Evidence for a Slow Tertiary Relaxation in the Reaction of *tert*-Butyl Isocyanide with Horseradish Peroxidase†

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ABSTRACT: The kinetics of *tert*-butyl isocyanide binding to the heme protein horseradish peroxidase (HRP) at 22 °C was examined on all time scales, from minutes to picoseconds, in aqueous borate buffer at pH 9.08. Unlike myoglobin (Mb) or hemoglobin, HRP shows two bimolecular ligand binding processes. For comparison, binding of the same ligand with Mb was measured under identical conditions. Ligand entry into the protein from the solvent in a mixing experiment is extremely slow in HRP: the bimolecular association constant is 0.04 M^{-1} s^{-1}, while in Mb it is 4×10^3 M^{-1} s^{-1}. Surprisingly, in view of that difference, picosecond and nanosecond photolyses reveal that once the ligand has reached the iron(II) site there is no difference in cage return or escape from the protein. The rate for the fastest cage return (from the contact pair) is close to 6×10^{10} s^{-1} in both proteins. The rates of escape from the contact pair to form a secondary protein-caged pair are also similar: for Mb, 10×10^{10} s^{-1}, and for HRP, 8.5×10^{10} s^{-1}. The rate of rebinding from the protein-separated cage is near 4×10^6 s^{-1} in both proteins, and the rate of escape from protein to solvent is close to 3.7×10^6 s^{-1} in both. The difference between the two proteins lies in the low-millisecond time domain. After flash photolysis of HRP, there is a concentration-dependent recombination not seen in mixing experiments. This bimolecular rate constant varies slightly for different HRP preparations, being 2.6×10^4 or 4.0×10^4 M^{-1} s^{-1} in two cases, both of which are much faster than is observed in mixing experiments, namely, 0.04 M^{-1} s^{-1}. In Mb, photolysis and mixing experiments consistently give the same combination rate, which is somewhat slower than the faster part of the HRP recombination. Similar measurements for the smaller ligand methyl isocyanide revealed no anomalous behavior. The interpretation proposed involves tertiary relaxation after ligand escape, which is significant in blocking the return of the large *t*-BuNC, but has no apparent effect on smaller ligands. Thus, HRP−*t*-BuNC reveals in dramatic fashion a phenomenon merely hinted at in earlier work involving the T-state binding kinetics of hemoglobin.

Ligand binding to myoglobin (Mb),[1] hemoglobin, and other heme proteins is much studied both because the proteins themselves are important and because they are relative simple systems, useful for testing experimental methods and developing theoretical models. Since the protein system is complicated, insight largely comes from comparing different heme proteins, as we are doing here, or the same protein from many species (Friedman, 1985), considering natural or deliberately engineered mutants (Rohlfs et al., 1990), or from comparing proteins with simpler, biomimetic models of the active site (Momenteau & Reed, 1994). Similarly, O_2 as a ligand may be contrasted with CO, NO, and a host of other chemical probes. In this work, we report kinetic studies on horseradish peroxidase (HRP) reacting with the large ligand *tert*-butyl isocyanide (*t*-BuNC). We use a combination of ultrafast kinetic studies, which explore what happens while the photodissociated ligand remains in the protein before it escapes to the solvent, along with conventional flash photolysis and mixing studies that monitor bimolecular association between protein and ligand. The novel finding, which came as a complete surprise, is that even though the kinetics of geminate rebinding at picosecond and nanosecond times are virtually identical in HRP and Mb, the bimolecular portion of the recombination of photolyzed *t*-BuNC shows two distinct phases on widely different time scales. Conventional mixing studies show only the slower process. Such behavior is well-known following (complete) photolysis of tetrameric Hb (Sawicki & Gibson, 1976; Martin & Parkhurst, 1990). In that case, some fraction of the escaped ligand recombines with Hb still in its high-affinity, fast-reacting R state prior to a transition from the R to the T state. After the R → T transition, which requires about 20−100 μs, further recombination occurs at a significantly slower rate. Such behavior in Hb involves quaternary structural changes,

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[1] Abbreviations: Mb, myoglobin (horse heart, unless otherwise specified); HRP, horseradish peroxidase (type VI-A, unless otherwise specified); Hb, hemoglobin (typically adult human); MeNC, methyl isocyanide; *t*-BuNC, *tert*-butyl isocyanide; *Q*(X), quantum yield of photolysis for ligand X, which is the probability that an absorbed photon will dissociate an iron−X bond and that the ligand will actually escape from the protein into the surrounding solvent.

