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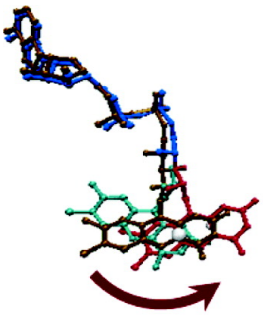
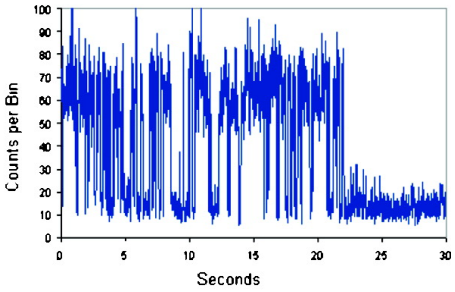


Conformational Change



Fluorescence Trajectory

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Conformational Dynamics of the Isoalloxazine in Substrate-Free *p*-Hydroxybenzoate Hydroxylase: Single-Molecule Studies

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Abstract: *p*-Hydroxybenzoate hydroxylase (PHBH) is a homodimeric enzyme in which each subunit noncovalently binds one molecule of FAD in the active site. PHBH is a model system for how flavoenzymes regulate reactions with oxygen. We report single-molecule fluorescence studies of PHBH in the absence of substrate that provide data consistent with the hypothesis that a critical step in substrate binding is the movement of the isoalloxazine between an "in" conformation and a more exposed or "open" conformation. The isoalloxazine is observed to move between these conformations in the absence of substrate. Studies with the Y222A mutant form of PHBH suggest that the exposed conformation is fluorescent while the in-conformation is quenched. Finally, we note that many of the single-molecule-fluorescence trajectories reveal a conformational heterogeneity, with populations of the enzyme characterized by either fast or slow switching between the in- and open-conformations. Our data also allow us to hypothesize a model in which one flavin in the dimer inhibits the motion of the other.

Introduction

Oxygen is a powerful and versatile oxidizing agent that can be used to drive a wide variety of chemical reactions. However, the very versatility of oxygen is also a danger to the cell, as its oxidation reactions are generally irreversible and side reactions can generate toxic byproducts. For oxygen to be a useful reagent to the cell, its reactions must be carefully controlled so that only the desired ones are carried out. The mechanisms by which biochemical reactions with oxygen are controlled represent an active area of research.

The family of flavoprotein monoxygenases represented by *p*-hydroxybenzoate hydroxylase (PHBH) offers insight into how such control is obtained.[1] PHBH is a homodimeric enzyme where each subunit noncovalently binds one molecule of FAD in the active site. The protein environment surrounding the bound flavin is crucial to the efficiency and regulation of the reaction. The flavin $C4_a$-hydroperoxide, which is the key oxygenating intermediate in the catalytic cycle, is unstable in protic solvents and must be shielded from solvent during the hydroxylation phase of the reaction to avoid the wasteful formation of toxic hydrogen peroxide. Nevertheless, the active site must be exposed to solvent in order for the substrate to bind. Different environments around the active site are therefore required for each of these steps of the overall reaction. Moreover, PHBH must distinguish between *p*-hydroxybenzoate and similar molecules such as *p*-aminobenzoate, which is an important intermediate in folic acid synthesis and must not be oxygenated by the enzyme.[2]

Crystallographic studies have suggested that PHBH achieves control over the reaction environment by forcing the flavin to adopt different conformations during different steps of the reaction. Crystal structures of PHBH reveal that the isoalloxazine of the flavin can exist in at least three different conformations, depending on the crystallization conditions (see Figure 1).

When the wild-type protein is crystallized with its natural substrate, *p*-hydroxybenzoate, the isoalloxazine is buried inside the protein and is shielded from solvent (the in-conformation of the flavin).[3] If *p*-hydroxybenzoate is replaced by 2,4-dihydroxybenzoate, the crystal structure shows the flavin adopting the out-conformation, where the isoalloxazine is tilted away from the substrate binding site toward the surface of the protein and is exposed to solvent.[4,5] Importantly, in the R220Q mutant of PHBH, without the substrate bound, the crystal structure shows the flavin in an intermediate position, and the enzyme adjusts to form a cleft to allow solvent access to the active site.[6] For the discussion below, we designate this conformation the open-conformation. Although the isoalloxazine

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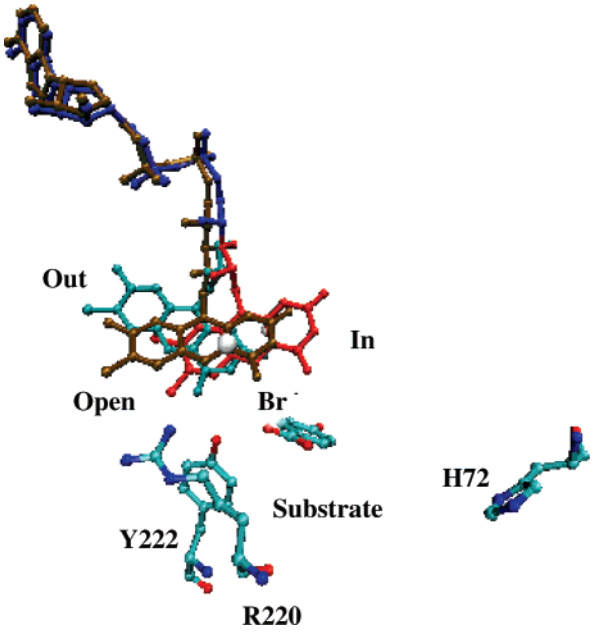

Figure 1. Flavin conformation in PHBH with key residues and the monovalent anion binding site marked. The in-conformation is shown in light blue, the open-confromation in ochre, and the out-conformation in red. Coordinates for this figure were formed from the superposition of PDB entries 1DOE (wild-type complex with 2,4 dihydroxybenzoate complex), 1K0L (R220Q mutant) and 1PBE (wild-type complex with *p*-hydroxybenzoate). Least-squares fitting was carried out to maximize the superposition of the FAD binding domain.

in both the open- and out-conformations is exposed to solvent, the open-conformation differs substantially from the out-conformation. The geometrical constraints in the open-conformation prevent the *re*-side of the isoalloxazine from making contact with NADPH, thus preventing reduction of the flavin. In addition, the open conformation provides a path for *p*-hydroxybenzoate to access the active site, in contrast to the out conformation.

