of oxy- and deoxyhemoglobin. *J. Am. Chem. Soc.* 100:4991–5001.
11. Greene, B. I., R. M. Hochstrasser, R. B. Weisman, and W. A. Eaton. 1978. Spectroscopic studies of oxy- and carbonmonoxyhemoglobin after pulsed optical excitation. *Proc. Natl. Acad. Sci. USA.* 75:5255–5259.
12. Cornelius, P. A., R. M. Hochstrasser, and A. W. Steele. 1983. Ultrafast relaxation in picosecond photolysis of nitrosylhemoglobin. *J. Mol. Biol.* 163:119–128.
13. Chantranupong, L., G. H. Loew, and A. Waleh. 1986. Quantum mechanical studies of the photodissociation of nitrosoheme complexes. *Am. Chem. Soc. Symp.* 321:2–19.
14. Shelnutt, J. A., D. L. Rousseau, J. M. Friedman, and S. R. Simon. 1979. Protein-heme interaction in hemoglobin: evidence from Raman difference spectroscopy. *Proc. Natl. Acad. Sci. USA.* 76:4409–4413.
15. Antonini, E., and M. Brunori. 1971. Hemoglobin and myoglobin in their reactions with ligands. *Front. Biol.* 21:2–6.
16. Maxwell, J. C., and W. S. Caughey. 1976. An infrared study of NO bonding to heme b and hemoglobin A. Evidence for inositol hexaphosphate induced cleavage of proximal histidine to iron bonds. *Biochemistry.* 15:388–409.
17. Perutz, M. F., J. V. Kilmartin, K. Nagai, A. Szabo, and S. R. Simon. 1976. Influence of globin structures of the state of the heme. *Biochemistry.* 15:378–387.
18. Szabo, A., and L. D. Barron. 1975. Resonance Raman studies of nitric oxide hemoglobin. *J. Am. Chem. Soc.* 97:660–662.
19. Stong, J. D., J. M. Burke, P. Daly, P. Wright, and T. G. Spiro. 1980. Resonance Raman spectra of nitrosyl heme proteins and of porphyrin analogues. *J. Am. Chem. Soc.* 102:5815–5819.
20. Perutz, M. R., and A. Szabo. 1976. Equilibrium between six- and five-coordinated hemes in nitrosylhemoglobin: interpretation of ESR spectra. *Biochemistry.* 15:4427–4428.
21. Nishikura, K., and Y. Sugita. 1976. The effect of inositol hexaphosphate on the absorption spectra of α and β chains in nitrosyl hemoglobin. *J. Biochem.* 80:1439–1441.
22. Nagai, K., C. Welborn, D. Dolphin, and T. Kitagawa. 1980. Resonance Raman evidence for cleavage of the Fe-N_ϵ (his-F8) bond in the α subunit of the T-structure nitrosylhemoglobin. *Biochemistry.* 19:4755–4761.
23. Friedman, J. M., T. W. Scott, G. J. Fisanick, S. R. Simon, E. W. Findsen, M. R. Ondrias, and V. W. MacDonald. 1985. Localized control of ligand binding in hemoglobin: effect of tertiary structure on picosecond geminate recombination. *Science (Wash. DC).* 229:187–190.
24. Findsen, E. W., J. M. Friedman, M. R. Ondrias, and S. R. Simon. 1985. Picosecond time-resolved resonance Raman studies of hemoglobin: implications for reactivity. *Science (Wash. DC).* 229:661–665.
25. Brault, D., and M. Rougee. 1973. Spectral studies of ferrous deuteroporphyrin in organic solvents. *Nat. New Biol.* 241:19–20.
26. Rougee, M., and D. Brault. 1975. Influence of trans weak or strong field ligands upon the affinity of deuteroheme for carbon monoxide. *Biochemistry.* 14:4100–4106.
27. Wayland, B. B., and L. W. Olson. 1974. Spectroscopic studies and bonding model for nitric oxide complexes of iron porphyrins. *J. Am. Chem. Soc.* 96:6037–6041.
28. Arnone, A., and M. F. Perutz. 1974. Structure of inositol hexaphosphate-human deoxyhaemoglobin complex. *Nature (Lond.).* 249:34–36.
29. Perutz, M. F. 1970. Haem-haem interaction and the problem of allostery. *Nature (Lond.).* 228:726–734.
30. Heidner, E. J., R. C. Ladner, and M. F. Perutz. 1976. Structure of horse carbonmonoxyhaemoglobin. *J. Mol. Biol.* 104:707–722.
31. Giacometti, G. M., M. Brunori, E. Antonini, E. E. DiIorio, and K. H. Winterhalter. 1980. The reaction of hemoglobin Zurich with oxygen and carbon monoxide. *J. Biol. Chem.* 255:6160–6165.

32. Doster, W., D. Beece, S. F. Bowne, E. E. DiIorio, L. Eisenstein, H. Frauenfelder, L. Reinisch, E. Shyamsunder, K. H. Winterhalter, and K. T. Yue. 1982. Control and pH dependence of ligand binding to heme proteins. *Biochemistry*. 21:4831–4839.
33. Collman, J. P., J. I. Brauman, and K. M. Doxsee. 1979. Carbon monoxide binding to iron porphyrins. *Proc. Natl. Acad. Sci. USA*. 76:6035–6039.
34. Baldwin, J., and C. J. Chothia. 1979. Haemoglobin: the structural changes related to ligand binding and its allosteric modeling. *J. Mol. Biol.* 129:175–220.