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but HRP is monomeric and cannot have a quaternary change. We infer that, with HRP and the large ligand *t*-BuNC, we have a system that displays a delayed and very significant transformation in the tertiary structure. We conjecture that the behavior illustrated in this system is not unique and unrelated to normal protein function, but rather is an exaggerated version of processes that may be widespread in more subtle forms.

Mechanistic studies of the enzyme functions of HRP were reviewed by Dawson (1988). The only ligand binding studies we are aware of all involve CO. A conventional kinetic study (Coletta et al., 1986) resolved earlier discrepancies between kinetic and equilibrium data and went on to suggest that HRP differs from Mb in both the steric and polar properties of its distal residues near the binding site. There is a detailed study at low temperature (Doster et al., 1987) and also one ultrafast kinetic study (Berinstain et al., 1990). There are no studies involving isocyanides.

In any kinetics study, it is important to have a clear view of the kinetic model that is postulated. We recently argued (with references to earlier work) that, after photolysis of the iron−ligand bond in Mb, or other heme proteins, even at room temperature, the probability of reforming the bond is a markedly nonexponential process, with the probability (or rate) of recombination progressively slowing on all time scales, from low picoseconds to many nanoseconds (Walda et al., 1994). Consequently, debates about how many intermediates might be called for do not address a fundamental question, but only an issue of appropriate approximation for particular circumstances. The factors affecting the kinetics of rebinding probably include both proximal and distal effects, as we argued elsewhere (again with references to prior literature) (Duprat et al., 1995). One may distinguish between two possible sources of the kinetic complexity: it could be that an ensemble of protein conformations exists prior to photolysis, some favoring rebinding and others not, or it could be that when a ligand is bound and for a short time after photolysis all of the ensemble exists in a conformation favorable for bond formation, but after bond breaking the protein relaxes over time through a series of conformational changes in which reforming the bond is progressively less favored. It is clear that in solid solutions at very low temperatures proteins exist in heterogeneous forms (Austin et al., 1975), which recombine with different kinetics, and it seems likely that, in aqueous solutions at ambient conditions, some heterogeneity persists immediately after photolysis. What is unclear is how long such a heterogeneous population persists. At the same time, it is also clear in some cases that there is a delayed conformational change after photolysis that profoundly affects the kinetics of recombination. The R → T transition in Hb is a persuasive example. To us it seems likely that there are conformational changes occurring in the tertiary structure as well, over a wide range of time scales, which have, at least in principle, some effect on kinetic behavior. An elaboration of such a model was presented by Agmon and Hopfield (1983). In our previous work, we had in mind tertiary changes over no more than a few nanoseconds, which would affect only geminate recombination.

For our present purpose, we simplify such a continually changing situation to a simpler "four-state" model (Jongeward et al., 1988), shown in eq 1, for any heme, Hm, and ligand, L. This is sufficient for the present argument and facilitates

$$\underset{\text{bound state}}{\text{H}-\text{L}} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{\text{contact pair}}{[\text{Hm L}]} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \underset{\text{protein-separated pair}}{[\text{Hm}|\text{L}]} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \underset{\text{separated species in solvent}}{\text{Hn} + \text{L}} \quad (1)$$

comparison with earlier work. It is also the case that isocyanides show rebinding that is concentrated in two very different time ranges, more so than the diatomic ligands. We suppose that the larger size of the isocyanides enforces a sharper distinction in the protein pocket between geometries in which bond formation is possible and those in which it is not. The time scale for ligand rebinding from the contact pair state falls in the picosecond time range, while rebinding from the protein-separated pair is observed on the nanosecond time scale. Both the pico- and nanosecond processes are concentration-independent and are described as cage processes. The last step is slow, bimolecular association−dissociation, which is concentration-dependent, and typically is investigated using a large excess of L. Our version of the four-state model is supported especially by studies on model compounds, which are just the iron(II) porphyrins with one axial base and another axial ligand L (Traylor et al., 1987); Bag et al., 1994; David et al., 1994; Grogan et al., 1994). As no protein is present, no nanosecond process is observed, yet there is recombination virtually identical to that ascribed to the contact pair. (One sometimes finds two or more fairly similar rates on either the pico- or nanosecond scale or both, leading to models with five or more states. We have reported such, but treated them as elaborations of the basic four-state model to incorporate additional heterogeneity. Ultimately, we became convinced that a continuum of rates is called for, as described earlier.)