On the basis of these structures, a model has been proposed wherein the flavin ring shuttles between different conformations as the reaction proceeds from substrate binding to flavin reduction to substrate hydroxylation with concomitant flavin oxidation in a tightly controlled process,[1,4−6] as illustrated in Figure 2.

According to the crystal structural data discussed above, the flavin is mainly in the open-conformation in the absence of substrate with an open channel to facilitate the binding of *p*-hydroxybenzoate. Binding of *p*-hydroxybenzoate shifts the equilibrium from favoring the open-conformation toward the in-conformation. Movement of the flavin from the in-position to the out-position is controlled by deprotonation of the substrate phenolic group, and this is facilitated by a proton-transfer network terminating in His72 at the solvent surface.[2,7] When in the out-position, the *re*-side of the isoalloxazine is exposed and NADPH can reduce the flavin by a hydride transfer to form the reduced flavin anion. Finally, because of the positive electrostatic field of the active site, the anionic reduced isoalloxazine of the flavin is pulled into the in-position, where it is shielded from solvent and can efficiently hydroxylate the substrate. This mechanism ensures that improper substrates, such as 4-aminobenzoate, which cannot be deprotonated in the

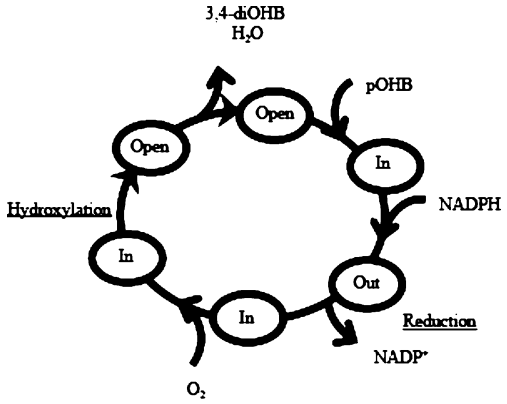

Figure 2. Conformational cycles of PHBH. The steps of the reaction cycle of PHBH. During the reaction cycle, the isoalloxazine of the flavin moves between conformations in which the flavin is exposed to solvent (designated "out" and "open") and ones in which it is sequestered from solvent ("in") as substrate (p-hydroxybenzoate, pOHB) is converted into product (3,4-dihydroxybenzoate, diOHB).

4-position, do not induce movement of the flavin to the out-conformation, where the flavin can be reduced by NADPH in preparation to hydroxylate the substrate. Several lines of evidence from kinetic studies, photochemical cross-linking, and geometrical considerations using crystal structures are consistent with this model.[2,4,7−9] However, there have been no direct observations of the transitions of the flavin between various conformational states in the substrate-free wild-type enzyme. In the present study, single-molecule fluorescence was used to examine the dynamic behavior of the flavin in the absence of substrate.

Earlier single-molecule fluorescence studies of flavoproteins revealed fluorescence blinking; i.e., the fluorescence switches between bright (on) and quenched (off) as the enzyme-bound flavin converts between the oxidized and reduced states.[10,11] The present study shows that, in contrast to the earlier reports on other flavoenzymes, the fluorescence fluctuations in PHBH occur even in the absence of oxidation−reduction reactions. The data suggest that the fluorescence fluctuations are due to PHBH moving between the in-conformation and the open-conformation. In contrast to the in-to-out transition of the flavin, the in-to-open transition has not been directly observed but only inferred from crystal structures and kinetic studies of mutants with impaired substrate binding. The data below show that the flavin conformational transition can be followed because the fluorescence of the in-conformation is quenched relative to that of the other conformations.

Materials and Methods

Mutant and wild-type (WT) PHBH were expressed in *Escherichia coli* containing the plasmid pIE130 and purified as described previously.[12,13]

Single-molecule experiments were run in 50 mM phosphate buffer at pH 6.5. To observe the fluorescence of a single molecule of PHBH, it is necessary to limit the translational motion of the enzyme so that

