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**Photochemical processes in flavo-enzymes as a probe for active site
dynamics: TrmFO of *Thermus thermophilus***

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Abstract

Quenching of flavin fluorescence by electron transfer from neighboring aromatic residues is ubiquitous in flavoproteins. Apart from constituting a functional process in specific light-active systems, time-resolved spectral characterization of the process can more generally be employed as a probe for the active site configuration and dynamics. In the C51A variant of the bacterial RNA-transforming flavo-enzyme TrmFO from the bacterium *Thermus thermophilus*, fluorescence is very short-lived (~1 ps), and close-by Tyr343 is known to act as the main quencher, as confirmed here by the very similar dynamics observed in protein variants with modified other potential quenchers, Trp283 and Trp214. When Tyr343 is modified to redox-inactive phenylalanine, slower and highly multiphasic kinetics are observed on the picosecond-nanosecond timescale, reflecting heterogeneous electron donor-acceptor configurations. We demonstrate that Trp214, which is located on a potentially functional flexible loop, contributes to electron-donor quenching in this variant. Contrasting with observations in other nucleic acid-transforming enzymes, these kinetics are strikingly temperature-independent. This indicates a) near-barrierless electron transfer reactions and b) no exchange between different configurations on the timescale up to at least 2 ns, despite the presumed flexibility of Trp214. Results of extensive molecular dynamics simulations are presented to explain this unexpected finding in terms of slowly exchanging protein configurations.

Keywords: flavoproteins, time-resolved fluorescence, electron transfer, protein dynamics

1. Introduction

Flavins are ubiquitous cofactors found in many proteins involved in the catalysis of electron transfer (ET) and proton transfer reactions. They can be present as the riboflavin (vitamin B2) derivatives flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD). Flavins are versatile redox partners as they can adopt three different redox states, each with different possible protonation states, overall including five physiologically relevant chemical forms [1]. In the oxidized state FAD_{ox}, which is the resting state of most flavoproteins, the flavin is intrinsically highly fluorescent, with a fluorescence lifetime of 4-5 ns [2,3]. In proteins, flavin fluorescence is often strongly quenched due to competing photo-induced ET reactions. This is the case in several functionally light-active flavoproteins, such as cryptochrome [4-6] and BLUF domain [7,8] blue-light sensors, where nearby aromatic amino acids may act as electron donors, and the recently discovered flavo-enzyme fatty-acid photodecarboxylase [9,10], where the substrate acts as electron donor, as well as in artificial polypeptide-flavin complexes mimicking such functions [11]. However, such photochemical processes also frequently occur in the vast majority of flavoproteins whose physiological functioning does not require light absorption. Here, population of the excited state FAD* leads to radical pairs of the form FAD[•]X^{•+}, where X is a close-by tryptophan or tyrosine residue, from which the charge pairs subsequently recombine, either directly or following further stabilizing electron and/or proton transfer processes [12]. These processes reduce the lifetime of the excited state, thus hindering formation of potentially harmful, reactive oxygen species producing long-lived flavin triplet states [13]. In this light, the presence of tryptophan and tyrosine residues in the flavin vicinity can be viewed as photoprotective.

Even in not-photocatalytic flavoproteins, characterization of intra-protein flavin photoreduction reactions can be highly informative. First, they can serve to populate and characterize intermediate states that do play roles in physiological reactions, but where they are

too short-lived to be detectably accumulated [14,12,15]. Second, the kinetic characteristics of the ET reactions are highly sensitive to the configuration of the redox partners and can be used as a probe for the active site [16,17], for their heterogeneity as well as for thermal dynamics [18,19]. In the simplest view, the ET rate depends on the distance between the reactants' ring systems in an exponentially decreasing manner with an average protein density [20], and barrierless ET can compete with intrinsic fluorescence decay (~ 3 ns) for distances up to ~ 11 Å. Finally, in very recent work also the spectral characteristics of the flavin and amino acid radical product states have been used to characterize their interaction [21].

TrmFO is a bacterial flavoenzyme that is not photocatalytic, but has recently become a model system for flavin photoreduction [14,15,22]. It is involved in post-transcriptional site-specific methylation of tRNA, further using C_2H_4 folate as a carbon donor substrate and NADPH as a flavin-reducing substrate [23,24]. In the available crystal structure, the closest residues to the flavin isoalloxazine ring system are a cysteine and a tyrosine residue (*T. thermophilus* numbering Cys51 and Tyr343, Fig. 1) [25]. The cysteine residue is thought to be forming a highly fluorescent [26] adduct with the flavin during the reaction cycle [27]. In the absence of the cysteine the flavin fluorescence is strongly quenched by ET from the close-by (3.3 Å ring-to-ring) tyrosine residue. This feature has allowed to show that tyrosine radicals can be formed and spectroscopically characterized in the visible [15] and infrared [22], in the elusive $TyrOH^{\bullet+}$ state, which has been invoked as a potential functional intermediate in biochemical reactions [28,29].