The isocyanides are a good choice for studying cage processes. They have the general formula RNC, and one can vary the size and the nature of R to study steric and electronic effects on cage return. Olson and co-workers have used them to good advantage (Johnson et al., 1989; Rohlfs et al., 1990). Here we report the kinetics of the reactions of HRP with *t*-BuNC, with brief comparison to MeNC. We find nothing unusual in the cage return from either the contact pair or the protein-separated pair, but we do have to modify eq 1 to a five-state model to accommodate unusual behavior in the bimolecular combination step. We use

$$\text{HRP}'-\text{L} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [\text{HRP}'\ \text{L}] \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} [\text{HRP}'|\text{L}] \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{HRP}' + \text{L} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \text{HRP} + \text{L} \quad (2)$$

In eq 2, L is specifically *t*-BuNC, HRP′ is an unrelaxed conformation that binds L, and HRP is a relaxed form that binds poorly or not at all to such a large ligand, but may bind smaller ligands. Given assumptions about widely different time scales, the elementary steps of the mechanism are related to observables as follows. Let k_{ps} and k_{ns} be the measured rates for the concentration-independent, geminate recombination phases, and ϕ_{ps} and ϕ_{ns} be the percentage

recombination observed on those time scales. Then,we have

$$k_{-1} = k_{ps}\phi_{ps}$$

$$k_2 = k_{ps}(1 - \phi_{ps})$$

$$k_{-2} = k_{ns}\phi_{ns}/\phi_{ps}$$

$$k_3 = k_{ns}(1 - \phi_{ns})$$

the effective quantum yield Q for photolysis to produce separated species

$$Q = (1 - \phi_{ps})(1 - \phi_{ns})$$

and the apparent rate k_{obs} observed in a mixing experiment is

$$k_{obs} = k_{off} + k_{on}[L]$$

with

$$\text{model:} \quad k_{off} = k_1 Q \quad \text{and} \quad k_{on} = k_{-2}(1 - Q)$$

$$\text{myoglobin:} \quad k_{off} = k_1 Q \quad \text{and} \quad k_{on} = k_{-3}(1 - Q)$$

$$\text{HRP:} \quad k_{off} = \frac{k_{-4}}{k_4} k_1 Q \quad \text{and} \quad k_{on} = \frac{k_{-4}}{k_4} k_{-3}(1 - Q)$$

This k_{off} for HRP pertains to the intercept of a plot of association rate *vs* concentration after mixing and not necessarily to other determinations.

MATERIALS AND METHODS

Materials. Horseradish peroxidase (types VI-A and XII) and horse heart myoglobin were purchased from Sigma. Sodium dithionite and *tert*-butyl isocyanide were from Aldrich. Methyl isocyanide was prepared from *N*-methylformamide by the literature procedure (Reisberg & Olson, 1980) and was stored under argon at 0 °C. A small quantity was passed through a small, dry alumina column just before use to remove impurities that might develop during storage. Sodium borate, potassium phosphate, and potassium hydrogen phosphate were obtained from Mallinckrodt. Buffers were prepared by dissolving quantities of solids in deionized water.

Sample Preparation. A slow stream of argon was passed for about 20 min over solid (approximately 4.8 mg) microcrystalline samples of HRP in a small, gas-tight glass vial. Degassed borate buffer (1.5 mL) was added to the protein, and the vial was shaken very gently. The resulting protein solution (1.0 mL) was transferred anaerobically into a quartz cuvette of 2 mm path length, and the final concentration of the protein was determined from the absorbance at 403 nm ($\epsilon = 1.02 \times 10^5$ cm^{-1} M^{-1}). Saturated sodium dithionite solution (3−5 μL) was added to the cuvette to reduce the ferric heme to ferrous heme. A known amount of isocyanide was added to the reduced heme using a microliter syringe. Absorbance at 431 nm was taken as the measure of the isocyanide adduct of HRP. The kinetic data were all collected within 30−45 min of making up the sample. A very fresh sample is always desirable and was essential in the case of MeNC, for which it was observed that any sample kept longer than 1−2 h developed a new band at 454 nm and lost the 431 nm band.

