Ultrafast real-time visualization of active site flexibility of flavoenzyme thymidylate synthase ThyX

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In many bacteria the flavoenzyme thymidylate synthase ThyX produces the DNA nucleotide deoxythymidine monophosphate from dUMP, using methylenetetrahydrofolate as carbon donor and NADPH as hydride donor. Because all three substrates bind in close proximity to the catalytic flavin adenine dinucleotide group, substantial flexibility of the ThyX active site has been hypothesized. Using femtosecond time-resolved fluorescence spectroscopy, we have studied the conformational heterogeneity and the conformational interconversion dynamics in real time in ThyX from the hyperthermophilic bacterium *Thermotoga maritima*. The dynamics of electron transfer to excited flavin adenine dinucleotide from a neighboring tyrosine residue are used as a sensitive probe of the functional dynamics of the active site. The fluorescence decay spanned a full three orders of magnitude, demonstrating a very wide range of conformations. In particular, at physiological temperatures, multiple angstrom cofactor-residue displacements occur on the picoseconds timescale. These experimental findings are supported by molecular dynamics simulations. Binding of the dUMP substrate abolishes this flexibility and stabilizes the active site in a configuration where dUMP closely interacts with the flavin cofactor and very efficiently quenches fluorescence itself. Our results indicate a dynamic selected-fit mechanism where binding of the first substrate dUMP at high temperature stabilizes the enzyme in a configuration favorable for interaction with the second substrate NADPH, and more generally have important implications for the role of active site flexibility in enzymes interacting with multiple poly-atom substrates and products. Moreover, our data provide the basis for exploring the effect of inhibitor molecules on the active site dynamics of ThyX and other multisubstrate flavoenzymes.

protein dynamics | flavoprotein | ultrafast fluorescence spectroscopy | quenching

Configurational flexibility is essential for enzyme function during catalysis. Binding of one or more substrates, accommodation of the transition state where the actual reaction takes place, relaxation to the product state, and release of the product (s) is possible because different configurations of the enzyme are continuously sampled, by thermal or reaction-driven motions, on timescales ranging from femtoseconds to microseconds. The configurational space sampled in a certain time range will depend on the local protein flexibility/energy landscape and the temperature (1). During these configurational changes, distances between constituents of the enzyme complex change. It has been recognized that the fastest (femtosecond to picosecond) localized motions exist alongside the slower motions that occur on the (typically millisecond) timescale of catalysis (2–4).

Various experimental techniques allow monitoring changes in interactions resulting from configurational changes. Long-range micro/millisecond domain motions in flexible proteins have been studied by NMR (5) and FRET techniques (6, 7). Molecular dynamics simulations and NMR experiments indicate that more localized structural fluctuations in these flexible regions occur on the picoseconds-to-nanoseconds timescale (6, 8). These fluctuations can only be directly investigated using very high time-resolution

optical techniques. In particular, the fluorescence properties of intrinsic or external fluorophores can be exquisitely sensitive to the protein environment. In flavoproteins, interaction of the flavin cofactor with the protein environment has been shown to diminish the lifetime of the flavin fluorescence from the intrinsic nanosecond timescale to the femtoseconds-to-picoseconds timescale, due to quenching by photooxidation of nearby aromatic residues (3, 9–15). In the present study, we use ultrafast fluorescence spectroscopy to investigate conformational flexibility of the flavoenzyme thymidylate synthase ThyX.

ThyX is a homotetrameric enzyme discovered a decade ago (16), which is essential for de novo synthesis of the DNA precursor 2'-deoxythymidine-5'-monophosphate (dTTP) in a large number of bacterial systems. ThyX shows no structural homology to thymidylate synthase ThyA, which is used in most eukaryotes (17). Because the ThyX pathway is used by a number of pathogenic bacteria and absent in humans, ThyX is considered a promising antimicrobial target (16, 18); it catalyses carbon transfer from *N*⁵,*N*¹⁰-methylene-5,6,7,8-tetrahydrofolate (MTHF or CH₂H₄folate) to deoxyuridine monophosphate (dUMP) using NADPH as a hydride donor and consequently has three substrates (dUMP, MTHF, NADPH) with the flavin adenine dinucleotide cofactor shuttling between the fully oxidized (FAD) and fully reduced (FADH₂) forms. dUMP binds in close interaction with the flavin group, displacing a nearby Tyr residue (19). A very recent study shows that folate derivatives may bind to the opposite side of the flavin cofactor with respect to dU(20). The binding site of NADPH has not been determined by structural studies, but inhibition studies indicate that folate and NADPH binding sites may partially coincide (21). Furthermore, reduction of flavin by NADPH appears gated by the presence of dUMP (21), further pointing at substrate-induced, functional structural rearrangements. Steady-state crystallographic studies of this enzyme also indicate substantial flexibility of the active site: in the substrate-free structure the flavin group and its close environment appear disordered (19); for the folate-bound form, multiple flavin configurations have been suggested (20).

