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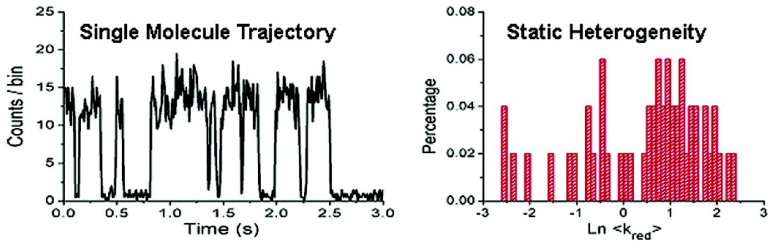

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Multiple States of the Tyr318Leu Mutant of Dihydroorotate Dehydrogenase Revealed by Single-Molecule Kinetics

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Abstract: Dihydroorotate dehydrogenase (DHOD) from *Escherichia coli* is a monomeric membrane-associated flavoprotein that catalyzes the oxidation of dihydroorotate to orotate. By using confocal fluorescence spectroscopy on the highly fluorescent Tyr318Leu DHOD mutant, we studied the catalytic turnover of single enzyme molecules through the characteristic on−off fluorescence signal, which corresponds to flavin mononucleotide (FMN) interconverting between the oxidized and reduced states during turnover. Our single-molecule data provide evidence of a distinct static heterogeneity in the enzymatic activity, with some molecules going through the on−off cycles 5-fold faster than others, however, there is no detectable dynamic disorder in DHOD turnover. When 0.1% reduced Triton X-100, a detergent that more closely simulates the natural membrane environment, is added, our data suggest the degree of static molecular heterogeneity is reduced. The observation of static heterogeneity suggests that the enzyme, which associates with the membrane in vivo, is present in distinct conformations that result in different catalytic efficiencies. The alternate conformations are most likely the result of the loss of van der Waals or other interactions between tyrosine 318 and FMN in the catalytic site with the mutation of Tyr318Leu, which disrupts the native structure of wild-type DHOD.

1. Introduction

Single-molecule spectroscopy offers a unique method for studying enzyme kinetics, providing information that can be very challenging to obtain by traditional ensemble methods.[1−8] By observing one molecule at a time, not only can the kinetic properties of individual molecules be obtained but also the dynamic behavior of enzyme molecules may be observed, including the presence of different forms of the enzyme and their interconversion. If kinetically distinct enzyme populations do not interconvert quickly, the population is said to exhibit static heterogeneity, in which the turnover of each enzyme population is governed by a single set of rate constants that do not change quickly over time. However, if a rate constant varies with time, there will be a correlation between the reaction rates in the sequential catalytic cycles. This has been called dynamic disorder and can also be detected in single-molecule studies because sequential turnovers of the same molecule are followed. If there is dynamic disorder, slow turnovers would be followed by turnovers on the same slow time scale, while fast turnovers would be more likely followed by turnovers on the same fast time scale. If there is no dynamic disorder, the turnover rate is randomly distributed. Therefore, studying enzymes at the single-molecule level may help answer questions that are beyond the scope of conventional ensemble studies.

Flavin-containing enzymes are good model systems for studying single-molecule turnover because the redox-active 7,8-dimethylisoalloxazine moiety is often fluorescent in the oxidized state and nonfluorescent in the reduced state; hence, studying the sequence of the fluorescence intensity gives information on catalytic rates, heterogeneity, etc. Single-molecule studies on the flavoenzyme cholesterol oxidase have already demonstrated a "memory effect" in catalysis[8] and shed new light on the dynamic behavior of the enzyme in turnover. A large number of flavoenzymes, catalyzing a large variety of reactions, are known.[9] Flavoenzymes most frequently use flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) as noncovalently bound prosthetic groups.

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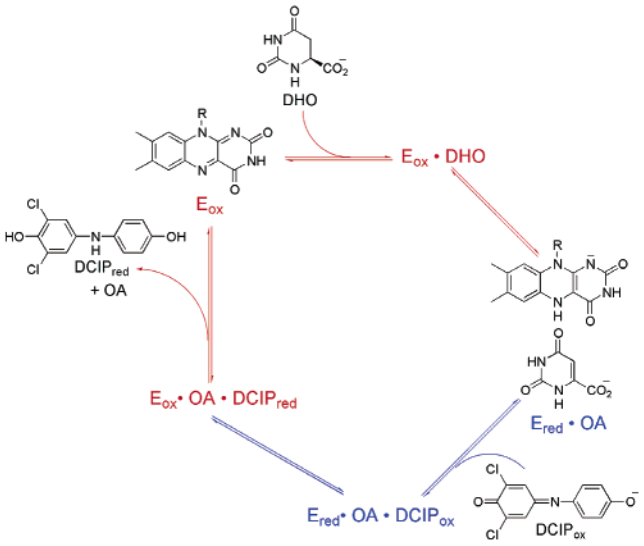

Figure 1. Catalytic cycle of DHOD with DCIP as the oxidizing substrate. Enzyme with oxidized flavin bound is abbreviated E_{ox} and enzyme with reduced flavin bound is abbreviated E_{red}. The reductive half-reaction is shown in red and the oxidative half-reaction is shown in blue.

In this paper we report studies on dihydroorotate dehydrogenase (DHOD) from *Escherichia coli*, which contains FMN. DHOD catalyzes the only redox reaction in de novo pyrimidine biosynthesis—the oxidation of dihydroorotate (DHO) to orotate (OA). *E. coli* DHOD is a membrane-associated monomer that uses ubiquinone as its natural oxidizing cosubstrate.[10] The catalytic cycle may conveniently be divided into two half-reactions (Figure 1). In the reductive half-reaction, the oxidized enzyme binds DHO and DHO reduces FMN, forming OA, which dissociates too slowly to allow the free reduced enzyme to be catalytically relevant.[11] Instead, in the oxidative half-reaction, the reduced enzyme−OA complex binds the quinone substrate, FMN is oxidized, and the products dissociate. A number of compounds can serve as effective oxidants, including the dye dichlorophenol indophenol (DCIP), which allows for convenient photometric assays and studies of DHOD kinetics.

