Photoswitching Behavior of Flavin–Inhibitor Complex in a Nonphotocatalytic Flavoenzyme

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Photo-Switching Behavior of Flavin–Inhibitor Complex in a Non-Photocatalytic Flavoenzyme

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ABSTRACT: An unprecedented photo-switching phenomenon of flavin–inhibitor complexes in a flavoenzyme was revealed by femtosecond transient absorption spectroscopy. The vast majority of flavoenzymes, including monomeric sarcosine oxidase (MSOX), perform non-light-driven physiological functions. Yet, the participation of flavin cofactors in photoinduced electron transfer reactions is widespread. MSOX catalyzes the oxidative demethylation of sarcosine; methylthioacetate (MTA) is a substrate analog inhibitor that forms a complex with MSOX exhibiting intense absorption bands over the whole visible range due to flavin–MTA charge transfer (CT) interactions. Here, we demonstrate that upon excitation, these CT interactions vanish during a barrierless high quantum yield reaction in ~300 fs. The initial complex subsequently geminately reforms in a few nanoseconds near room temperature in a thermally activated way with an activation energy of 28 kJ/mol. We attribute this hitherto undocumented process to a well-defined photoinduced isomerization of MTA in the active site, as corroborated by experiments with the heavier ligand methylselenoacetate. Photoisomerization phenomena involving CT transitions may be further explored in photocatalytic and photo-switching applications of flavoenzymes.

Flavin cofactors are found in a large variety of enzymes and photoreceptors. They are accommodated in proteins essentially in the form of flavin adenine dinucleotide (FAD) or flavin mononucleotide, with the isoalloxazine moiety (Fig. 1a) acting as chromophore and displaying highly versatile redox and protonation properties.[1–4] The vast majority of flavoproteins perform non-light-driven physiological functions ("non-photoactive"). Yet some display natural photo-activity based on photoinduced electron transfer (ET) or triplet formation. In particular this is the case in the photoenzymes DNA photolyase and fatty acid photodecarboxylase, as well as blue-light photoreceptors. Investigation and modification of these systems for photocatalytic and photo-switching applications is blooming.[5–8]

In "non-photoactive" flavoproteins, photoreactions also frequently occur. The singlet excited state of the resting oxidized flavin state is often quenched by photoinduced ET from nearby aromatic residues with time constants down to hundreds of femtoseconds, followed by charge recombination usually on the picosecond timescale.[9–13] These processes do not accumulate any photoproducts but rather avoid them (i.e., photoprotective self-quenching),[14,15] thus thermally dissipating the photon energy. These systems are therefore not very suitable as templates for bioengineering new photocatalysts or photo-switches based on the oxidized flavins. However, a recently emerging approach is to employ fully reduced flavins,[16,17] whose excited states are not quenched by nearby residues, as inspired by the native photocatalytic process in DNA photolyase. The photochemistry of yet other flavin forms, including charge-transfer (CT) complexes, may also await practical applications, but is largely unexplored.

The well-documented ability of flavin cofactors to establish CT interactions with a variety of external ligands[18,19] may have functional roles, such as regulating the midpoint potentials[20] or stabilizing small ligands in the active site.[18] Many flavoproteins also involve catalytic intermediates with CT characteristics.[21–23] Investigating the photophysical properties of flavin CT complexes can be informative on the interactions of the involved substrates or inhibitors with flavin.[19,24] Moreover, flavin CT complexes usually exhibit significantly red-extended absorption bands that cover the whole visible range and in some cases extend to the near-infrared.[20,25] This property may contribute to biotechnological applications that require long-wavelength excitation.[26,27] In this broader perspective, we present a first full-spectral investigation on the photochemical processes of a flavin–inhibitor CT complex in a flavoenzyme, monomeric sarcosine oxidase (MSOX).