In previous work on another nucleotide-transforming bacterial flavoenzyme, thymidylate synthase ThyX, heterogeneous quenching kinetics were used to investigate the conformational dynamics of the active site environment [18], showing a remarkable flexibility of the flavin. In the present work we extend our work on TrmFO variants along similar lines, by investigating longer range and heterogeneous ET dynamics.

2. Materials and Methods

Construction of site-directed mutants of TrmFO from *Thermus thermophilus* and protein expression were performed analogous to the procedures described in Ref. [15]. Experiments were performed with purified proteins suspended in 50 mM Hepes buffer, at pH 8.0, containing up to 250 mM NaCl. All experiments were performed in quartz cells of 1 mm path length at flavin concentrations in the range of 100-150 μ M. The sample was kept in a thermostatable sample holder. Unless indicated otherwise, the temperature was 20 °C.

Steady state absorption spectra were taken at room temperature using a Shimadzu UV-Vis 1700 spectrometer. Time- and spectrally-resolved fluorescence experiments were performed using a Kerr gate-based setup that was described before [18,30]. Very briefly, the setup operates at a repetition rate of 500 Hz and employs excitation pulses centered at 390 nm. The temporal resolution is determined by the Kerr-gate medium, and was either \sim 200 fs (suprasil, used for C51A, C51A/W214F and C51A/W283F mutants) or \sim 1 ps (CS₂, used for C51A/Y343F and C51A/Y343F/W214F mutants); in the latter case the signal is higher due to higher efficiency of the medium. Time-resolved absorption experiments were performed as described [15,31], with pump pulses centered at 390 nm. All time-resolved data were globally analyzed in terms of species associated spectra (SAS) using the Glotaran package [32]. For transient absorption data, strong cross phase modulation artifact around $t=0$ in association with the probe beam spectral dispersion, can lead to modulations of the short time-scale SAS as in some traces in Fig. 2C.

For molecular dynamics (MD) simulations, an atomic model of the TrmFO C51A variant was constructed. With the three structures of TrmFO (3G5Q, 3G5R and 3G5S) currently available in the PDB [25] as templates, we used MODELLER [33] to change Cys51 to Ala51 and to construct missing residues of the flexible loop (213-219) and of the C-terminal region

(439-443) that are unresolved in all three crystal structures. A set of ten structures was generated and the best structural model was chosen with Procheck [34]. In addition to crystal waters, a cubic box of water was overlaid and waters overlapping the protein and crystal water molecules removed. The size of the water box was chosen so that the shortest distance between protein atoms and the box edges was 20 Å. Hydrogen atoms were added using CHARMM [35]. The final system contained a 443 amino acids polypeptidic chain, an FAD cofactor, 27594 water molecules, and 31 Cl⁻ and 36 K⁺ ions corresponding to a 0.15 M ion concentration. Protonation states of histidines were assigned by visual inspection and ideal stereochemistry. Long-range electrostatic interactions were computed using the particle mesh Ewald method [36] and periodic boundary conditions were assumed using a real-space cutoff of 16 Å. After energy minimization, the structure was heated from 50 K to 300 K, equilibrated at 300 K for 500 ps and eventually a constant-temperature and pressure 500-ns dynamics trajectory was performed with NAMD [37] without any restraints. Structures were saved every 1 ps for further analysis. The CHARMM36 force field was used for the protein [38] and the TIP3P model for water [39,40]. The flavin cofactor was modelled using the force field developed in previous work [41].

Replica Exchange Molecular Dynamics (REMD) [42] simulations of TrmFO C51A were performed using the NAMD software. The model used for these simulations contained the same protein/cofactor complex and was surrounded by 14534 water molecules and 41 Cl⁻ and 46 Na⁺ ions. A number of 16 replicas with a temperature range of 300 to 330K was chosen in order to reach a replica exchange transition probability of about 0.1 [43]. An exchange between replicas was attempted every 1 ps and a 1 fs integration time step was used. Long-range electrostatics were calculated using the Particle-Mesh Ewald summation. Periodic boundary conditions were employed using a rectangular water box with dimensions 94 Å, 77 Å, and 68 Å. A production run of 30 ns was performed, leading to an ensemble of 480,000 conformations.