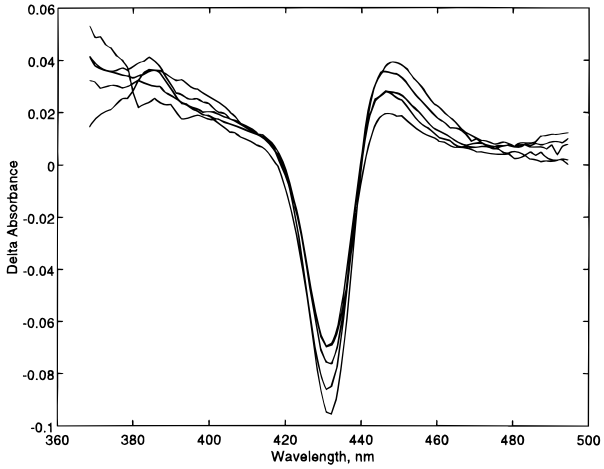

FIGURE 1: Typical absorbance difference spectra following subpicosecond photolysis of HRP−*t*-BuNC in 0.05 M borate buffer at pH 9.06 and 22 °C. Time delays are 1, 3, 5, 7, and 9 ps, increasing upward at 433 nm. These are extracted from a single run, which would typically include traces every 0.5 ps for 50 ps. Final kinetic results are averaged over many such runs. Spectral filtering reduces light at both extremes of this plot, such that data are so noisy as to be meaningless.

Picosecond and Nanosecond Kinetics. The apparatuses were described recently (Walda et al., 1994). Conventional spectra and kinetics were recorded with a Kontron Uvikon 810 spectrophotometer. Measurements were made at 22 ± 1 °C.

RESULTS AND DISCUSSION

In its native state, HRP has a 5-coordinated Fe(III) with a Soret band maximum near 406 nm. When it is reduced to Fe(II), it reacts with both MeNC and *t*-BuNC. A broad Soret maximum for deoxy-HRP−Fe(II) at 437 nm changes to a sharp, intense feature at 430 nm upon the addition of any isocyanide in borate buffer at pH 9.08. All of these spectral features are very similar to what is observed with Mb under corresponding conditions. The isocyanide spectra are slightly shifted, but are otherwise similar to those of the CO analogs. It is important to note that, in the case of HRP−MeNC, the Soret maximum at 430 nm slowly decreases in amplitude, concomitant with the growth of a broad feature at 454 nm. This change, which is characterized by very nice isosbestic features, is noticeable in as little as 1 h, depending on the MeNC concentration. This deserves further study, but we report here only the results collected under conditions for which the Soret intensity at 430 nm remained unaltered within 2−3% uncertainty.

Typical transient difference spectra for the picosecond rebinding of *t*-BuNC are presented in Figure 1. Spectra are displayed at intervals of 2 ps. The rate constant for rebinding was derived from data at both the 6-coordinate bleach (430 nm) and the 5-coordinate transient absorption (450 nm). The observed rate constant k_{ps} and percentage return ϕ_{ps} are (1.4−1.7) $\times$ 10^{11} s^{-1} and 45%, respectively.