MSOX is a bacterial flavoenzyme containing a covalently bound FAD cofactor, with the oxidized form (FAD_{ox}) being the resting state.[28] It catalyzes the oxidative demethylation of sarcosine (*N*-methylglycine), a process that does not involve light absorption.[25,29] In MSOX, FAD_{ox} forms CT complexes with various inhibitors, including the sarcosine analog methylthioacetate (MTA, Fig. 1a).[25,30] Binding of MTA induces an intense new absorption band centered at 532 nm and extending to ~700 nm (Fig. 1b). This band is presuma-

bly due to interactions between the MTA S atom and the isoalloxazine ring (Fig. 1c),[31,32] leading to charge delocalization from the ligand to FAD_{ox}.[33]

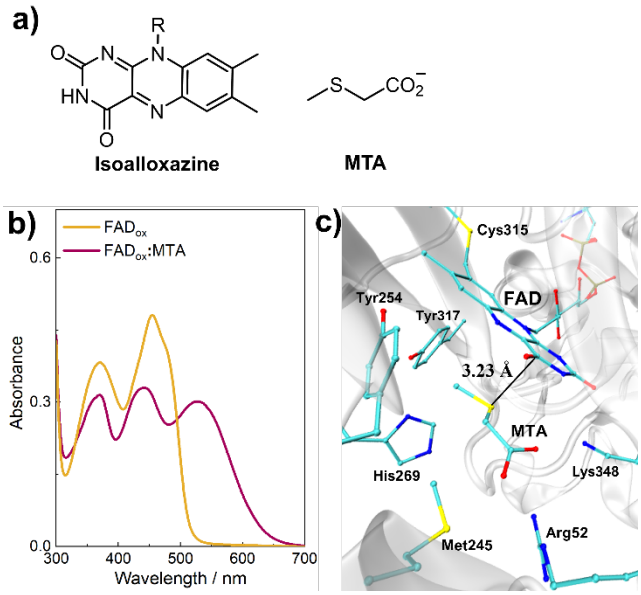

Figure 1. (a) Chemical structures of the flavin ring and MTA. (b) Steady-state absorption spectra of FAD_{ox} and the FAD_{ox}:MTA complex in MSOX. The complex spectrum is corrected by removing the contribution from FAD_{ox} (~15%; see also Fig. S1). (c) Active site of MSOX from *Bacillus sp.* in complex with MTA (PDB entry: 1EL9).

The excited-state properties of the FAD_{ox}:MTA complex were investigated using femtosecond transient absorption (TA) spectroscopy, with green-light excitation pulses (maxima at 520 nm or 560 nm) to avoid exciting the small fraction of uncomplexed FAD_{ox} (*SI* Sections 1, 2). On the picosecond timescale, we observed profound ground-state bleaching (GSB) signals at ~520 nm, as well as marked positive bands at ~400 and ~470 nm suggesting photoproduct formation (Fig. 2a). These induced absorption features are similar to those in the ground state absorption spectrum of FAD_{ox} (*cf.*, Fig. 2a, *inset*, and Fig. 1b). Global analysis reveals that two distinct kinetic components, with time constants of 300 fs and 2.5 ns at 10 °C, are required to fit the data. The results (Fig. 2b) are depicted in terms of evolution associated spectra (EAS), which correspond to intermediate states assuming a sequential reaction scheme. The initially populated state is characterized by a pronounced induced absorption band at ~400 nm and GSB in the 430–610 nm range. Remarkably, the EAS of the subsequent state populated in 300 fs is almost identical to the FAD_{ox} minus FAD_{ox}:MTA difference spectrum (Fig. 2c). This indicates that upon initial population of the excited state in the Franck Condon (FC) region, the CT complex effectively dissociates in 300 fs, leading to the population of uncomplexed FAD_{ox}. We determined the quantum yield (*QY*) of the dissociation to be ~80% (*SI* Section 3). The CT complex reforms on a nanosecond timescale, and there is no other spectral evolution prior to or during this recovery. The photochemical processes of the CT complex are very distinct from those of uncomplexed FAD_{ox} in MSOX: here, the excited state predominantly decays in 200 fs (91%) by photoinduced ET from a nearby tyrosine residue (likely Tyr317), the self-quenching phenomenon typically observed in "non-photoactive" flavoproteins (*SI* Section 4).