Using a newly developed, ultrafast fluorescence spectrometer with full spectral resolution, we performed studies on the dynamics of FAD fluorescence in wild-type and genetically modified ThyX enzymes from several bacterial species. In the present work we focus on the enzyme from the hyperthermophilic bacterium *Thermotoga maritima* that allows studies over a wide temperature range. We identified a close-lying tyrosine as well as the substrate dUMP itself as fluorescence quenchers. The observed fluorescence decay revealed the presence of a wide range of conformations, whose

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Fig. 4. Effect of dUMP binding on fluorescence decay of WT and Y91F *TmThyX* at 520 nm. The time axis is linear until 4 ps and logarithmic thereafter. A global analysis in terms of DAS of WT *TmThyX* fluorescence spectra in the presence of dUMP is shown in Fig. S3F.

included all four subunits were based on the X-ray structure of dUMP-bound *TmThyX* that was obtained at -173°C (19). Fig. S6 shows that after warming and equilibration of the model, the rmsd of the backbone atoms of the model from the X-ray structure remained roughly constant and $\sim 1\text{ \AA}$ for the 2.5-ns free dynamics trajectories. In the absence of dUMP, the deviation is significantly larger for the trajectories at 70°C than at 27°C , indicating a larger conformational heterogeneity. Interestingly, such a difference is not observed in the absence of dUMP, indicating that this substrate effectively rigidifies the protein.

We now focus on the vicinity of the FAD. Fig. 5 compares time-averaged simulated structures of the four active sites; a corresponding full tetramer is shown in Fig. S7. Though the structures in the presence of dUMP are similar to the X-ray structure (19), in the absence of dUMP the flavin is rotated in all subunits by $\sim 50^{\circ}$ with respect to the X-ray structure and shows substantial orientational freedom (Fig. S8; Fig. S8). Similar observations were made with models based directly on the dUMP-devoid structure. Interestingly, these rotated conformations are similar to those observed recently in structural studies in some of the active sites of mutant *TmThyX* (20). As in the crystal structures of the *TmThyX* WT proteins (19), in all subunits, the presence of dUMP results in a shorter FAD–Tyr-91 distance.

Fig. 6 shows the fluctuations of the FAD–Tyr-91 distance during the free dynamics in each of the four subunits. The shortest edge-to-edge distance is thought to be relevant for the electronic coupling determining the electron transfer rate. Therefore, at each instant, this distance is plotted rather than the distance between two fixed atoms. In the crystal structure the shortest distance is between the C6 atom of FAD and the C_{e1} atom of Tyr-91. In our simulations, other atom pairs, such as FAD N5–Tyr-91 C_{e1} , also occur as shortest distance. The active site structures of Fig. 5 indicate that the distance fluctuations in the dUMP-devoid model are rather the result of fluctuations of the flavin cofactor than of Tyr-91.

In the dUMP-devoid model, the FAD–Tyr-91 distance fluctuates substantially in all four subunits, at 27°C from ~ 3 to $\sim 8\text{ \AA}$. Larger fluctuations of $2\text{ \AA}$ or more appear infrequently (intervals of several nanoseconds) on the simulation timescale, and the statistics, especially at 27°C , are clearly undersampled, indicating that substantial conformational changes also take place on a longer timescale. These observations are in agreement with our analysis of the broad range of fluorescence decay times that indicate that FAD–Tyr-91 distance changes take place on the timescale of $\sim 1\text{ ns}$ or more. At 70°C the fluctuations become substantially more frequent, both the smaller, high-frequency and the larger, low-frequency fluctuations.

Interestingly, our simulations also indicate that the presence of dUMP drastically reduces the distance fluctuations for both

temperatures. This finding implies that substrate binding leads to a rigidification of the active site, and in particular of the FAD cofactor positioning, and is generally consistent with the better resolution of the active site in the X-ray crystallography studies at cryogenic temperatures (19) and our finding of dUMP-induced thermal stabilization of the FAD–protein complex (Fig. S5). Similar analyses for other residues involved in substrate binding and catalysis also indicate substantial distance variation specifically in the absence of dUMP. For instance Arg-147, Arg-174, and Ser-88, which all H-bond to dUMP, were all found to be able to intermittently H-bond to FAD in the absence of dUMP.