Catalysis may be studied by classical steady-state kinetics, stopped-flow methods, or single-molecule techniques. Rates of substrate consumption or product production are measured in steady-state studies of turnover. The kinetic parameters obtained, such as k_{cat} and K_m, depend on many or all of the steps in the catalytic cycle and therefore can be complicated algebraic functions of many rate constants. This can make it difficult to obtain information about individual catalytic steps. Stopped-flow studies of half-reactions are more informative because they directly monitor the reactions of the enzyme starting from a chosen state. For instance, the reductive half-reaction of DHOD may be studied by mixing anaerobic solutions of oxidized DHOD with anaerobic solutions of DHO in a stopped-flow spectrophotometer/fluorometer and recording the changes in flavin absorbance or fluorescence. This approach offers a convenient means for determining rate constants for individual reactions but has two potential weaknesses: noncatalytic side reactions, if present, will be observed but might not be distinguished from normal behavior, and the enzyme might behave differently during turnover than during the approach to steady-state in a half-reaction. Single-molecule studies of turnover can follow the reaction cycle in a repetitive manner under conditions similar to steady-state studies. When observed by single-molecule spectroscopy during turnover, a flavoenzyme, which is fluorescent in the oxidized state and nonfluorescent in the reduced state, would display a blinking fluorescence signal as the enzyme cycles between the oxidized and reduced states. The distributions of durations of the on-states provide the lifetimes of the oxidized state, while the distributions of durations of the off-states provide the lifetimes of the reduced state. Because the lifetimes are the waiting time for flavin reduction and oxidation, they are directly related to the rate constants for the reactions, enabling kinetic analysis by use of single-molecule data.

The FMN in wild-type *E. coli* DHOD is heavily quenched by contact with Tyr 318 and therefore is not fluorescent, preventing single-molecule studies on this enzyme. Therefore, we made the Tyr318Leu mutant to remove the quenching residue. The mutant enzyme was highly fluorescent in the oxidized state with an emission maximum at 520 nm and a quantum yield 90% that of free FMN ($Q_{FMN} = 0.26$[12]). The mutant enzyme was not fluorescent in the reduced state. Therefore, it is suitable for single-molecule studies of catalysis, which are described in this paper. Our results reveal distinct static heterogeneity, but no dynamic disorder, in the activity of the mutant enzyme. The static heterogeneity is reduced with the presence of a detergent mimicking cell membranes. Interestingly, we have obtained kinetic information on the dissociation of FMN from the holoenzyme and show that this process is retarded under turnover conditions.

2. Methods

Materials. The plasmid pAG1 was used as a template for polymerase chain reaction (PCR) to generate the Tyr318Leu mutant by the method described in ref 4. The mutant enzyme was expressed in *Escherichia coli* strain SØ6645 and purified as previously described.[10] Dihydroorotate (DHO), dichlorophenol indophenol (DCIP), and reduced Triton X-100 (hydrogenated to reduce UV absorbance and the resulting fluorescence) were purchased from Sigma.

Gel Electrophoresis. A nondenaturing gel (4−15% Tris-HCl, Bio-Rad) was run and then stained for enzyme activity with 1 mM DHO, 0.023 mg/mL phenazine methosulfate, and 0.4 mg/mL *p*-nitro blue tetrazolium in 0.1 M Tris-HCl, pH 8.0.

Assay of DHOD Activity. DHOD was assayed spectrophotometrically at 22 °C. Turnover was monitored in a Shimadzu UV-1601PC spectrophotometer at 600 nm, where DCIP loses absorbance upon reduction ($\Delta\epsilon = 20$ mM^{-1} cm^{-1}). Assay mixtures contained 0.5 mM DHO, 0.1 mM DCIP, and 50 nM DHOD in a 50 mM sodium phosphate buffer, pH 7.0, with or without 0.1% reduced Triton X-100.

Stopped-Flow Measurements. The reductive half-reaction of DHOD by DHO was studied by use of a Hi-Tech SF-61 stopped-flow instrument. The loss of FMN fluorescence at 520 nm upon reduction was followed to determine the reaction kinetics at 22 °C in a 50 mM sodium phosphate buffer, pH 7.0, with or without 0.1% reduced Triton X-100. Enzyme solutions were made anaerobic in a glass tonometer by repeated cycles

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of evacuation and equilibration over an atmosphere of purified argon.[13] Substrate solutions were made anaerobic by bubbling with purified argon within the syringes that were to be loaded onto the stopped-flow instrument.

Confocal Fluorescence Microscopy. Fluorescence time traces of single DHOD molecules were collected by use of an inverted confocal microscope designed and built by our group. It was equipped with a 1.65 NA ×100 oil-immersion objective (Optical Analysis APO 1-UB615). Light at 457 nm from an Ar^+-ion laser (Melles-Griot 532-AP) was passed through a dichroic beam splitter and then focused on a glass slide coated with the sample. 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 image in this geometry was built up by raster-scanning. A count rate threshold was set in the control program for initiating the collection of trajectories. Time traces were collected in time steps of either 20 or 10 ms.

Preparation of Single-Molecule Samples. To physically confine enzyme molecules in the pores of a 1% agarose gel,[8] agarose was melted in 50 mM phosphate buffer, pH 7.0 (with or without substrates) with gentle stirring and heating. DHOD was diluted into the agarose solution to 1 nM just above the gelling temperature (~30 °C), and the mixture was spin-coated onto a glass slide, forming a smooth thin layer of gel containing the DHOD molecules. After the slide was mounted on the microscope stage, a small volume of buffer (~60 μL) was applied on the sample to keep it moist, which also helped to reduce background noise. In turnover experiments, the substrates were premixed with the agarose solution with or without 0.1% reduced Triton X-100. On average, at enzyme concentrations on the order of 1 nM, no more than one molecule would reside in the confocal field of view.

Simulation of the Stochastic Enzymatic Turnover. A program for the stochastic simulation of enzyme turnover was written in Mathematica. Turnover was assumed to consist of a reductive and an oxidative half-reaction controlled by the experimentally determined rate constants. The rate constants k were used to compute the probability of reduction or oxidation in unit time, $\alpha = [1 - \exp(-k\Delta t)]$, where Δt is the time step of simulated data collection. α was compared to a random number p, generated by the Random function in Mathematica, whose value ranged between 0 and 1. Reactions occurred when $p < \alpha$; if $p \geq \alpha$, the molecule remained unreacted and another value of p was generated. The number of iterations of random number generation before the occurrence of a reaction, N, is a random variable and has a geometric probability distribution as $\alpha(1 - \alpha)^N$. The on- or off-time was approximated by $N\Delta t$.

3. Results

FMN Dissociation. Figure 2a presents typical fluorescence trajectories of single DHOD molecules, in which the fluorescence signal was constant over time until it dropped to background level. The one-step fluorescence loss suggested we were observing single molecules, not aggregates of several molecules. The histogram of fluorescence on-times of 110 DHOD molecules fits a single-exponential decay (Figure 2b). To investigate whether the abrupt disappearance of fluorescence of single DHOD molecules was the result of photobleaching, the laser power was increased from 1 to 2.5 μW. Exponential fits of the on-time distributions for 1 and 2.5 μW laser power gave a similar k_{off}, the rate constant of fluorescence loss, around 0.8 s^{-1} (Figure 2b), showing that photobleaching is not the origin for the loss in fluorescence. A more plausible mechanism is that FMN dissociates from the protein and rapidly diffuses away from the field of observation.[14] This is consistent with our observation that the fluorescence signal would occasionally return after the molecule being observed lost its initial fluorescence during the 30-s data collection time.