The kinetics of ligand rebinding on the nanosecond time scale were derived from data (not shown) collected at 445 nm with a different instrument. The observed rate constant k_{ns} and the percentage return ϕ_{ns} for the nanosecond process after photolysis of *t*-BuNC are 8.0 $\times$ 10^6 s^{-1} and 53%,

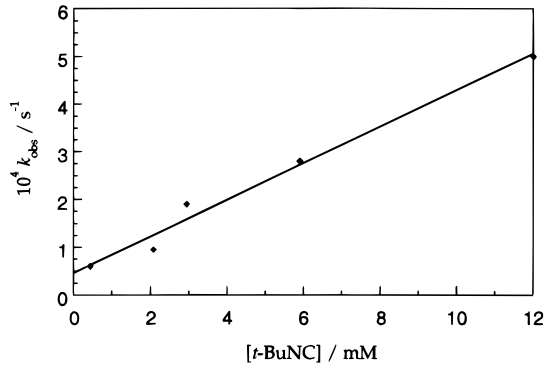

FIGURE 2: Reaction rate constant k_{obs} as a function of concentration for the combination of HRP with *t*-BuNC, measured at 445 nm in 0.05 M borate buffer at pH 9.06 in a mixing experiment.

respectively. Both the pico- and nanosecond processes were found to be independent of ligand concentration and, consequently, were attributed to recombination from protein-caged geminate pairs, the faster involving intimate pairs and the slower involving protein-separated pairs.

For comparison, similar experiments with horse heart MbFe(II)−*t*-BuNC on both pico- and nanosecond time scales were also conducted under the same reaction conditions in the same buffer medium. The observed rate constant and percentage return for *t*-BuNC in Mb in the picosecond regime are 1.63×10^{11} s^{-1} and 36%, respectively. The rate and percentage return for *t*-BuNC in Mb on the nanosecond time scale are 7.6×10^6 s^{-1} and 52%, respectively. It is clear that the concentration-independent, geminate recombination processes involving the postulated contact pair and protein-separated pair are virtually identical for *t*-BuNC in the two different proteins HRP and Mb. One may calculate that Q(HRP) = 0.26 and Q(Mb) = 0.31.

The bimolecular association of deliganded HRP with *t*-BuNC was measured in a simple mixing experiment in a spectrophotometer as a function of concentration over the range $(0.44-12) \times 10^{-3}$ M, with the results shown in Figure 2. From the slope one obtains the overall bimolecular $k_{on} = 0.039 \pm 0.004$ M^{-1} s^{-1} and from the intercept $k_{off} = (4.5 \pm 2) \times 10^{-5}$ s^{-1}. These data imply an association equilibrium constant of $\sim 10^{-3}$. The corresponding values for the same reaction with sperm whale Mb at pH 7 are completely different. We found $k_{on} = 4 \times 10^3$ M^{-1} s^{-1}, and one literature source has $k_{on} = 2 \times 10^3$ M^{-1} s^{-1} and $k_{off} = 1$ s^{-1} (Gibson et al., 1986). That study found $Q = 0.09$, which is somewhat smaller than our value of 0.31. Even in Mb the association reaction for *t*-BuNC is much slower than that of smaller isocyanides, as is reasonable for any reaction that requires the entry of a large ligand into the protein pocket, and the equilibrium constant for association is less. But in HRP, association with *t*-BuNC is orders of magnitude slower yet. The implication could be that HRP undergoes a significant conformational change to accommodate *t*-BuNC. This is indicated in eq 2 by the notation HRP′ to suggest that only a special conformation can react with *t*-BuNC. A more direct argument for this conjecture appears in the bimolecular portion of recombination in a photolyzed HRP−*t*-BuNC solution, as described next.

In HRP there is a recombination process observed on the low-millisecond time scale that is much faster than the process observed in the mixing experiment. Nothing like this is seen in Mb, in which the bimolecular phase following