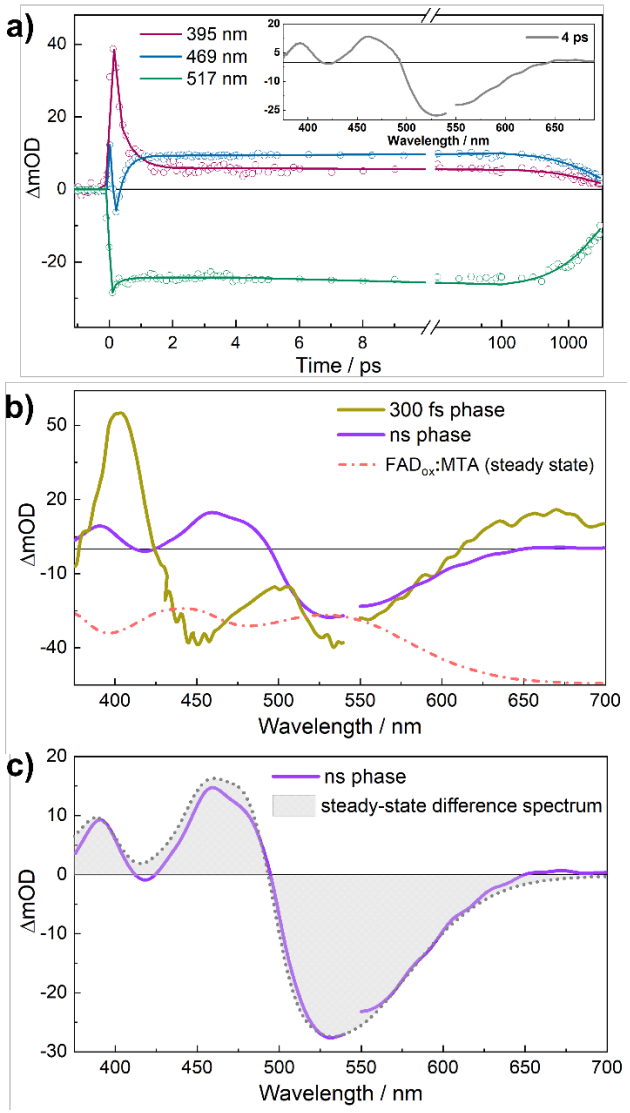

Figure 2. (a) TA kinetics of the FAD_{ox}:MTA complex at selected wavelengths upon excitation at 560 nm. The inset shows the transient spectrum recorded at 4 ps after the excitation. (b) EAS obtained from global analysis. The spectra at < 550 nm and > 550 nm were obtained under 560-nm and 520-nm excitation, respectively. The steady-state absorption spectrum of the FAD_{ox}:MTA complex is also shown. (c) Comparison of the ns-phase EAS with the steady-state difference spectrum that corresponds to dissociation of the FAD_{ox}:MTA complex. The spectra are normalized on the minima of the bleaching bands.

We then investigated the effect of temperature on the involved processes in the range from 5 to 30 °C. There is no noticeable temperature dependence on the sub-picosecond phase, consistent with a barrierless excited-state relaxation.

In contrast, the rate of the long-lived phase markedly increases with rising temperature (Fig. 3a and Table S1), corresponding to an activation energy of 28 ± 4 kJ/mol (Fig. 3b). Extrapolation of the Arrhenius plot suggests that at 77 K the complex should remain in the dissociated form (> ~100 hours). Indeed, as shown in Fig. 3c, we observed that when the pinkish FAD_{ox}:MTA complex immersed in liquid N_2, was illuminated with green light, it quasi-permanently switched color to yellow (uncomplexed FAD_{ox}).