The rapid loss of FMN from the holoenzyme suggests that our single-molecule samples contained a mixture of free FMN, holoenzyme, and apoenzyme in a dynamic equilibrium. To better quantify the equilibrium, the concentration of holoenzyme was estimated by comparing to a fluorescent standard. At a concentration of 1 nM, the number of fluorescent DHOD molecules was comparable to that observed for 0.1 nM plasminogen activator inhibitor 1 covalently labeled with fluorescein. Therefore, the amount of apoenzyme due to FMN dissociation is significant at nanomolar concentrations compared to holoenzyme. By comparing the number of fluorescent DHOD molecules to the number of fluorescent plasminogen activator inhibitor 1 molecules, we estimate that there was ~0.1 nM holo-DHOD in a ~1 nM total protein sample. This allows the estimation of a K_d of FMN from DHOD of ~10^{-8} M. By assumption of a simple one-step dissociation of FMN from the holoenzyme and use of the observed dissociation rate constant of ~0.8 s^{-1}, an association rate constant of 8×10^7 M^{-1} s^{-1} can be estimated, which is near the diffusion limit. Since the free FMN concentration is subnanomolar, the reassociation of FMN is a rare event.

In the presence of DHO and DCIP, FMN fluorescence blinked on and off as the flavin cycled between the oxidized and reduced states during turnover. However, eventually FMN dissociated from the holoenzyme, as indicated by the long time scale of loss of fluorescence. A histogram of the duration of the fluorescence signal before the final loss of FMN gave an FMN dissociation rate constant of 0.3 s^{-1} (Figure 2c), which is 2.5-fold slower than that without substrates.

Steady-State Turnover. Steady-state turnover of the Tyr318Leu mutant was measured in assays at 22 °C, by monitoring the reduction of DCIP. A turnover rate of 5.7 s^{-1} was measured with 500 μM DHO and 100 μM DCIP (both saturating concentrations). With the addition of 0.1% reduced Triton X-100, a turnover number of 9.3 s^{-1} was measured.

Stopped-Flow Studies on the Reductive Half-Reaction. The reduction of the enzyme-bound flavin was studied by mixing anaerobic solutions of enzyme and DHO at 22 °C in a stopped-flow instrument and recording the loss of either the absorbance at 450 nm (characteristic of flavin), or the absorbance at 550 nm (characteristic of a reduced flavin−orotate charge-transfer complex), or the flavin fluorescence (excitation at 450 nm, emission at >520 nm). The fluorescence traces were fit well to

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(14) If we assume the diffusion rate of FMN in agarose gel is similar to that in sucrose (whose molecular weight is similar), $D = 4.59 \times 10^{-6}$ cm^2 s^{-1}. The pore size of 1% agarose gel, $\langle x \rangle$, is around 0.5 μm in diameter. Therefore, the time t for FMN to diffuse away from our field of observation can be derived as 0.3 ms with $t = \langle x \rangle^2/2D$, which is much faster than the resolution in our data collection time (10 ms). So the drop of fluorescence is very sharp.

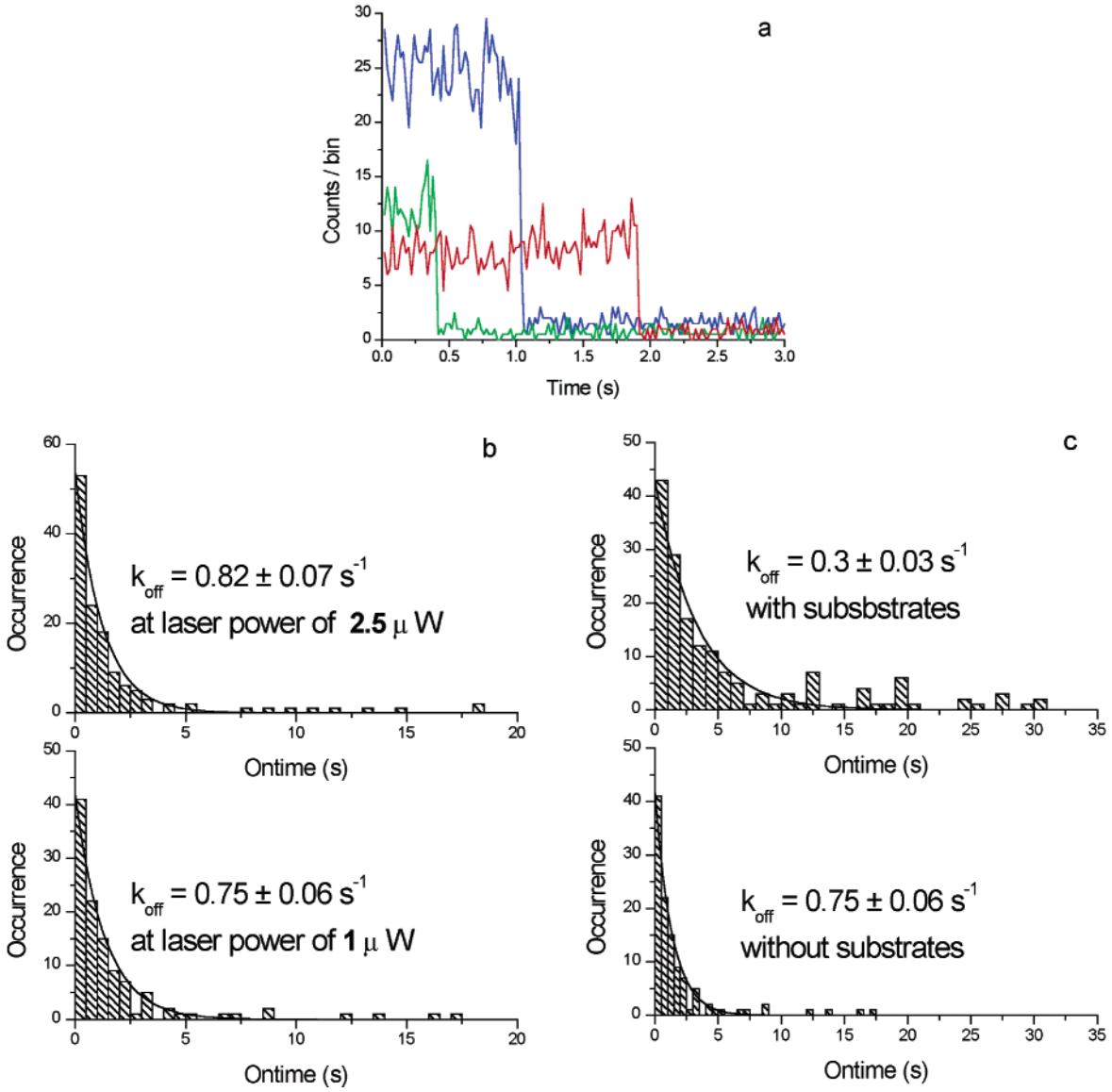

Figure 2. (a) Fluorescence trajectories of single DHOD molecules. Note that the fluorescent intensities of different molecules varied depending on their vertical displacement from the focal point. (b) On-time distribution of one-step trajectories from single DHOD molecules at laser power of 1 and 2.5 μW. k_{off} did not show a dependence on the laser intensity, indicating the fluorescence drop is not due to photobleaching. (c) On-time histogram of DHOD molecules with substrates present compared to the case without substrates at the same laser power of 1 μW.