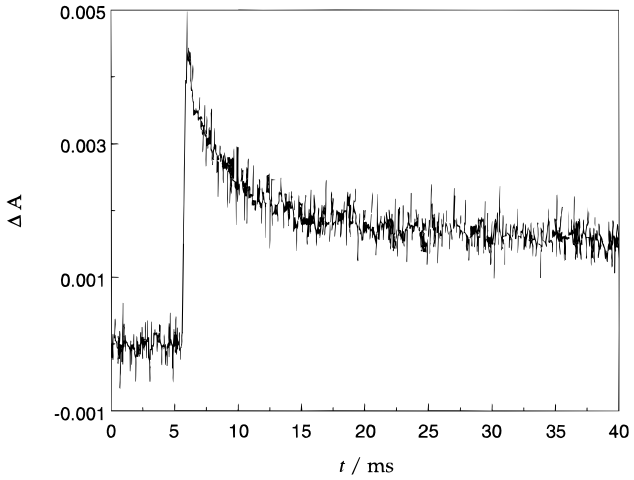

FIGURE 3: Absorbance changes on the millisecond time scale for HRP recombining with *t*-BuNC after photolysis, in a concentration-dependent process: [*t*-BuNC] = 0.0089 M, λ = 445 nm, photolysis energy = 2 mJ. The flat "plateau" eventually returns to the baseline after some seconds in a much slower process. At higher concentrations of *t*-BuNC, the millisecond partial decay of the transient absorption is faster and the "plateau" is lower. When a similar measurement is made on photolyzed Mb−*t*-BuNC, the decay is somewhat slower and returns all the way to the baseline in a single-exponential process.

photolysis shows behavior that has always appeared to be very similar to that observed in mixing, for *t*-BuNC as well as for smaller ligands. This millisecond recombination of photolyzed *t*-BuNC is shown in Figure 3. The reality of this process was verified for different types of HRP (types VI-A and XII), using old as well as fresh batches received from Sigma. Type VI-A is less homogeneous, predominantly consisting of two isozymes. Type XII is further purified to be essentially a single isozyme. (A large number of preliminary studies were carried out over some years by different investigators, under different measurement conditions and at various concentrations of ligand, before we became convinced. We report the most definitive measurements here, but all showed the same surprising process.) By using type VI-A, the observed decay rates for the transient absorption are 1100 s^{-1} at a *t*-BuNC concentration of 0.0443 M and 243 s^{-1} at 0.008 86 M. We infer a bimolecular association rate constant of 2.3×10^4 M^{-1} s^{-1} and a dissociation near 80 s^{-1}. For type XII, the rate constant was 4×10^4 M^{-1} s^{-1}. The difference is enough that it probably reflects a real difference between isozymes, but it is insignificant for our main point here. We use 3×10^4 for further calculations and deduce that $k_{-3} = 4 \times 10^4$ M^{-1} s^{-1}. Ultimately, essentially all ligand does rebind; permanent photochemical changes, if any, have a very low quantum yield. What appears as a plateau in Figure 3 is recombination continuing at a markedly reduced rate. The asymptotic value of the recombination rate seems to be near 0.04 M^{-1} s^{-1}, that is, about the same as in the mixing experiment, although that measurement was difficult to make on the apparatus used. Although one could not be certain from Figure 3, there is, in fact, some heterogeneity within the fast phase and, probably, also the slow phase. This implies that reality is even more complicated than can be described by the mechanism in eq 2, but the key observation is that, in the photolysis experiment, over half the bimolecular recombination occurs with a rate constant 6 orders of magnitude greater than that that accounts, so far as we can tell, for all of the

Table 1: Elementary Rate Constants for the Reaction of L = *t*-BuNC with Various Fe(II)−Heme Compounds

models: $\mathrm{Hm-L} \underset{5\times10^{10}}{\overset{3}{\rightleftharpoons}} [\mathrm{HmL}] \underset{2.2\times10^{8}}{\overset{3\times10^{10}}{\rightleftharpoons}} \mathrm{Hm} + \mathrm{L}$ $\quad k_{on} = 1.4 \times 10^{8}$

Mb: $\mathrm{Mb-L} \underset{6\times10^{10}}{\overset{3-10}{\rightleftharpoons}} [\mathrm{MbL}] \underset{11\times10^{6}}{\overset{10\times10^{10}}{\rightleftharpoons}} [\mathrm{Mb|L}] \underset{5\times10^{3}}{\overset{3.6\times10^{6}}{\rightleftharpoons}} \mathrm{Mb} + \mathrm{L}$

$k_{on} = 1.8 \times 10^{3}$

HRP: $\mathrm{HRP'-L} \underset{7\times10^{10}}{\overset{200}{\rightleftharpoons}} [\mathrm{HRP'L}] \underset{9.4\times10^{6}}{\overset{8.5\times10^{10}}{\rightleftharpoons}} [\mathrm{HRP'|L}] \underset{4\times10^{4}}{\overset{3.8\times10^{6}}{\rightleftharpoons}} \mathrm{HRP'} + \mathrm{L}$ $(k_{on} = 3 \times 10^{4})$

$10^{-4} \uparrow\downarrow 10^{2}$ $\quad k_{on} = 0.04$
HRP

association in a mixing experiment (and the last portion of the photolysis experiment). The fast process, assigned as recombination with unrelaxed HRP′, is quite facile, being more than an order of magnitude faster than combination with Mb.