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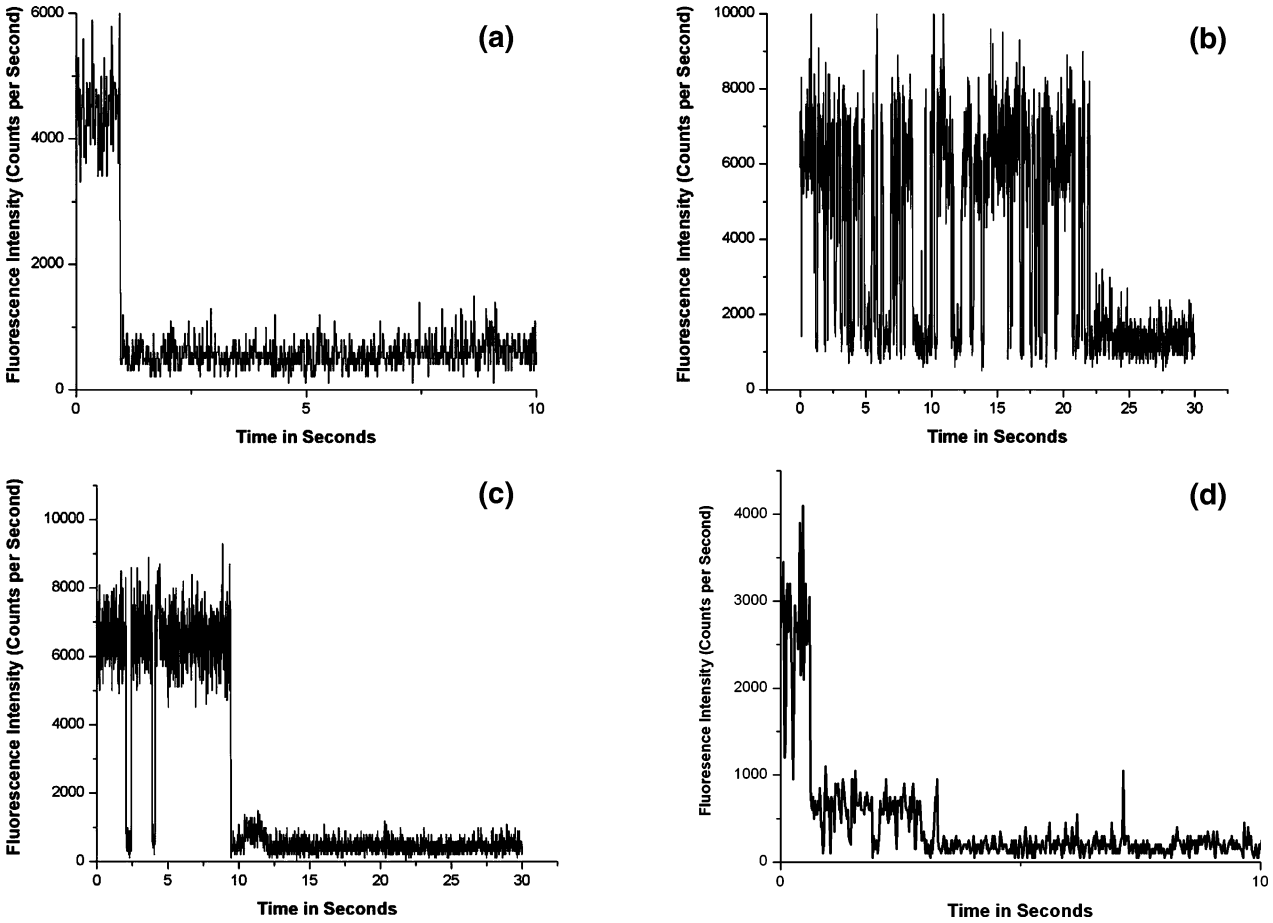

Figure 3. (a) Typical fluorescence of a single WT PHBH molecule. The signal remains at a constant level and then drops to the background in a single step. (b) Fluorescence trajectory of a WT PHBH molecule showing frequent fluctuations. The fast fluctuations probably correspond to the movement of the flavin between the open and in conformations. The loss of fluorescence at ~22 s is presumably due to loss of FAD. (c) Fluorescence trajectory of a WT PHBH molecule showing infrequent fluctuations. Individual molecules of PHBH showed a great difference in the frequency of fluorescence fluctuations (compare to part b). (d) Fluorescence trajectory of WT PHBH showing dimeric PHBH. The two levels of fluorescence indicate that two FAD molecules are bound initially to the PHBH dimer, a rare event at the concentrations of enzyme used. The first level of fluorescence (from $t = 0$ to ~1 s) corresponds to the combined signal of both FAD molecules bound to the PHBH dimer. The second level of fluorescence (from $t = \sim 1$ to ~3.5 s) corresponds to the signal from the enzyme with a single FAD molecule bound to the dimer after the other FAD molecule has been lost from the enzyme due to dissociation. The third level (no fluorescence, from $t = \sim 3.5$ s to the end of the trajectory) corresponds to the signal from an enzyme molecule that has lost both FADs due to dissociation.

it remains in the focal spot of the microscope for an appreciable period of time. This was accomplished by immobilizing the enzyme in a thin film of 1% agarose.[10] The pores of the agarose gel are small enough (~300 nm in diameter) to prevent the enzyme from leaving the focus of the microscope but are large enough to allow free rotation of the enzyme within the pore. The "chicken wire" structure of the gel pores permits the entry and exit of free FAD dissociated from the enzyme. A 100 nM solution of enzyme was centrifuged through a 30 000 MW cutoff filter (Centricon-30) to remove free FAD and then diluted 1:10 into 1% low-melting point agarose heated to just above the gelling temperature (26 °C). The enzyme−agarose solution was then spun-cast onto a clean glass slide with the agarose−enzyme solution gelling during spinning. Glass slides were cleaned by sonication in successive applications of water, detergent solution, ethanol, aqua regia, and water before use with the enzyme.

Fluorescence time traces of single PHBH molecules were observed at room temperature using a home-built inverted confocal microscope with a 1.65 NA × 100 oil-immersion objective (Optical Analysis APO 1-UB615). The 457-nm line from an Ar^+ ion laser (Melles-Griot 532-AP) was passed through a dichroic beam splitter and then focused to the diffraction limit on a glass slide coated with the sample in agarose. The average power used ranged from 5 to 20 μW. The fluorescence emission was detected by a single-photon-counting avalanche photodiode (Perkin-Elmer Optoelectronics SPCM-AQ 161) after a notch-plus filter (Kaiser 38988) and a 520-nm band-pass filter (Chroma D520/60M). The surface was raster-scanned and traces over time were collected whenever the fluorescence intensity exceeded a threshold (typically 3−4 times the background signal). Traces were collected in intervals of 10 ms over a 30 s data collection time for each spot.

The rate constant for a conformational transition can be determined by analyzing the distribution of residence times of the fluorescent and nonfluorescent states. We define the time spent in the fluorescent state between two consecutive transitions as the on-time and the time spent in the nonfluorescent state between two consecutive transitions as the off-time. The on- and off-times correspond to the time required for the thermal activation of the transition between states; the actual transition between states occurs on a time scale much faster than can be measured here. On- or off-times shorter than 10 ms are not resolved with our apparatus and appear only as smaller amplitude dips or peaks in the trajectory and were not taken into account in the analysis.