the sum of three exponentials. The observed rate constants did not vary with DHO concentration above 25 μM, indicating tight DHO binding. The observed rate constants were 21 s^{-1}, 4 s^{-1}, and 0.7 s^{-1}. Inclusion of 0.1% reduced Triton X-100, a detergent that simulates the hydrophobic membrane environment, altered the time scale of reaction with observed rate constants of 25 s^{-1}, 6 s^{-1}, and 0.7 s^{-1}.

As expected, the absorbance traces obtained at 450 nm gave results identical to the fluorescence traces. Absorbance traces obtained at 550 nm showed an increase in absorbance for the first reaction phase, followed by two phases of decreasing absorbance. The increase in absorbance corresponds to the formation of a reduced flavin−orotate charge-transfer complex, as described for the wild-type enzyme.[11]

Turnover of Single DHOD Molecules. The turnover of single DHOD molecules was monitored with 50 μM DHO (the reductant) and 10 μM DCIP (the oxidant) in a 50 mM phosphate buffer, pH 7.0 at a laser power of 1 μW. Turnovers of single molecules were observed as traces that alternated between fluorescent (on, oxidized) and nonfluorescent (off, reduced) states (Figure 3a).[15] Because bound FMN generally dissociated from the protein within seconds, most of the trajectories of single DHOD molecules were short and contain less than 10 turnovers. Histograms of the duration of the on- and off-states of 50 molecules, including 247 turnovers in total, were generated to obtain the kinetics of the reductive and oxidative half-reactions.

Without detergent, the fluorescence on-time distribution for 50 molecules was fit to two exponentials, with observed rate constants of 4.2 s^{-1} and of 0.9 s^{-1} (Table 1 and Figure 3b). The values of these rate constants are similar to those obtained for the two slower phases (4 s^{-1} and 0.7 s^{-1}) in the stopped-flow studies of the reductive half-reaction given above, indicat-

(15) On−off blinking could conceivably be the result of triplet-state formation. However, the lack of on−off blinking in the absence of substrates (Figure 2a) rules out the possibility of triplet-state formation as the cause of blinking. Furthermore, in the absence of deoxygenation, the lifetime of the FMN triplet state is 1−4 μs (Berg et al. *Spectrochim. Acta Part A* **2001**, *57*, 2135−2144), which is much shorter than the millisecond time scale of the chemical kinetics we were following in our single-molecule experiment.

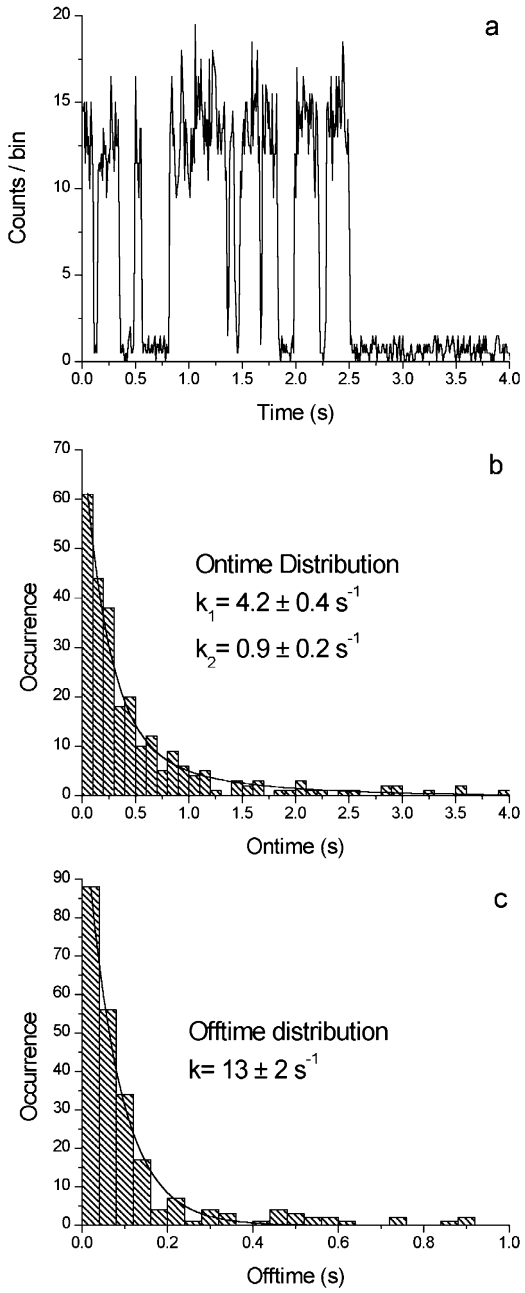

Figure 3. (a) Fluorescence trajectory of a single DHOD molecule during turnover (50 μM DHO and 10 μM DCIP in 50 mM phosphate buffer, pH 7 at 22 °C). At 2.5 s, FMN dissociated from the enzyme and diffused away from the field of view, resulting in the irreversible loss of fluorescence. (b) On-time and (c) off-time distributions of an ensemble of 50 molecules in the absence of detergent.

ing that the single-molecule measurement reports on the two slower phases seen in the stopped-flow results. The off-time distribution was well fit by a single exponential with an observed rate constant of 13 s^{-1}.