Since this process has not been observed with ligands of any size in Mb or Hb, we wondered whether it is common in HRP or unique to *t*-BuNC. We investigated the smaller ligand, MeNC. All observations that we have made on that system so far, however, can be explained with only one bimolecular rate constant of 5.7×10^{3} s^{-1}, observed both upon mixing and after photolysis. If there is anything like what we observe with *t*-BuNC, it is at least a much smaller effect. This observation, that there is some threshold size for the ligand, probably indicates that the path through which ligands enter or leave HRP to reach to the active site has a narrow neck comparable in size to that of *t*-BuNC. Unfortunately, there are as yet no structural determinations from X-ray diffraction for HRP.

To explain in even an approximate way the major features of these data, one must postulate at least one more step than is shown in eq 1 and use the kinetic scheme in eq 2. From the data for the two association processes above, we calculate the ratio $k_{-4}/k_4 = 10^{-6}$. This, together with the overall dissociation, implies that $k_{-1} = 160$ in surprisingly good agreement with the value from the concentration dependence of the fast process alone, which is $80/Q = 320$ s^{-1}. To the extent that eq 2 is complete, one may vary the concentration of ligand and measure the change in percentage of recombination in the fast and slow bimolecular processes to determine k_4, as has been done for the Hb R → T transition (Martin & Parkhurst, 1990). This leads to $k_4 \cong 100$ and, therefore, $k_{-4} \cong 10^{-4}$. We are not, however, completely satisfied with that analysis. A wide range of ligand concentrations was not practical, and the heterogeneity within kinetic phases made it difficult to settle on precise amplitude ratios. The fact that the amplitude varies at all, however, leads us to prefer a kinetic model with a conformational change after a long delay, rather than the alternative explanation for heterogeneous kinetics, namely, two conformations, one fast-reacting and one slow-reacting, both of which exist prior to photolysis and persist throughout the entire measurement.

All the different elementary rate constants are collected in Table 1. It is interesting to observe that the values of k_{-1} (the elementary step involving only bond formation from the contact pair) are almost the same in model compounds, myoglobin, and HRP. Apparently, it does not matter for bond formation itself whether the heme iron is embedded in the solvent or in different proteins. The constant k_2 describes the breakup of the rapidly reacting contact pair. This process is poorly understood (and almost certainly cannot be represented by a single-barrier process), but presumably involves a sort of diffusion of the ligand away from the iron; it may also incorporate some proximal relaxation in the protein tension. [It does not involve the major part of the iron motion out of plane, which is now thought to be subpicosecond (Franzen et al., 1995).] It seems to be 2−3 times faster in proteins than in model compounds. This may be explained by the fact that the photolyzed ligand in the model compound has to diffuse through well-packed, randomly oriented solvent molecules, while the protein pocket is more ordered and somewhat open, but k_2 could also be influenced, in part, by changes in the amount of tension the protein exerts on the proximal ligand after it is already out of plane. The nanosecond return to form the fast-reacting contact pair k_{-2} and the ultimate escape out of the protein altogether k_3 are very similar for the two heme proteins Mb and HRP′. Model compounds, of course, show no such nanosecond processes. This indicates that the nature of the second cage, so far as the diffusion of *t*-BuNC is concerned, is very similar in these two proteins.

The concentration-dependent millisecond process that we observe in HRP−*t*-BuNC after photolysis, but not in simple mixing, was very surprising, unprecedented to our knowledge. Because of the size effect, we hypothesize that tertiary relaxation is influencing ligand entry into the protein. We infer that the path through which *t*-BuNC escapes remains available for reentry for a large fraction of a millisecond, even after the ligand has escaped, but then a conformational change blocks reentry. This represents both a larger effect on binding kinetics and a slower time scale for relaxation than are typically expected for tertiary structural changes at room temperature. The inference that ligand entry is critical, however, is not completely rigorous. It is logically possible that any of the elementary kinetic steps could be subject to a slow relaxation.