3. Results and Discussion

Previously [15] we showed that Tyr343, located at 3.3 Å from the FAD ring, is the main flavin fluorescence quencher in C51A TrmFO. Other residues that could potentially serve as electron donor to excited flavin are located further away and include the tryptophan residues Trp283 and Trp214. These are respectively located at 8.6 Å in the *T. thermophilus* crystal structure and on a flexible loop that is not resolved in the crystal structure (Fig. 1). These residues are therefore not expected to participate in quenching when Tyr343 is present. This prediction was tested by examining the photochemistry of the C51A/W283F and C51A/W214F mutant proteins.

Fig. 2A compares the ground state flavin absorption spectra of these constructs with that of the C51A mutant. The spectra are very similar suggesting that the direct environment of the flavin cofactor is not significantly perturbed by these changes.

Fig. 2B compares the fluorescence decay of the three constructs. As in the C51A single mutant [15], in both double mutants the fluorescence predominantly decays with a time constant of ~1 ps. This implies that the rate of ET to the flavin is very similar.

Transient absorption experiments on the C51A mutant have shown that following excited state decay a $\text{FAD}^{\bullet-}\text{TyrOH}^{\bullet+}$ product state is formed that decays back to the ground state by charge recombination in ~3 ps [15]. In the double mutants, a spectrally very similar product state is formed that also decays in ~3 ps (Fig. 2C, D). Altogether, these results confirm that Tyr343 is responsible for quenching FAD fluorescence and show that mutation of Trp214 and Trp283 to phenylalanine does not strongly influence the active site conformation.

When Tyr343 is replaced by phenylalanine (C51A/Y343F mutant) the decay of FAD fluorescence is dramatically slowed ([15], Fig. 3, cf. Fig. 2B). Yet, the decay is not fully slowed down to rates expected for unquenched flavin fluorescence ($(4-5 \text{ ns})^{-1}$ [2]). This finding

indicates that quenching by other intra-protein electron donors plays a role in this mutant. Notably, the fluorescence decay of the C51A/W283F mutant also is strongly multiphasic; in a multiexponential fit at least three time constants are required, of 30 ps, 400 ps and >2 ns. This kinetic heterogeneity suggests that multiple configurations are involved that do not interchange faster than the timescale of the fluorescence decay. Trp214 and Trp283 are among the potential electron donors (see above). In view of the heterogeneity, Trp214 is an interesting candidate, as it is located in a region that is not resolved in the crystal structure and thought to be intrinsically flexible [25], thus potentially adopting different configurations corresponding to different inherent rates of ET to flavin. We therefore constructed the C51A/Y283F/W214F triple mutant and investigated its fluorescence decay. Fig. 3 shows that the alteration of Trp214 to redox-inactive phenylalanine further slows down the fluorescence decay, confirming that this tryptophan residue acts as a fluorescence quencher in the C51A/Y283F mutant (without excluding others).

The heterogeneity of the kinetics along with the presumed flexibility of the electron donor environment raises the question whether configurations with significantly different ET characteristics exchange within the picoseconds to early nanoseconds timescale of the fluorescence decays. In principle, this issue can be investigated by monitoring the temperature dependence of the fluorescence decay. Such an approach was for instance successfully used to demonstrate the extreme flexibility of the active site (and in this case presumably in particular the flavin cofactor) of thymidylate synthase ThyX from a hyperthermophilic bacterium [18].

Fig. 4 shows that the multiphasic fluorescence decay kinetics in C51A/Y343F TrmFO are actually strikingly temperature-independent in the range of 20-40 °C. This observation implies that 1) the intrinsic ET between the electron donor(s) and the flavin takes place in an effectively barrierless regime and 2) exchange between conformations exhibiting significantly different ET rates does not take place on the timescale of the fluorescence decay (< 2 ns). The first

assessment is in general agreement with earlier studies on photo-induced ET between flavin-aromatic residue pairs [16] and further validates the interpretation of the temperature dependence of such kinetics in terms of configurational dynamics [18]. Yet, the second assessment was *a priori* unexpected in view of the above-discussed involvement of Trp214 as electron donor and the presumed location of this residue on a flexible loop.