In the presence of 0.1% reduced Triton X-100, a biphasic on-time distribution was again obtained, with observed rate constants of 30 s^{-1} and 6.2 s^{-1} (Table 1). These rate constants correspond roughly to the first two phases (25 s^{-1} and 6 s^{-1}) observed in the stopped-flow experiments. The third component (0.7 s^{-1}) observed in the stopped-flow measurement was not identifiable because there was insufficient signal-to-noise in our single-molecule data to allow for meaningful fit to three

Table 1. Kinetic Results of Steady-State Assay and Stopped-Flow and Single-Molecule Measurement[a]

	without detergent	with detergent
steady-state assay	$k_{cat} = 5.7 \pm 0.4\ s^{-1}$	$k_{cat} = 9.3 \pm 0.7\ s^{-1}$
stopped-flow measurement for reductive half-reaction	$k_1 = 21 \pm 0.2\ s^{-1}$ $k_2 = 3.9 \pm 0.1\ s^{-1}$ $k_3 = 0.7 \pm 0.1\ s^{-1}$	$k_1 = 25 \pm 0.6\ s^{-1}$ $k_2 = 6 \pm 0.3\ s^{-1}$ $k_3 = 0.7 \pm 0.1\ s^{-1}$
single-molecule study: on-time reductive half-reaction	$k_1 = 4.2 \pm 0.4\ s^{-1}$	$k_1 = 30 \pm 4\ s^{-1}$
single-molecule study: off-time oxidative half-reaction	$k_2 = 0.9 \pm 0.2\ s^{-1}$ $k = 13 \pm 2\ s^{-1}$	$k_2 = 6.2 \pm 0.5\ s^{-1}$ $k = 23 \pm 3\ s^{-1}$

Kinetics were measured in 50 mM phosphate buffer, pH 7 at 22 °C.

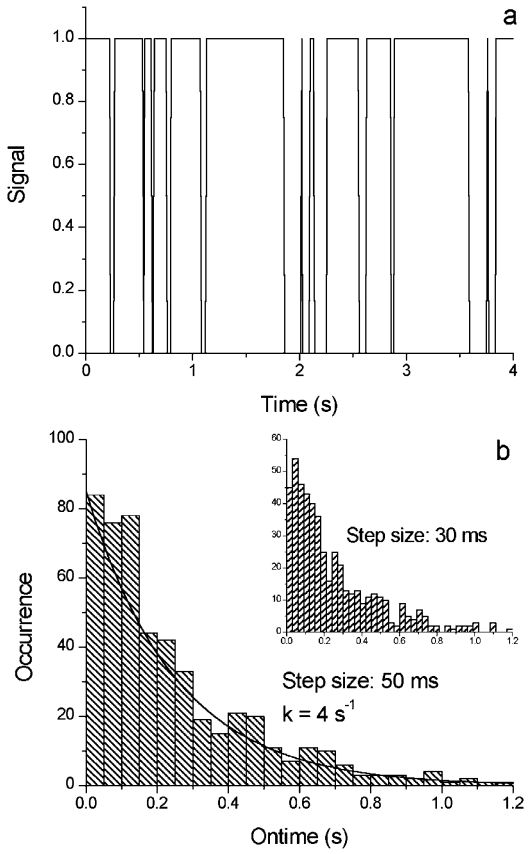

Figure 4. (a) Simulated time trace of a single-molecule turnover. (b) On-time histogram of the simulated data. Data binned in 30 ms time step (inset) show a rise phase.

exponentials. The off-time distribution was fit to a single exponential, with an observed rate constant of 23 s^{-1}, about twice the value obtained without detergent.

It was not clear whether data binning could lead to systematic errors in the analysis of the single-molecule data. Therefore, we compared our data to the simulation results of the stochastic process of a single molecule turning over, obtained by randomly generating reductive and oxidative events. The time trace of a random catalytic process with a single reduction rate constant of 4.2 s^{-1} and an oxidation rate constant of 13 s^{-1} was simulated with a time step of 10 ms, as shown in Figure 4a (500 turnovers in total). By definition, in this simulation there was neither static heterogeneity nor dynamic disorder. Both the simulated on-time (Figure 4b) and off-time distributions (data not shown) fit to single exponentials, giving the rate constant values used in the simulation. Interestingly, when we binned the data in steps of 30 ms (or 20 ms, data not shown), a distinct phase with a rise

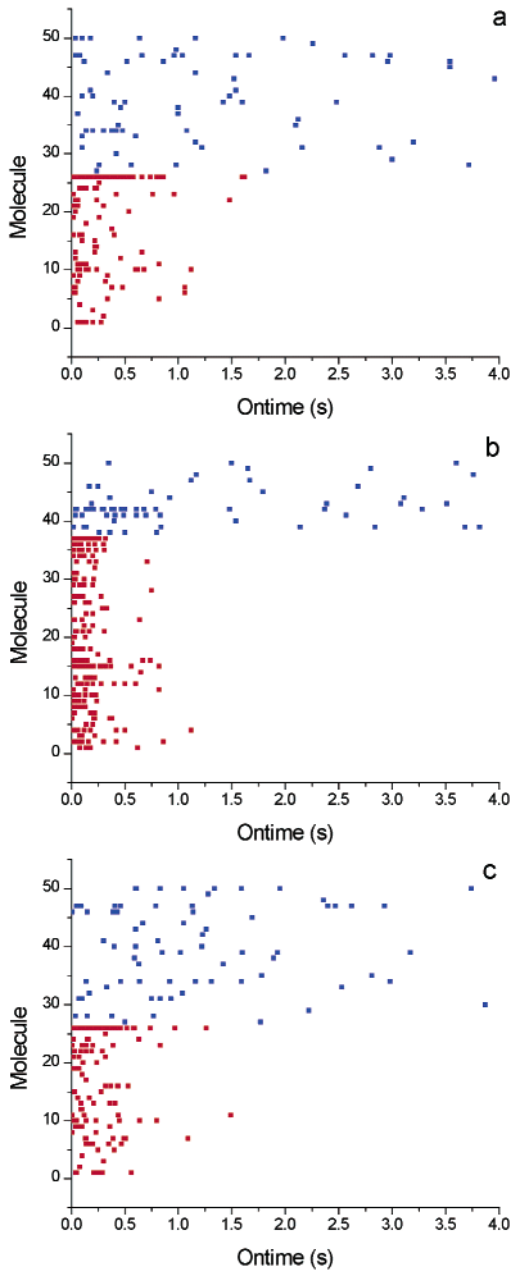

Figure 5. Individual on-time distributions of 50 different single molecules (a) without detergent and (b) with detergent. (c) Simulated results of on-times of 50 different molecules with reductive rate of either 4.2 s^{-1} or 0.9 s^{-1}. Slow molecules, shown in blue, have less than 10% on-times shorter than 0.1 s, while fast molecules, shown in red, have few on-time longer than 1 s. Different molecules were grouped according to their kinetic behavior and then numbered.

time was observed preceding the exponential decay (Figure 4b, inset), which could be misinterpreted as a distinct kinetic step. This is due to the fact that the time resolution of 10 ms results in no data between 0 and 10 ms and thus places few counts in the first (and sometimes in the second) bin. The way to avoid such an artifact is either to generate a histogram with a time step that is the same as the time resolution in the experiment or to bin data with a large time step, possibly sacrificing information on the fast reactions.