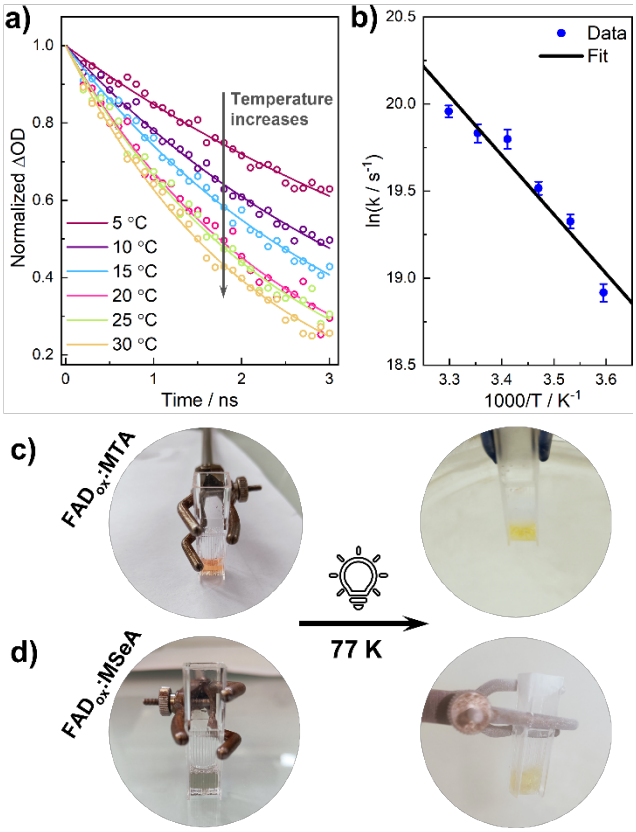

Figure 3. (a) Temperature dependence of the ps–ns decay kinetics at 456 nm of the FAD_{ox}:MTA complex. Data were fitted with single-exponential decays. (b) Arrhenius plot for the reformation of the FAD_{ox}:MTA complex. The fit corresponds to an activation energy of 28 kJ/mol. Error bars correspond to ±2σ. (c, d) FAD_{ox}:MTA and FAD_{ox}:MSeA complex before (*left*) and after (*right*) continuous light illumination at 77 K.

MTA accommodation in MSOX from solution to form the CT complex occurs in a few milliseconds at 5°C.[30] Yet, at near-physiological temperatures, the recovery of the complex upon photodissociation occurs on the nanosecond timescale. Therefore, throughout the photoinduced processes, MTA must remain in the protein. Considering the modest energy barrier (~28 kJ/mol), the most likely explanation for our observations is that, upon excitation, MTA undergoes a conformational change in the active site, so that the strong noncovalent interactions between the S atom of MTA and the flavin ring are abolished, resulting in the GSB of the CT complex and appearance of a pure FAD_{ox} spectrum.

In addition to MTA, MSOX can also bind methylselenoacetate (MSeA) and methyltelluroacetate (MTeA) by CT complexation, where the S atom of MTA is replaced by Se and Te, respectively.[25] The corresponding absorption bands are red-shifted compared with that of the FAD_{ox}:MTA complex (Te > Se > S), in agreement with CT interactions between the X atom (X = S, Se, Te) and the flavin ring. Interestingly, in the crystal structures, MSeA and MTeA both bind to MSOX in two discrete conformations (Fig. 4 shows those of MSeA; those of MTeA are similar).[25] In one conformation, the coplanar atoms C1–C2–X3–C4 lie nearly parallel to the flavin ring and superimpose well on the MTA structure (Fig. S7), where the X3 atom is located very close to the flavin (3.32 Å to flavin C4 atom; *X3-in* conformer). In the alternative conformation, the C2–X3–C4 plane is rotated ~70°, with the X3 atom moving away from the flavin (4.81 Å to flavin C4 atom; *X3-out* conformer), with the remaining inhibitor atoms essentially staying at the same positions. Given the structural similarity between MTA, MSeA and MTeA, we propose that MTA can also adopt the two discrete conformations (*X3-in* and *X3-out*) in MSOX. It predominantly adopts the *X3-in* conformation in the steady state; absorbing a photon causes MTA to switch from the *X3-in* to the *X3-out* conformation, via a crossing region on the potential energy surfaces of the excited state and the *X3-out* ground state. It then reconverts to the *X3-in* conformation by a thermally activated transition. The proposed reaction pathways are depicted in Fig. 5, along with the involved energetics and time constants. In this scheme, the reaction coordinate thus involves isomerization of the MTA ligand.

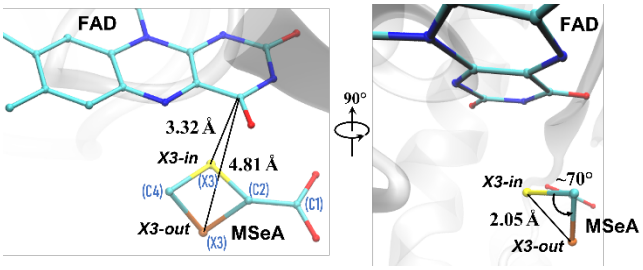

Figure 4. Two discrete conformations of MSeA (*X3-in* and *X3-out*) in the active site of MSOX (PDB entry: 1EL8).