Results

Typical time courses for the fluorescence intensity of a single spot are shown above (Figure 3a−d).

In general, the fluorescence intensity drops abruptly to the background level in a single step, corresponding to the loss of fluorescence either from a single FAD bound to a dimeric

Table 1. Equilibrium Constants and Rates of Conformational Transition (E_{open} to E_{in}; See Figure 8)

	$K_{open-in}$	% showing fluctuations	rates of in-to-open transition (s^{-1})	% of molecules	rates of open-to-in transition (s^{-1})
WT	3.3	45	21.0 ± 1.3		6.4 ± 0.3
H72N	1.0	24	16.2 ± 2.1	73	18.0 ± 0.7
				27	3.1 ± 0.2
R220Q	7.2	32	26.6 ± 3.1		3.7 ± 1.2

enzyme (with no FAD bound to the other subunit) or from FAD bound to a PHBH monomer. Rarely, a trajectory with two distinct levels can be seen (cf. Figure 3d), which corresponds to a dimeric enzyme with FAD bound to each subunit. At such low concentrations (5−100 nM) we believe there is a substantial population of monomers, the decrease of fluorescence to the mid-level when it does not return to the upper level is indicative of the loss of FAD from one of the subunits. Trajectories that lose fluorescence in one or two steps are only seen when FAD is bound to the enzyme. Control slides prepared with free FAD but without PHBH only showed an increased background fluorescence, confirming that the trajectories as observed in Figure 3 are only due to the enzyme and not free FAD.

Because the loss of fluorescence that persists over an extended time frame (Figure 3a) is independent of the excitation intensity, this loss is not likely due to photobleaching but is instead due to dissociation of FAD from the enzyme. A histogram composed of dissociation data observed from many individual PHBH molecules (data not shown) gives an FAD dissociation rate of $0.63 \pm 0.04\ s^{-1}$. Occasionally, but rarely, the fluorescence is recovered as another FAD molecule binds to the enzyme, confirming that the enzyme remains intact during observation.

While many of the trajectories show no detectable fluorescence fluctuations beyond that in Figure 3a, a number of trajectories show frequent fluctuations in the fluorescence between a high and a low level before the fluorescence is lost entirely (Figure 3b−d). Table 1 shows the relative fraction of single-molecule data sets exhibiting this fast fluctuation behavior for the WT and mutant forms of PHBH.

Such fluctuations are documented in studies of catalysis by flavoproteins in the presence of substrate, where they correspond to the oxidized and reduced states of the flavin as the flavoprotein undergoes turnover.[10,11] However, the fluctuations seen in Figure 3b−d are unique because they are seen in the absence of a reducing substrate. Oxidation−reduction is not a possible cause of the fluctuations because PHBH is not reduced in the absence of NADPH.[14] Separate measurements also confirm that this is not due to photochemical reduction (below). As discussed later, we attribute these fast fluctuations to repetitive motions of the flavin between the in-and open-conformations, with the flavin being strongly quenched in the in-conformation relative to the open-conformation.

To test our hypothesis that the blinking behavior seen in PHBH is associated with the conformational transition between open- and in-conformations of the flavin, we ran several control experiments. The most common cause of on−off fluorescence blinking is reversible photochemically induced transitions to dark states. Photochemically induced changes in the fluorescence will increase with increasing excitation intensity. For PHBH,

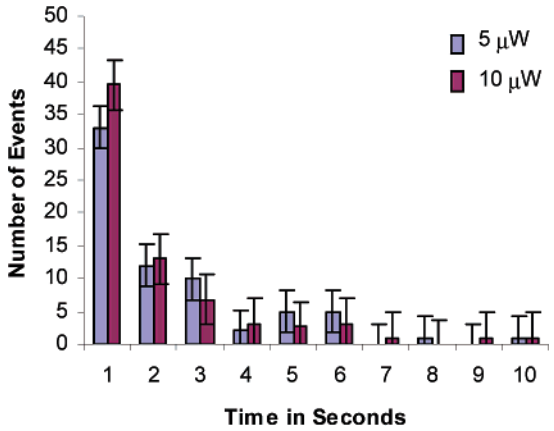

Figure 4. Comparison of the on−off transition times for the H72N mutant at two incident intensities. The on−off transition rate is the same at each intensity ($10.1 \pm 0.7\ s^{-1}$ at 5 μW and $9.7 \pm 0.4\ s^{-1}$ 10 μW), suggesting that the on−off transition is not caused by a photochemical effect but rather by the motion of the flavin from the fluorescent open position to the quenched in position.

measurements of the fluctuation rates at excitation intensities of 5 and 10 μW show, within experimental uncertainty, identical decay kinetics for the on-times (Figure 4).

Furthermore, as discussed below, blinking is completely suppressed in the Y222A mutant. Other experimental artifacts could also induce blinking behavior in single-molecule experiments. Blinking could be caused by interactions with the surface of the glass coverslip or the agarose matrix. However, PHBH does not adsorb to the glass surface because coverslips with samples including PHBH but without the agarose matrix do not show the single-molecule-type transition seen in Figure 3. PHBH was also shown not to adsorb onto the surface of the agarose pores, as the ensemble fluorescence anisotropy of the flavin fluorescence was the same inside an agarose gel as in free solution.