Discussion

In this work, the quenching of flavin fluorescence in an enzyme involved in DNA synthesis, associated with MD simulations, was used to probe the dynamic properties of the active site. Experimentally, we exploited the fact that photoexcited FAD (FAD^*) can accept an electron from the aromatic residues Tyr and Trp on the femtosecond-to-nanosecond timescale, and showed that in the case of *TmThyX* from, Tyr-91 is the principal electron donor. It has been argued that the $\text{FAD}^*\text{Tyr} \rightarrow \text{FAD}^-\text{Tyr}^{\text{ox}}$ reaction occurs with a driving force in the range of $0.7\text{--}1\text{ eV}$ and in a near-activationless regime (3, 11). Indeed, the rates of this electron transfer reaction were found to be temperature independent in flavoproteins with a much less heterogeneous fluorescence decay distribution than *TmThyX* (11, 13). Moreover, in *TmThyX*, increase in temperature leads to a shift of slow-decaying populations to fast-decaying populations rather than an increase of the rates itself. For these reasons we interpret the temperature dependence observed in this work as originating from changes in the distance-dependent electronic coupling between the reactants, rather than from activation barriers. We note that temperature-dependent fluorescence decay times have been reported for the flavoprotein lipoamide dehydrogenase (29). The FAD cofactors in this protein have an extremely high

Fig. 5. Superimposed structures of elements of the four active sites, time averaged over the last 500 ps of the free trajectories of MD simulations of *ThyX* in the presence (A) and absence (B) of dUMP. The superposition is on the entire backbone of each subunit. The His53 residues associated with the active sites belong to different subunits as the other elements. The carbon atoms are color-coded with the respective subunits.

Fig. 6. Dynamics (Left) and distribution histograms (Right) of the distance between FAD and Tyr-91 (shortest distance between an atom on the isoalloxazine ring and the tyrosine aromatic ring) in the four subunits during the 2.5-ns free dynamics.

fluorescence quantum yield and are not quenched; here, the activation of the fluorescence decay was due to distinct conformational changes that are static on the nanosecond timescale (29). The analysis in the present work by contrast highlights dynamic conformational changes occurring on the picosecond timescale.

Our analysis is based on the widely used (3, 11, 14) notion that excited-state quenching in flavoproteins is due to electron transfer from aromatic residues. The validity of this notion has been demonstrated for tryptophan quenchers (9, 30) and strongly suggested for tyrosine quenchers (31, 32). In both cases, the radical pair can be subsequently stabilized by proton transfer from the residue. Simultaneous proton and electron transfer, avoiding high-energy charge-separation intermediates, has been observed for phenol oxidation reactions in aqueous solutions (33, 34). Though we cannot fully exclude that such simultaneous processes (and hence motions between Tyr-91 and potential proton acceptors) could play a role in FAD^{*} quenching in ThyX, we consider it unlikely in view of the evidence from other flavoproteins and the fact that the high-driving force generated by FAD^{*} formation (see above) energetically allows formation of the FAD[•]-Tyr^{•+} intermediate by electron transfer only.

We observed that not only aromatic residues, but also the substrate dUMP quenches FAD fluorescence, strongly suggesting that bound dUMP also donates an electron to FAD^{*}. dUMP indeed binds very close to FAD, and the isoalloxazine and uracil rings are actually in, or close to, van der Waals contact (distance ~ 3.5 Å in the crystal structure). The relevant redox properties of dUMP have not been determined, but the light-induced oxidation of uracil in the presence of a photoreducing material has been reported (35), suggesting that the reaction is energetically possible. We will further investigate this hypothesis by transient absorption spectroscopy of the photoproducts formed in the ThyX-dUMP complex.

The FAD^{*} decay dynamics of ThyX are strikingly multiphasic, spanning three orders of magnitude in time. We assign the large and continuous lifetime distribution in ThyX fluorescence to conformational heterogeneity in the active site; this assignment is strongly supported by the MD simulation that shows large variations of active site configurations. In itself, nonmonoexponential decay of the excited state of quenched flavin cofactors in flavoproteins is frequent (15). Biexponential decay has been documented spanning shorter time spans (14, 36, 37), and in flavodoxin multiexponentiality has been associated predominantly with partial flavin dissociation from the protein (26). In blue light-sensing using FAD (BLUF) photosensor proteins (13, 31, 32) and in flavin

reductase (3), broader and more heterogeneous lifetime distributions have been interpreted in terms of ground-state heterogeneity. The lifetime distribution in *TmThyX* is considerably larger than in the abovementioned flavoproteins, and the associated donor-acceptor distance distribution is substantially wider (~ 2 Å FWHM at 20 °C; Fig. 3D) than the ~ 1 Å FWHM distribution in Yang et al. (3).