Although the two concentration-dependent association processes pointing to distinct conformational states are seen, so far, only with HRP−*t*-BuNC, there are lesser anomalies even for binding CO to HRP. That small ligand also exhibits very slow dissociation ($\sim 10^{-4}$ s^{-1}) and association [$(3-6) \times 10^{3}$ M^{-1} s^{-1}, depending on pH] rate constants. This was interpreted, partly on the basis of analogies with the known crystal structure of cytochrome *c* peroxidase, as in part reflecting considerable steric crowding on the distal side, but even more to a key role played by charged propionates (Coletta et al., 1986). Soon afterward, low-temperature flash photolysis uncovered a number of differences between Mb−CO and HRP−CO (Doster et al., 1987), leading to the conclusion, in our terminology, that *Q* was not at all unusual and, consequently, "the slow recombination rate at 300 K can thus be explained by the large Gibbs energy of the conformational change necessary to let CO move into the heme pocket". Of course, some conformational change concomitant with ligand entry might well be necessary for many protein−ligand pairs. What is different about HRP−*t*-BuNC is that the relaxation back to the unreactive form is dramatic enough and slow enough even at ambient temper-

ature to have forced us to recognize its presence. Presumably, the requisite conformational change needed for the large ligand *t*-BuNC is more extreme and temporarily locks the protein into a metastable arrangement.

A number of time-resolved spectroscopic measurements have observed transients interpreted as revealing tertiary changes in proteins, but few were correlated with ligand entry or escape. A tertiary change approximately concomitant with ligand escape from Hb−CO was advanced to account for subtle UV−vis spectral changes (Murray et al., 1988a), but the same group concluded that there is almost no effect on kinetics due to such relaxation (Murray et al., 1988b). Possibly related is the detection by special methods of kinetically distinct species in Mb (Tian et al., 1993), which interconvert in 1−10 μs, too fast to affect bimolecular combination. Dual rates in cytochrome P450 may reflect slow interconversion between conformations (Tian et al., 1995), but the authors are tentative. We should also mention our own work with Hb(carp) reacting with MeNC in the presence of inositol hexaphosphate (IHP), conditions thought to induce T-state behavior. We did not identify any spectral signature for a conformational change, but we found it impossible to reconcile all of our kinetic data without some novel mechanism and concluded that a conformational change affecting association probably occurs simultaneous with or shortly after ligand escape (Bandyopadhyay et al., 1990).

In both our earlier observations on Hb(carp) and the present data on HRP, proteins were reacting with ligands under circumstances in which the protein is "trying" to discriminate against binding. Perhaps it is not so surprising that part of the discrimination occurs at entry into the protein and that this discrimination involves a conformation that is fully developed only when the ligand is not present. Perhaps the surprise is that this effect has not been seen more often.

The observation reported here suggests a large number of additional experiments. A study with more isocyanides of different sizes is needed. It could be, for example, that up to ethyl or *n*-propyl isocyanide there is no barrier and that there is a small effect for isopropyl isocyanide and a very large effect for ortho-substituted aromatic isocyanides. Perhaps most diagnostic would be different diagnostic assays, such as a time-resolved circular dichroism measurement. It might be useful to reexamine data even for well-characterized systems, in which subtle indications of slow tertiary changes affecting bimolecular recombination may have been overlooked or ignored.

We note that the tertiary relaxation that we postulate has a relaxation time even slower than the quaternary transition in Hb. There is no question that the R → T transition in Hb does critically depend upon the fact that Hb is a multi-subunit protein, but perhaps the tetramer is not so much able to endow a novel property as to enhance a property that monomeric proteins already have. After all, quaternary changes can hardly have the effects on ligand binding attributed to them except by modulating tertiary structures (Friedman, 1985).

CONCLUSION

The present study has demonstrated a time-dependent barrier to bimolecular recombination following flash photolysis of *tert*-butyl isocyanide binding with horseradish peroxidase. This is attributed to a slow (for ambient conditions) tertiary relaxation in the protein, which we suggest affects ligand entry.

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