Static Heterogeneity. The observation of static heterogeneity in the reduction rate is easily seen in Figure 5a. The on-times of molecules from a sample of 50 DHOD molecules having few (<10%) on-times shorter than 0.1 s are plotted in blue, and others are plotted in red. Interestingly, the molecules represented by red rarely have on-times longer than 1 s. In other words, the slow molecules account for nearly all of the slow on-times. Similar behavior could be identified in the off-time distributions as well. There is a discernible population of long off-times (>1 s) in the off-time distribution (data not shown). However, the molecules with long on-times do not necessarily have long off-times and vice versa. Therefore it appears that some molecules are intrinsically fast (denoted in red), while some (denoted in blue) are intrinsically slow in either the reductive half-reaction (on-time) or the oxidative half-reaction (off-time) and sometimes in both, indicating static heterogeneity in enzymatic activity of DHOD. This heterogeneity could account for the multiple phases observed in the stopped-flow experiments. The addition of 0.1% reduced Triton X-100 clearly increases the reduction rate and reduces the number of slow molecules as seen in Figure 5b.

The static heterogeneity illustrated in Figure 5a can be better understood by comparing it to the simulated results of the on-times of 50 single molecules with either the fast (4.2 s^{-1}) or the slow (0.9 s^{-1}) reductive rate, as shown in Figure 5c. The number of fast and slow molecules as well as the number of turnovers of each individual molecule was simulated to be the same as in the experimental data without detergent. The simulation results of static heterogeneity showed that the slow molecules with reductive rate of 0.9 s^{-1} had few on-times shorter than 0.1 s, while the fast molecules with reductive rate of 4.2 s^{-1} had few on-times longer than 1 s.

As an alternate demonstration of static heterogeneity, the distribution of the average on-times during turnover for these 50 DHOD molecules is plotted in Figure 6a. The average on-time of each molecule is calculated as $\langle t_{on} \rangle = (1/n)\sum_{i=1}^{n} t_{on,i}$, where n is the number of turnovers. The overall reduction rate constant $\langle k_{red} \rangle$ is thus calculated as $1/\langle t_{on} \rangle$. As shown in Figure 6, the average rate observed in our single-molecule measurements was clearly more widely distributed than that from the simulation results without heterogeneity (data plotted in red in Figure 6b). The experimental data also appeared to be more distributed than the results simulated with heterogeneity (data plotted in black in Figure 6b), but this is most likely the result of having trajectories of only 50 molecules in the experiment or because the heterogeneity in the experiment was greater than that assumed in the simulation.

A nondenaturing gel was run in an attempt to identify two populations of DHOD mutant molecules with different activities. However, the DHOD Tyr318Leu mutant appeared as a single band on the nondenaturing gel, when stained for enzyme activity. Therefore, nondenaturing gels were not sensitive enough to detect the subtle difference between the two populations of DHOD mutant molecules identified in our single-molecule experiments.

Dynamic Disorder and Memory Effect. Single-molecule measurements can detect dynamic disorder, the fluctuations of the reaction rate for a single molecule. If there is no dynamic disorder, long and short on-times would be randomly distributed in the trajectories. However, if dynamic disorder exists, the duration of a given on-time would show a dependence on previous on-times. Similar considerations also apply to the off-times.

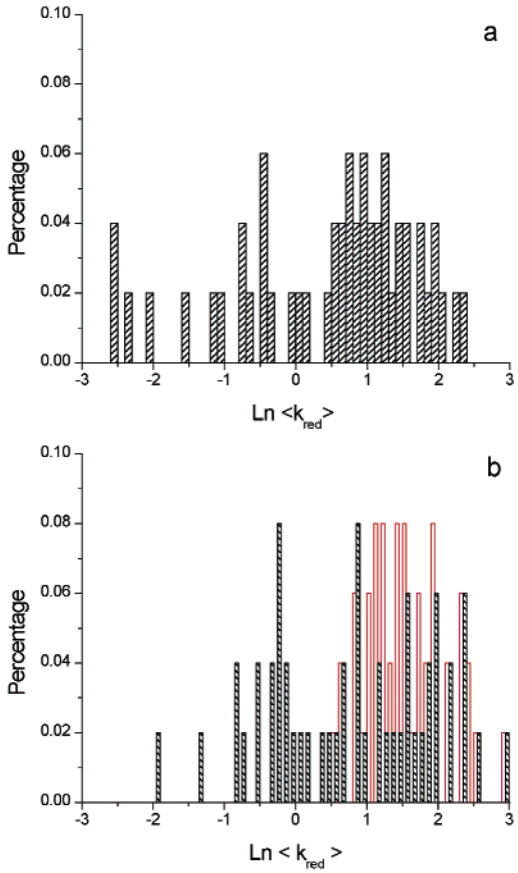

Figure 6. Distribution of average turnover rate $\langle k_{red} \rangle$ in a log scale for 50 different DHOD molecules (a) from the experimental data without detergent and (b) from the simulated data. Results in red in the simulated plot were from simulation with one reductive rate, 4.2 s^{-1} (homogeneous), while results in black were from simulation with two reductive rates, 4.2 s^{-1} and 0.9 s^{-1} (heterogeneous). The average rate is clearly more widely distributed in our experimental data set than that from the simulation results without heterogeneity.

The correlation between a series of events is best described by the autocorrelation function. The autocorrelation function for on-times (or off-times) of single-molecule trajectories is calculated with a covariant parameter defined as[16]

$$r(m) = \frac{\frac{1}{n-m}\sum_{i}^{n-m}(t_i - \langle t \rangle)(t_{i+m} - \langle t \rangle)}{\frac{1}{n}\sum_{i}^{n}(t_i - \langle t \rangle)^2}$$

where m is the number of turnovers separating the pairs of on- (off-) times, n is the total number of turnovers, i is the index number for the state being considered in a trajectory, t_i is the on- (off-) time, and $\langle t \rangle = (1/n)\sum_i^n t_i$ is the average on- (off-) time. Results of on-time autocorrelation derived from a long single-molecule trajectory consisting of 87 turnovers are shown in Figure 7. The randomness suggests there is no dynamic disorder in the activity of the mutant DHOD on the time scale of the single-molecule measurement.

The dynamic correlations between sequential turnover rates can be also probed with conditional distribution plots of the pairs of sequential on- (off-) times, which revealed the existence of the memory effect observed by Lu et al.,[8] in their study of single cholesterol oxidase. The conditional distribution of consecutive on-times from the data simulated without a memory effect, as discussed in Figure 4, shows no pattern (Figure 8a), indicating no memory effect. Both with and without detergent, the 2D conditional distribution from our experimental data demonstrates similar randomness comparable to the simulated results, indicating no correlation between the consecutive on-times. Therefore, there is no observable memory effect in DHOD catalysis (Figure 8b).