Our results show that a very broad range of configurations is accessible for *TmThyX* in the absence of substrate, and that these do not fully interconvert on the fluorescence timescale and at sub-catalytic temperatures. These findings can be considered consistent with an early crystallographic study of substrate-free *TmThyX*, where fractions of the protein, and in particular the flavin ring, appear disordered, in contrast to the dUMP-containing protein (19), and appear general for ThyX enzymes (38, 39). More specifically for *TmThyX*, our MD simulations indicate the presence of substantial variation in the distance between FAD and the Tyr-91 quencher, with large changes (up to ~ 4 Å) occurring infrequently on the timescale of up to several nanoseconds. This flexibility is no longer apparent in the presence of dUMP. Single-molecule studies of flavin reductase have demonstrated that interconversion of the lifetime-distinguishable configurations for a substantial part takes place on the timescale of milliseconds and beyond (3). Our temperature dependence studies of ThyX show that the distribution does not broaden at higher temperature, as could be expected from pure increase of accessible configurational space, but narrows and shifts to shorter effective distances. We assign this finding to acceleration of the interconversion rates of configurations on the same timescale as fluorescence decay. This interpretation is supported by our MD simulations that indicate that substantial fluctuations in donor-acceptor distance do occur on the picosecond-to-nanosecond timescale, and that these, and in particular the less-frequent large amplitude fluctuations, occur substantially more frequent at higher, physiologically relevant, temperatures.

We suggest that the flexibility in the active site of this enzyme, as reflected in the picosecond-nanosecond multiangstrom conformational fluctuations, is related to its capacity of binding multiple different aromatic substrates, of which at least two (NADPH/dUMP and MTHF/dUMP) simultaneously. The picture that emerges is that the extremely rapid conformational sampling of the protein allows dUMP to enter the active site and be accommodated close to the flavin. The binding of dUMP then stabilizes the flavin cofactor in the active site, as indicated by our MD simulations, presumably in a configuration favorable for NADPH binding and subsequent flavin reduction. This mechanism is in agreement with the reported drastic acceleration of flavin reduction by NADPH in the presence of dUMP (21). We stress that our observations have further functional consequences. Indeed, low stereospecificity and a very high K_m for NADPH have been observed for the *TmThyX* protein at 37 °C (40, 41). Moreover, this protein turns over substantially faster at 65 °C (within the physiological range) than at 37 °C (41, 42).

Our results indicate that a selected fit-type mechanism applies, where the substrate binding does not necessarily change the enzyme configuration, but rather stabilizes a favorable configuration among many sampled. The accelerated and more extensive sampling at higher temperature does not substantially influence the equilibrium binding of the substrate, but may allow faster exchange with the solvent of substrate and product.

In conclusion, we exploited ultrafast time-resolved FAD fluorescence quenching by a neighboring aromatic residue in ThyX and show that, in combination with temperature dependence studies, it is a sensitive probe for conformational fluctuations. We have shown that the active site of ThyX, which has to accommodate multiple substrates, is highly flexible and, in particular, samples configurations with very high speed, with large, multiple angstrom displacements occurring on the picoseconds timescale in the physiological temperature range. We suggest that this structural plasticity allows efficient binding of the dUMP substrate.

Binding of dUMP, which acts as a very efficient FAD fluorescence quencher itself, arrests these movements and stabilizes the enzyme in a configuration allowing FAD reduction by the NADPH substrate. We conjecture that the rapid large amplitude motions of the active site monitored in ThyX enzymes may be related to their capacity of interacting with multiple different aromatic and relatively rigid substrates and products. The method that we have explored here can be used to study the effect of other substrates and, in particular, inhibitors, including potential antimicrobial agents (18), on the dynamics of the active site of ThyX. More generally, our results emphasize the important role of enzyme dynamics in the interaction with the substrates. Whereas flavoenzymes provide an intrinsically well-suited tool for real-time monitoring of configurational evolution, extensive high-speed sampling of active site configurations on the intrinsic picosecond timescale of global protein

motions is likely to be a general feature for flexible protein domains and important for understanding enzyme substrate reactivity (2).

Materials and Methods

Proteins were expressed using a codon-optimized expression vector, and purified following standard procedures. Time-resolved fluorescence experiments were performed using a recently developed spectrally resolved Kerr-gate femtosecond fluorometer (43). The models for the MD simulations (performed with CHARMM) in the presence and absence of dUMP shown in the main text were based on the structure of the dUMP-containing protein (PDB ID code 1O26) (19); a model based on the substrateless structure (PDB ID code 1O2A) gave similar results (Fig. S9). A detailed description of the experimental procedures, data analysis, and simulation protocols is given in *SI Materials and Methods*.

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