Assignment of Fluorescent States to Conformations. We interpret the on−off blinking in trajectories as the conformational fluctuation of FAD in the enzyme. In order for such fluctuations to be responsible for the blinking of fluorescence seen in single molecules, the quantum yield of the two conformations must differ substantially. Crystal structures show that the aromatic ring of Tyr222 is positioned to quench the flavin fluorescence differently in the open- and in-conformations, providing a plausible origin for the change in the fluorescence signal. Tyrosine has been shown in other studies on the proteins flavodoxin and glutathione reductase to directly quench the fluorescence of a favorably positioned proximal FAD by photoinduced electron transfer,[15−17] quenching which could be reversed by the addition of a ligand that intercalates between the FAD molecule and the tyrosine.[15] This hypothesis was supported with single-molecule spectroscopy studies of the Y222A mutant, which showed that the flavin of this mutant enzyme was fully fluorescent and did not fluctuate in single-molecule trajectories.

The open-conformation of the WT was assigned as the fluorescent state, and the in-conformation was assigned as the

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nonfluorescent state on the basis of the effects of Cl^- binding. It has been shown that Cl^- binding quenches the flavin fluorescence of WT and that monovalent anions shift the flavin conformation to the in-position.[4] Neither free FAD nor free FMN is quenched by Cl^-. Therefore, the loss of flavin fluorescence is caused by the movement of the isoalloxazine to a quenching environment and not direct quenching by Cl^- itself. In the Y222A mutant, Cl^- does not quench flavin fluorescence, consistent with the absence of an aromatic quencher in proximity to the isoalloxazine (data not shown).[18]

We note that, in the context of the present work, our data do not distinguish between the open-conformation and the out-conformation, both seen in crystal structures of PHBH. Nevertheless, we believe the fluorescent state corresponds to the open-conformation because, according to the current model,[2,7] a critical step in ensuring the selectivity of the reaction is the movement of the flavin to the out-position upon substrate binding. If the flavin existed in the out-position prior to substrate binding, the enzyme would react with NADPH in the absence of substrate, which is not seen.[14]

Flavin Dynamics of WT PHBH. Figure 3a−c shows two general classes of fluorescence trajectories for WT. In the case of Figure 3a (this type of behavior is seen for about 55% of the total observations) no in/open bursts are seen and the fluorescence remains at a constant level before being lost due to dissociation of FAD. In other trajectories (45% of the total observations in WT) sharp transitions are observed that we associate with transitions from the fluorescent open-conformation to the nonfluorescent in-conformation (see Figure 3b,c). Figure 3b,c also shows the behavior of two different types of molecules: one that frequently interconverts between the in- and open-conformations and another that infrequently interconverts. The class of trajectories in which no fluctuations in fluorescence are seen (see Figure 3a) probably corresponds to PHBH molecules that are in the slowly interconverting class and in the open-conformation but lose FAD before adopting the in-conformation ($k_{diss} = 0.63\ s^{-1}$).

For WT PHBH, we generated histograms of the on-times and off-times recorded during the period of rapid interconversion (see Figure 5).[10]

The first on-time and the last on-time were excluded from analysis because in the former case the real-time duration of the open-state is not known and in the latter case it is not known if the last transition is due to loss of the flavin molecule or a transition to the in-conformation. The statistics showed a simple exponential decay (within experimental uncertainty) for both on and off states, corresponding to first-order reactions during the period of rapid interconversion, with the rate constants for open-to-in and in-to-open transitions being $6.4\ s^{-1} \pm 0.3$ (open-to-in) and $21.0\ s^{-1} \pm 1.3$ (in-to-open), respectively. The equilibrium constant for this transition is 3.3, favoring the open state (see Table 1). The total equilibrium constant may be higher because molecules in the slowly interconverting population are under-represented in this analysis, since their first and last on-times were not included.

Flavin Dynamics of the R220Q Mutant of PHBH. The crystal structure of the R220Q variant has been determined in the substrate-free form.[6] Single-molecule studies of the R220Q variant show a similar pattern of bursts to the nonfluorescent state (data not shown), as seen with the wild-type enzyme (cf. Figure 3b). We note that the equilibrium constant calculated from the single-molecule trajectories favors the open form of the R220Q enzyme ($K_{eq} = 7.2$) more strongly than does the WT ($K_{eq} = 3.3$), a result also suggested by the R220Q crystal structure. The change in the equilibrium constant arises from a slowing of the open-to-in-transition ($k = 3.7\ s^{-1}$ for the mutant compared to $6.4\ s^{-1}$ for the WT enzyme) and an increase of the in-to-open-transition ($k = 26.6\ s^{-1}$ for the mutant compared to $21.0\ s^{-1}$ for the WT).

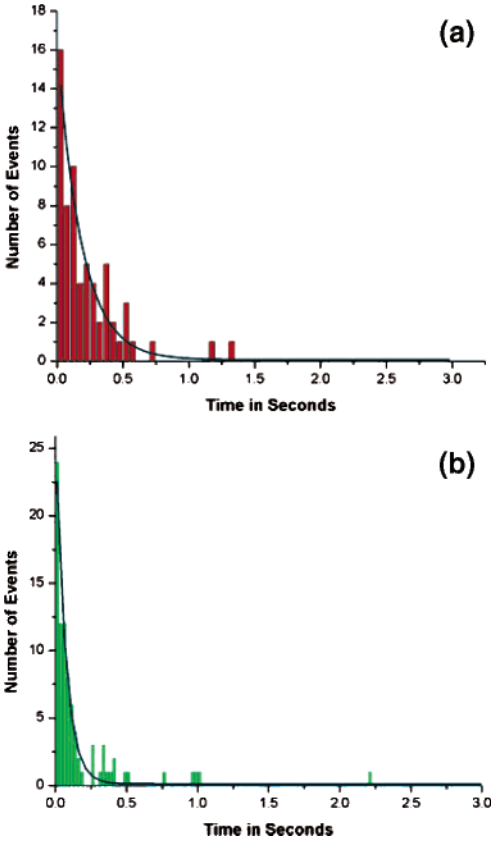

Figure 5. Histogram of the on-times (Figure 5a) and off-times (Figure 5b) for 112 molecules of WT PHBH. The on-times correspond to the length of time spent in the open state before converting to the in state and the off-times correspond to the time spent in the in state before converting to the open state. The decay constant for the exponential fit to the distribution corresponds to the rate of transition between states ($6.4 \pm 0.3\ s^{-1}$ for the open-to-in and $21.0 \pm 1.3\ s^{-1}$ for the in-to-open).