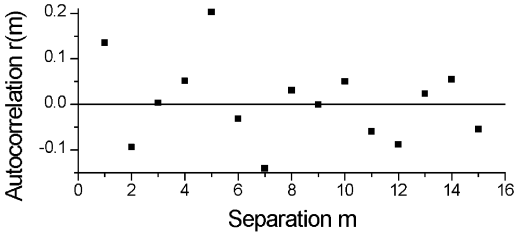

Figure 7. Autocorrelation function $r(m)$ of on-times of a single DHOD molecule without detergent. m is the separation between the pairs of on-times being correlated. The random distribution suggests no dynamic disorder.

Figure 8c depicts the 2D conditional histogram of pairs of on-times and the consecutive off-times. The absence of a correlation pattern demonstrates that the on-times and off-times are not correlated, indicating the reductive and the oxidative half-reactions are kinetically independent.

4. Discussion

The unique strength of single-molecule enzymology lies in the fact that kinetic information that is hidden in ensemble methods can be obtained. Our single-molecule results clearly demonstrate static heterogeneity in the activity of the Tyr318Leu DHOD mutant. There is a distinct population of enzyme molecules (23 of the total 50 observed molecules) with slow reduction rates, and this population was diminished when detergent was added to more closely simulate the cellular environment. More than one reaction phase was also observed in stopped-flow experiments of the ensemble catalysis. At least two explanations are possible. Either the complex reaction traces are caused by a homogeneous enzyme population sequentially reacting through a series of intermediates with decreasing fluorescence, or the enzyme is a mixture of forms of different reactivity. The single-molecule data clearly show that the enzyme is heterogeneous. If instead there were several intermediates, each with a different intrinsic fluorescence, then the single-molecule fluorescence trajectories would not have behaved as two distinct states but would have included one or more states of intermediate fluorescence. This was not observed. Because wild-type DHOD did not show a significant third phase in stopped-flow experiments, this static heterogeneity is the result of the mutation of tyrosine to leucine.

While the focus of the above measurements is on understanding the heterogeneity detected by single-molecule spectroscopy, it is interesting to note that, in Table 1, three different assays done under significantly different conditions all report on various aspects of the oxidation−reduction reaction identified in Figure 1. Both the steady-state assays and the single-molecule measurements report on the enzyme as it goes cyclically through

(16) Kendall, M.; Ord, J. K. *Time Series*; Hodder and Stoughton Educational: Kent, U.K., 1990; Chapt. 6.

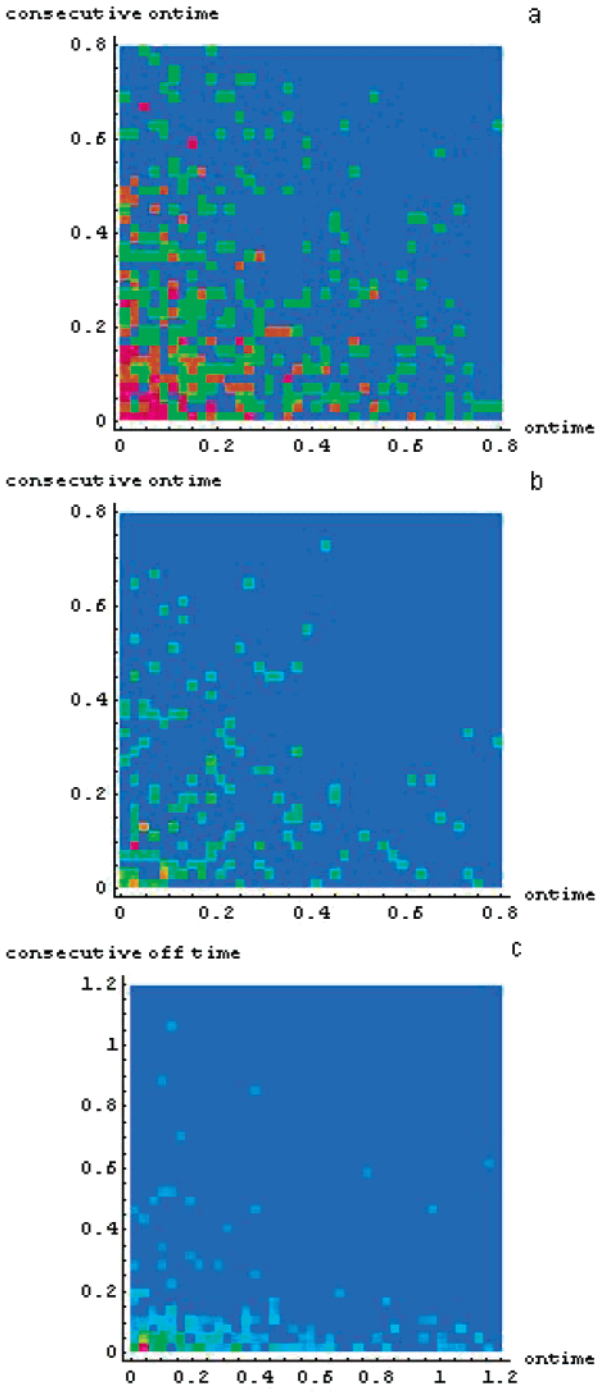

Figure 8. (a) Two-dimensional conditional distribution of consecutive on-times without detergent. There is no correlation that could signify a memory effect. (b) The correlation graph of on-times and the consecutive off-times showed no correlation pattern, indicating the reductive and oxidative half-reactions are not correlated. (c) Two-dimensional conditional distribution of consecutive on-times from simulated data.

oxidation and reduction, though they report on different aspects of the reaction. The stopped-flow experiments report on the reductive half-reaction only, where turnover is not possible. While there are some qualitative features of agreement, it is difficult and beyond the scope of this paper to make a quantitative comparison. We note that in the presence of detergent the steady-state assays show an increase in the reaction rate, in agreement with the single-molecule studies, but the stopped-flow experiments are more complicated. The stopped-flow measurements show a slow phase ($k < 1\ s^{-1}$) with and without detergent. However, in the single-molecule experiments, we are only able to clearly see this in the case without detergent. With detergent, the single-molecule data of the type in Figure 3 do not have sufficient signal-to-noise to enable a meaningful fit of three exponentials to see this third phase, though a simple visual examination of the same single-molecule data as displayed in Figure 5 does suggest the existence of this slow phase even in the detergent data. Taken together, we are able to hypothesize that the slow phase seen in the single-molecule measurements is due to heterogeneity of the DHOD and that detergent seems not only to speed up the reaction but also (from Figure 5) to reduce the number of DHOD molecules that are contributing to the slow phase. Interestingly, the fastest phase seen in the stopped-flow experiment without detergent is completely missing in the single-molecule experiments, which suggests that it arises from a transient species in the stopped-flow measurement that is not present in the steady state.

Our single-molecule data showed no identifiable dynamic disorder for the Tyr318Leu DHOD mutant in turnover. Therefore, conformational changes, which account for the fluctuations in the reaction rate, do not occur on the same time scale as the single-molecule measurement. Fluctuations that are more rapid than the measurement will modulate the reactivity of the enzyme according to a weighted average of the states, while fluctuations slower than the time scale of the measurement will not be observed during our experiments but will be detected as static heterogeneity.