The proposal that the loop harboring Trp214 is very flexible arises from the observation that this region is not ordered enough to be resolved in the crystal structure [25]. To investigate whether this region is actually disordered in solution we performed MD simulations of a C51A variant model, based on the wild type crystal structure (Fig. 1) and an initial loop configuration constructed for the unresolved residues 213-219. To optimally explore the configurational space, REMD simulations were performed in the temperature range 300-330K, and widely varying configurations were observed, confirming the flexibility of the loop. Fig. 5A shows two extreme cases of the range of observed loop conformations; in one case Trp214 is relatively close to the flavin ($\sim 5\text{\AA}$), in the other case it extends well into the solvent. For the first conformation, ET to the flavin is possible within the intrinsic flavin fluorescence decay time, in the other conformation ET is extremely unlikely due to the large distance between Trp214 and flavin.

A classical MD simulation was performed at a fixed temperature (300K) to investigate the dynamics of the flexible loop without accelerating conformational changes as in the REMD simulations presented above. Fig. 5B shows the closest distance between the rings of the flavin and of Trp214 during the 500-ns trajectory. This distance varies dramatically over this timespan, also fully consistent with the proposed flexibility and macroscopic disorder of the loop. However, the exchange between different configurations occurs mostly on timescales $> 10\text{ ns}$ (spectral density $< 0.1\text{ ns}^{-1}$, Fig. 5C), well beyond the window of fluorescence decay. This implies that the configurations, although heterogeneous, are quasi-stable on the 2-ns

experimental window of the fluorescence decay. Therefore, the simulations are in agreement with the experimental observations of heterogeneous and strictly temperature-independent fluorescence decay kinetics.

4. Concluding remarks

Altogether, the present work has explored the role of potential quenchers of flavin fluorescence in the nucleic-acid transforming enzyme TrmFO from *T. thermophilus*. We show that in the presence of Tyr343, this residue, which closely interacts with the flavin, is virtually the sole effective electron donor to photoexcited flavin. In the absence of this tyrosine further-lying residues may quench ET, leading consistently to overall slower and also remarkably multiphasic kinetics. The latter property has also been previously observed in the flavoenzyme thymidylate synthase ThyX that, similarly to TrmFO, has to accommodate the relatively large substrates folate, NADPH and nucleotide/nucleic acid [18]. As in ThyX the multiphasic kinetics could be quantitatively analyzed in terms of rapidly (<2 ns) exchanging ET donor-acceptor configurations, in TrmFO we focused on the identified Trp214 ET donor, located on a flexible loop. As discussed above, the properties of the decay kinetics imply that the Trp214-flavin pairs, although heterogeneous, do not rapidly exchange configurations. Subsequent molecular dynamics simulations indeed indicate that the region containing Trp214 is highly flexible, as suggested by the crystal structure [25], but can be considered quasi-static on the timescale of fluorescence decay. This regime of conformational fluctuation may be functionally relevant as it has been suggested that the rearrangement of this loop is involved in tRNA substrate binding [25]. The comparison between the ThyX and TrmFO protein systems illustrates the power of the employed photochemical technique to experimentally discern regimes of protein dynamics, even in non-naturally photoactive flavoproteins. In future work, we foresee to further explore

the role in the quenching dynamics of TrmFO of other potential quenchers, including Trp283, which has been suggested to interact with the tRNA substrate [23,25].

5. Declarations

Conflicts of interest/Competing interests Not applicable

Availability of data and material Not applicable

Code availability Not applicable

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Figure captions

Fig. 1 Close-up of the structure of TrmFO from *T. thermophilus* (PDB ID 3G5Q). Residues critical to this work are shown in orange. The red spring represents the loop consisting of residues 208-222 that is not resolved in the crystal structure

Fig. 2 Photochemical properties of C51A, C51A/W283F and C51A/W214F TrmFO. A: ground state spectra. B: fluorescence decay at 520 nm. C: species-associated spectra of the ~3 ps decay phase in transient absorption. D: transient absorption at 500 nm. All panels are normalized. Color coding is the same for all panels

Fig. 3 Effect of mutation of Trp214 to Phe on the fluorescence decay at the 520-nm emission maximum of C51A/Y343F TrmFO. Time scale is linear up to 10 ps and logarithmic thereafter

Fig. 4 Temperature dependence of fluorescence decay of C51A/Y343F TrmFO. Time scale is linear up to 10 ps and logarithmic thereafter

Fig. 5 MD simulations of C51A TrmFO. A: two of the configurations of the loop containing Trp214 observed during 300-330K REMD simulations. FAD and Trp214 are shown as lines; B: The shortest distance between atoms of the Trp214 and isoalloxazine ring systems during a 500-ns MD simulation performed at 300K. C: Fourier transform spectrum of the distance in B