Flavin Dynamics of the H72N Mutant of PHBH. In WT PHBH, His72 shuttles protons to the aqueous solvent from the substrate through an internal hydrogen-bonding network. The ionization of the substrate in turn repels a dipole along the backbone of the protein, which enables the flavin to move to the out-position for reduction.[2,7] Because this proton network to the flavin is disrupted in the H72N variant, this enzyme has a substantially different reactivity compared to WT. Specifically, the H72N variant is reduced only slowly by NADPH at neutral pH due to the lack of a facile ionization mechanism for the substrate. If the in-open control mechanism is disrupted in a manner similar to the in-out control mechanism, then the H72N mutant should show slower switching from the in-conformation to the open-conformation. In the single-molecule data this would be reflected in longer off-times compared to WT. As shown in

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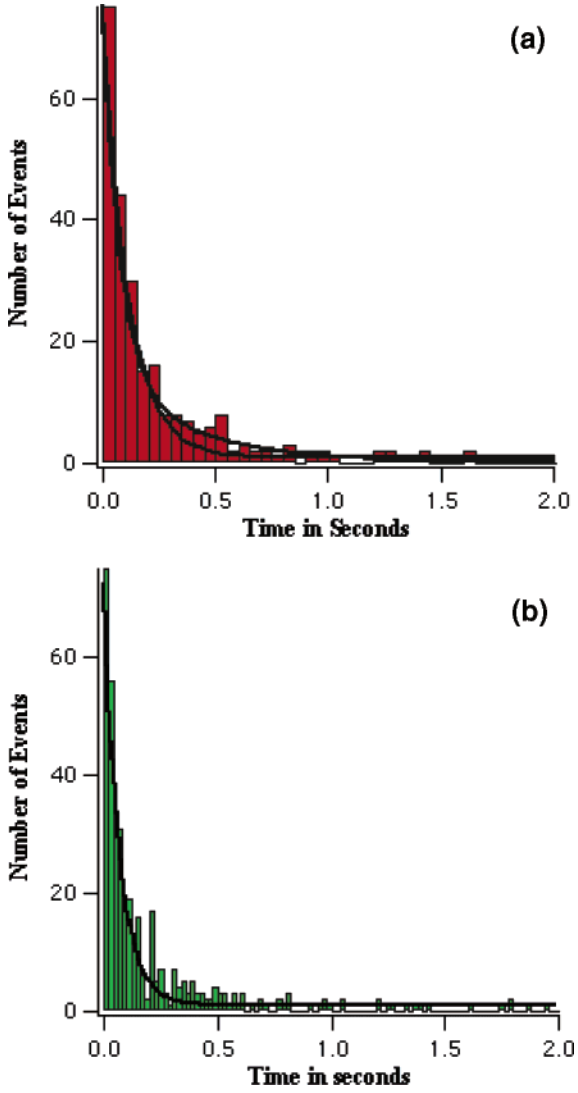

Figure 6. Histogram of the on-times (a) and off-times (b) for 167 molecules of H72N PHBH. The on-time distribution corresponding to the open-to-in transition is shown in part a with a monoexponential ($k = 9.0 \pm 0.3\ s^{-1}$) and biexponential (73% $k_1 = 18.0 \pm 0.7\ s^{-1}$ and 27% $k_2 = 3.1 \pm 0.2\ s^{-1}$) fit. The off-time distribution corresponding to the in-to-open transition is shown in Figure 6b with a monoexponential ($k = 16.2 \pm 2.1\ s^{-1}$) fit.

Figure 6b, the decay curve obtained from the histogram of off-times of the H72N mutant is characterized by only a slightly smaller eigenvalue than the off-time decay curve obtained from the WT ($16.2 \pm 2.1\ s^{-1}$ vs $21.0 \pm 1.3\ s^{-1}$ in WT).

This suggests that the H72N mutant is not deficient in its ability to move from the in-position to the open-position but rather in its ability to move from the in-position to the out-position and the mechanisms for the two types of flavin and protein dynamics are different.

In contrast to the single exponential on-time distributions found in the WT and R220Q versions of the enzyme, the on-time distribution of H72N averaged over many molecules is biexponential (Figure 6a). This indicates that there is heterogeneity arising from two distinct conformations: one that rapidly moves from the open-to-in state and one that is significantly slower to change from the open-to-in state. The majority of the enzyme exists in the rapid form (73%) with an eigenvalue of $18.0 \pm 0.7\ s^{-1}$ for the open-to-in transition. The remaining slow component has an eigenvalue of $3.1 \pm 0.2\ s^{-1}$ for the open-to-in transition. Because this heterogeneity is observed in data sets that have been added together from the trajectories of individual molecules, we cannot determine if the heterogeneity is static (each enzyme molecule has a single rate constant that does not change over time) or dynamic (the rate constant for each enzyme molecule fluctuates with time). If the heterogeneity is due to switching between slow and fast forms, it is clear that the trajectories that can be measured from individual molecules before FAD dissociates are not long enough for the data to detect the change.