Interestingly, in the single-molecule trajectories, long on-times are not necessarily followed by long off-times (Figure 8c), so the reductive half-reaction (probed by the on-time) and the oxidative half-reaction (probed by the off-time) do not appear to be correlated. The independence of the reductive and oxidative half-reactions may be explained by the fact that DHO (the reductant) and DCIP (the oxidant) bind and react at distinct sites. Distortion in these two distinct sites could account for the uncorrelated slow reductive and oxidative half-reactions, respectively. Our results indicate that there is a significant population with a distorted DHO binding site and a significant population with a distorted quinone binding site, and because there was no correlation between a slow reductive and oxidative half-reaction, there must also be a significant population that has both sites distorted.

Stopped-flow studies of wild-type DHOD did show a third reaction phase during reduction,[10] corresponding to the slow phase discussed above, but the relative fraction of this third phase was insignificant compared to that of the Tyr318Leu mutant. With the single-molecule results we conclude that this slow phase is the result of heterogeneity. Therefore, mutating tyrosine 318 to leucine not only caused a large increase in the fluorescence of the flavin but also created a significant degree of heterogeneity in the mutant enzyme, which we attribute to alternate enzyme conformations.

Although we do not know what the differences are between these conformations, several possibilities are noted here. The structure of wild-type DHOD[17] shows that the phenolic moiety of tyrosine 318 is in van der Waals contact with the xylene ring of the *re* face of FMN, and it partially separates the

(17) Norager, S.; Jensen, K. F.; Bjornberg, O.; Larsen, S. *Structure* **2002**, *10* (9), 1211−1223.

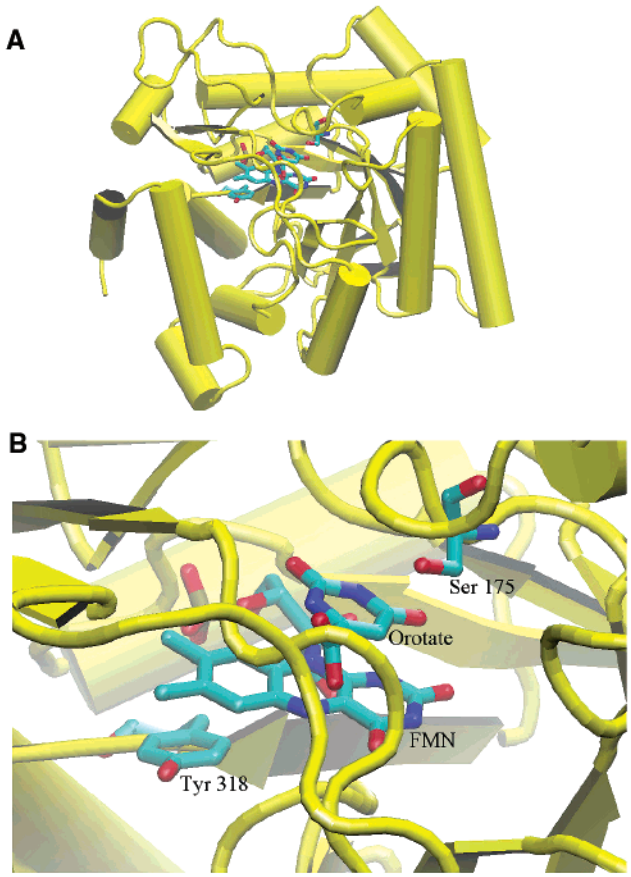

Figure 9. (Top) Structure of wild-type DHOD from *E. coli*. (Bottom) Environment of FMN in the catalytic site.

isoalloxazine ring from direct contact with the presumed quinone binding site (Figure 9). The conformational perturbation could be localized in the site of the mutation. If that is the case, then the two conformations could simply be two orientations of the leucine side chain. The tetrahedral β-carbon of leucine may conceivably orient itself with the β-hydrogen pointing away from or toward the face of the flavin. However, even buried side chains are often observed to rotate relatively rapidly by NMR, so the two conformations should interconvert very quickly, which would not explain our observation of static heterogeneity. Furthermore, it is not clear how the two hypothetical leucine conformations could influence the rate of reaction between DHO and the enzyme-bound flavin, because residue 318 is not in contact with this reaction site.

A more plausible scenario is that, instead of a local effect, the Tyr318Leu mutation has a more global effect on structure. This might also explain the effect of detergent in reducing the heterogeneity in enzyme activity because the DHO binding site presumably is not accessible to the reduced Triton X-100. DHOD is not an integral membrane protein but binds to membranes via a hydrophobic surface on the N-terminal domain of the protein. A cleft in this domain, lined by a number of hydrophobic residues, is the site of quinone binding. Tyr 318 lies between this cleft and the flavin. It has been shown that disruptions in the N-terminal domain decrease the stability of the protein and the reactivity of DCIP.[17] Therefore we propose that the Tyr318Leu mutation partially disrupts the N-terminal domain and that Triton X-100 binds to the N-terminal domain, partially restoring its proper structure. Changes in the N-terminal domain caused by the mutation would also need to be propagated through the structure to the DHO binding site in order to slow the reductive half-reaction, but it is as yet unclear how this happens.

In our experiments, we observed FMN dissociating from the enzyme as a single process, without heterogeneity. However, we observed significant heterogeneity in catalysis, which we attribute to alternate conformations. Therefore, our data suggest FMN dissociates identically from the distinct DHOD conformations. This behavior is consistent with minimal disruption of the FMN binding site.

Our data showed that the presence of substrates slows down the loss of flavin from Tyr318Leu DHOD. However, the interpretation of this observation is complicated by the fact that in the presence of substrates the enzyme is turning over—the oxidation state of the enzyme cycles, and ligands (substrates and products) are intermittently bound to the DHO and quinone binding sites. Therefore, the FMN dissociation rate constant of 0.3 s^{-1} during enzymatic turnover may not be directly comparable to that obtained in the absence of substrates, in which FMN is always in the oxidized state. When a histogram of the on-times was constructed with the off-times excluded, a rate constant $\sim$ 0.32 s^{-1} was obtained. This is 2-fold slower than that without substrates. Therefore, the presence of substrates, products, or the reduced state of the flavin slows the dissociation of FMN from the mutant enzyme.

5. Conclusion

The presence of improperly folded protein molecules that are not competent catalysts could account for the strong static heterogeneity observed in the DHOD mutant. Because DHOD is a membrane-associated protein, the addition of detergent might promote correct folding of this hydrophobic protein. Results of both steady-state assays and single-molecule measurements confirm the rate increase, which could be the result of an increased fraction of DHOD molecules in the native conformation.

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