Heterogeneity of the Conformational Transition Rates. Apparent static heterogeneity for the WT and mutant versions of the enzyme can be seen in some trajectories such as shown in Figure 3b,c. Under the same conditions, one molecule of the wild-type enzyme undergoes two transitions in 9 s (Figure 3c), while another undergoes 26 transitions in 8 s (Figure 3b). The static heterogeneity of the system can be analyzed by comparing the experimental distribution of reaction rates to what is expected for a simulated sample of identical molecules. Because only a limited number of transitions are observed for each molecule due to the finite observation time, there is a statistical distribution of reaction rates in both the experimental and simulated distributions that is proportional to the length of the trajectory. However, the variation of rates in the simulated sample of identical molecules reflects only the statistical variation in the measurement of the reaction rate, while the variance of reaction rates in the experimental distribution reflects both the statistical variability of the measurement of the reaction rate and any variability of the reaction rate itself among different molecules. The distribution of reaction rates for the H72N mutant plotted in Figure 7 is compared to a Monte Carlo simulated distribution of rates from a sample of identical molecules.[11]

Compared to the simulated distributions, the actual distributions of the mutant (Figure 7a,b) and WT (data not shown) versions of the enzyme showed a wider variation in reaction rates with a clear fast and slow population of both the in-to-open and open-to-in transition rates, with the in-to-open transition showing a greater difference from the simulated distribution than the open-to-in transition. This indicates PHBH molecules are heterogeneous with respect to both the in-to-open and open-to-in transitions on the seconds time scale, with the in-open transition being more heterogeneous than the open−in transition.

An unresolved question is whether the fraction of enzyme molecules that do not show any transitions from the fluorescent open state in the observation time ever undergo transitions to the in state. These molecules may form a separate population that exists in the open state only or may undergo transitions on a slower time scale. The observation that in-to-open transitions occur in bursts, with a time lag before and after several rapid in-out transitions with the molecule locked in the fluorescent open state, suggests that the unchanging molecules are not a separate population but that the FAD molecules in these trajectories were lost before the enzyme switched to the rapidly converting form.

Correlation between the In/Open Positions of the Two Flavins in the PHBH Dimer. PHBH is a dimer and usually has two FAD molecules bound to the enzyme. The majority of the molecules we observed lost fluorescence in a single step, indicating that only one FAD was bound to the enzyme at that

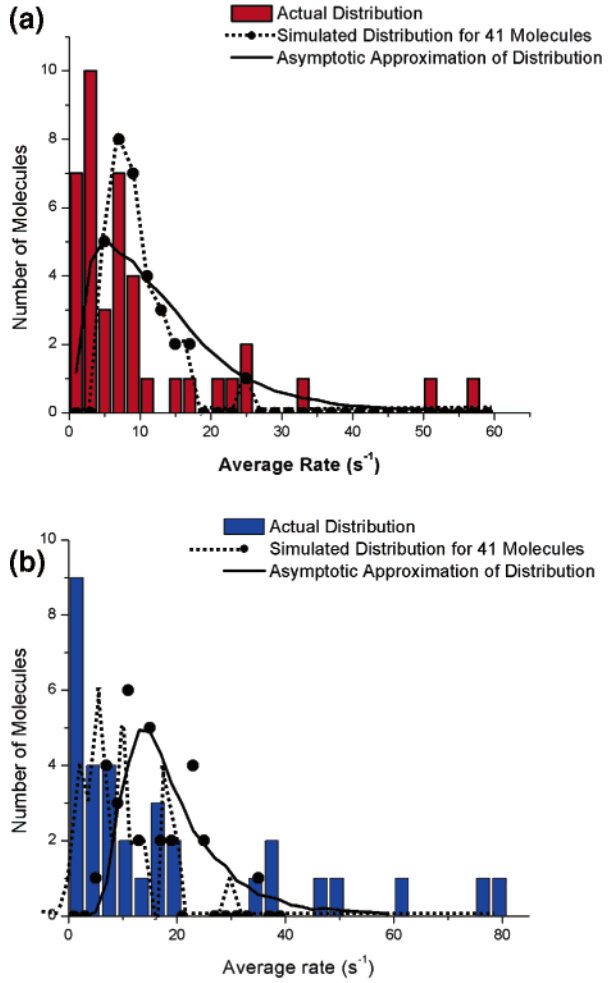

Figure 7. Distributions of the transition rates derived from 41 individual molecules of the H72N mutant for the open-to-in transition (Figure 7a) and the in-to-open transition (Figure 7b) compared to simulated distributions of 41 identical molecules with the same average rate as the experimental sample and asymptotic approximations to the theoretical distributions. Compared to the simulated distributions of identical molecules, the experimental distributions show clear fast and slow populations of both the in-to-open and open-to-in transition rates.

point. This is most likely due to the very dilute solution of PHBH required for these single-molecule studies. Occasionally, a trajectory was recorded in which the fluorescence was lost in two steps, indicating that the molecule in question was an intact PHBH dimer with an FAD molecule bound at each binding site (Figure 3d). It is possible that trajectories that lose fluorescence in a single step represent intact dimers with only one FAD bound or else a monomer within the agarose pore.

For molecules that show a two-step trajectory, full fluorescence indicates that both flavins are in the open position. Partial fluorescence indicates one flavin is in the open-conformation and one is in the in-conformation or else it has dissociated from the enzyme. The absence of fluorescence indicates that both flavins are in the in-conformation or dissociated. The small number of trajectories of dimers with two FADs bound prevented a quantitative calculation of rates. However, an interesting effect can be seen in Figure 3d. The fluorescence of dimeric PHBH fluctuates between the full and partial level and only rarely decreases to the background level. This suggests that movement of the flavin to the in-conformation on one subunit of the dimer inhibits the movement of the flavin to the in conformation on the other subunit.

Discussion

The results presented here suggest a dynamic picture with the flavin oscillating between the in-conformation and what we believe is the open-conformation seen in the crystal structure of the R220Q enzyme. Intermediate positions, which would be indicated by states with intermediate levels of fluorescence, were not seen. The equilibrium constant measured here for the R220Q mutant ($K_{eq} = 7.2$) favored the open-position more than did the WT enzyme, and this is consistent with the X-ray data.[6]

The dynamic picture of flavin motion in the absence of substrate suggested here has implications for the binding of substrate to the enzyme. Substrate binding can only occur when PHBH is in the open-conformation, because the in-conformation does not have an open channel to the solvent through which the substrate can approach the binding site. The single-molecule measurements show an interconversion between conformations on the time scale of tens of milliseconds at 25 °C—a time scale that should be detectable in stopped-flow measurements—as PHBH molecules that were formerly in the in-conformation convert to the open-conformation and rapidly bind substrate. However, when measured with a stopped-flow spectrophotmeter, wild-type PHBH binds *p*-hydroxybenzoate within the dead time of stopped-flow mixing (i.e. ~3 ms at 4 °C). The difference between the single-molecule and stopped-flow experiments may provide additional insight into the binding of substrate by PHBH, as highlighted below.

Ensemble kinetic techniques such as stopped-flow spectrophotometry measure the relaxation to equilibrium upon a perturbation such as the addition of substrate to an enzyme. Single-molecule techniques directly measure fluctuations at equilibrium. In ensemble techniques some effects, such as how conformations of the flavins on each subunit of the PHBH dimer correlate, can be masked. In the minority of trajectories where FAD was observed to be bound to each subunit of the dimer, the fluorescence was found to only infrequently decrease to a background level reflective of a state in which both flavins of the dimer are in the in-position. Such a state would prevent the enzyme from binding substrate. The fluorescence mostly oscillated between a level corresponding to both flavins being in the open-position and a level reflecting one flavin being in the open-position and the other in the in-position (see Figure 3d). This suggests that one subunit of the PHBH dimer is almost always in position to bind substrate.

A reaction scheme that would explain the stopped-flow results for binding of substrate in the dimeric enzyme in the context of the data presented here is shown in Figure 8.

The binding of substrate according to this reaction scheme will occur within the dead time of a stopped-flow instrument provided (i) the binding of substrate to the open-conformation is fast, (ii) the binding of substrate on one subunit induces the flavin on the other subunit to rapidly change conformation from the in-conformation to the open-conformation, and (iii) one subunit is always in position to bind substrate (it should be noted that the conformation with both flavins in the in-conformation is infrequent, as supported by data shown in Figure 3d in the time frame between 0 and ~1 s from the start of recording when

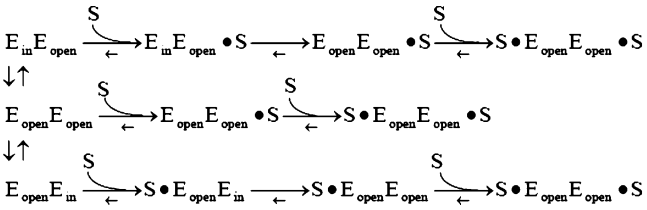

Figure 8. Proposed reaction scheme for the dimeric enzyme binding substrate. The $E_{in-open}$ to $E_{open-open}$ transition is observed in this study, while the remainder of the reactions happen quickly (<1 ms) in the presence of substrate. The E_{in-in} state is not a major species on the basis of our observations of the dimeric enzyme (the fluorescence rarely fluctuates to the background level). This reaction scheme assumes binding of substrate on one subunit shifts the equilibrium of the other subunit to the open state.

there are two flavin molecules bound to the dimer). Such a model has some support based on studies of the A45G mutant.[1,19]

The small number of trajectories that have FAD bound on both subunits prevented us from testing this model directly by comparing the experimental occurrence of both flavins being in the in-position to the theoretical probability of both flavins being in the in-position. At the very low concentrations of both enzyme and flavin that were used here, it is expected that very few enzyme molecules are PHBH dimers populated with two flavins. Perhaps recent advances in immobilization of enzymes (for example immobilization in lipid vesicles) for single-molecule work will allow the properties of dimeric PHBH to be studied in greater detail.[20]

Besides a correlation between conformations of the flavins in the PHBH dimer, a fast interconversion of open- and in-conformations occurring within less than 1 ms, in addition to the slower interconversion seen in our experiments, would also explain the absence of a phase that reflects the in-to-open transition in stopped-flow measurements. However, no evidence of such a fast interconversion was found in our experiments. A PHBH molecule undergoing fast (relative to the 10 ms resolution of the data) interconversion between the open- and in-conformations would exhibit a mean fluorescence intensity that was intermediate between the intensities expected of the open- and in-conformations. The fluorescence intensity of the off state was found to be the same as the background, essentially nonfluorescent. It can therefore be concluded that once in the in-conformation the flavin stays in that position for at least several milliseconds and the enzyme is not able to bind substrate during this period. Interconversion between states occurring within the 10 ms resolution of our data is therefore not likely to be the reason for there not being a phase that reflects the in-to-open transition when observing with stopped-flow spectroscopy.

Heterogeneity in reaction rates has been observed before in single-molecule measurements of a number of systems.[10,11,21−31] The fast in-to-open conformational change for PHBH was also found to be heterogeneous, with individual H72N molecules showing as much as a 5-fold variation in reaction rates (see Figure 7). The open-to-in conformational change showed less heterogeneity, but this may be due to the finite time resolution of our experimental system. Because the open-to-in conformational change is faster than the in-to-open change, it is more likely that small differences in reaction rates of different populations of molecules will be obscured.

In summary, we have found evidence of dynamic motions of the isoalloxazine ring of the flavin between the in- and open-conformations. The structural mechanism for switching between in- and open-conformations in the absence of ligand is not known; however, data from studies of the H72N mutant suggest that the in-to-out transition, which is required for reduction of the flavin by NADPH, is triggered by the internal proton-transfer network.[7] Analysis of the single-molecule fluctuations shows a hierarchy of conformational states, with states in which the open-conformation rapidly interconverts to the in-conformation and other states in which the flavin interconverts more slowly. The complexity of the prebinding conformational kinetics of PHBH found here has not been resolvable previously by stopped-flow techniques with WT enzyme and suggests that the single-molecule approach may be profitable for the detailed resolution of steps in enzymatic reactions.

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