We hypothesized that similar photo-switching also takes place in the FAD_{ox}:MSeA and FAD_{ox}:MTeA complexes. Fig. 3d shows that this is indeed the case at 77K for the FAD_{ox}:MSeA complex. Whereas MTA can efficiently flip back to the *X3-in* conformation in a few nanoseconds near room temperature (*RT*), after photo-switching the larger and heavier Se and Te atoms may induce more steric effects to trap MSeA and MTeA in the *X3-out* conformation. Preliminary TA experiments (*SI* Section 7) near *RT* demonstrate similar ~300-fs photodissociation of MSeA and indeed as expected markedly slower recovery of the initial state (our time window presently hinders precise determination of the activation barrier for MSeA). Furthermore, the broader and more intense absorption bands (Fig. S8)[25] of the FAD_{ox}:MSeA and FAD_{ox}:MTeA complexes presumably facilitate photo-switching. These two factors likely explain that in the (100 K) crystal structures of MSOX complexed with MSeA and MTeA,

two distinct conformations are discerned but in that complexed with MTA only one.[25]

In summary, we have investigated the excited-state and photoproduct properties of flavin CT complexes in MSOX. We show that upon green-light excitation, the FAD_{ox}:MTA complex dissociates (i.e., loses the CT interactions) in a barrierless way in 300 fs following the relaxation of the excited state, and recombines (recovers the CT interactions) on nanosecond timescales near physiological temperatures. Similar phenomenon was also observed for blue-light excitation although here the spectral and kinetic characterization are complicated by a small FAD_{ox} fraction in the sample (*SI* Section 8). We make a strong case that these processes involve switching between two isomers by the movement of the X atom. Our results also provide direct evidence demonstrating that upon excitation of these CT complexes, the charge displaces from flavin to the ligands, the opposite direction to the CT in the ground state of the complexes. This assessment is distinct from many other cases where exciting a CT band is believed to lead to complete charge separation,[24,34,35] in the same direction as the ground-state charge delocalization. Furthermore, although mostly occurring as the resting form in flavoproteins, here FAD_{ox} is involved as an intermediate with nanosecond lifetimes. An interesting parallel can be made with the recently reported ultrafast photooxidation of protein-bound $FAD^{\bullet-}$, where FAD_{ox} is also transiently formed following the photoexcitation.[36]

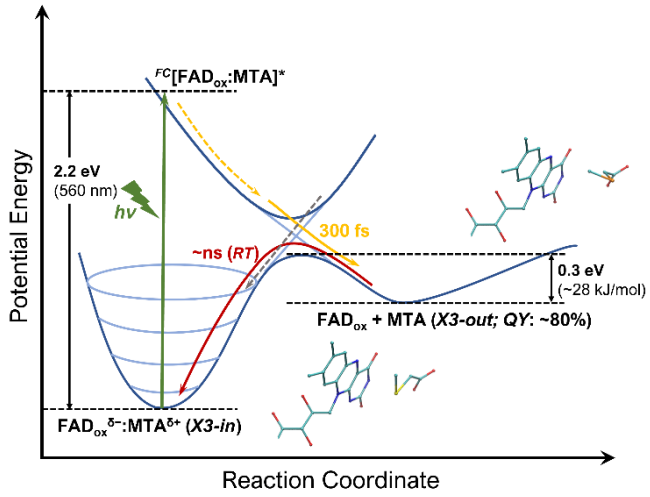

Figure 5. Proposed reaction pathways following the excitation of the FAD_{ox}:MTA complex in MSOX.

In this work, we have uncovered a hitherto undocumented photochemical process in flavoproteins. In particular, our results strongly suggest a novel photoisomerization reaction, not of the chromophore itself, but of electronically interacting ligands non-covalently bound to the protein. This process thus expands the repertoire of photoisomerization reactions in proteins that include retinal and coumarin-containing proteins and more generally that of protein-based photo-switches. We anticipate that our findings will open a new avenue for the exploration of flavin photochemistry with ultimately possible practical implications as photo-switches and optogenetic tools.

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available free of charge on the ACS Publications website.

Experimental methods; composition of the MTA containing MSOX sample; quantum yield of the photoinduced dissociation of the FAD_{ox}:MTA complex; photoinduced processes of uncomplexed FAD_{ox} in MSOX; effect of temperature on the rates of the complex reformation; steady-state properties of the FAD_{ox}:MSeA and FAD_{ox}:MTeA complexes; photoinduced processes of the FAD_{ox}:MSeA complex; transient absorption measurements under 390-nm excitation

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Notes

The authors declare no competing financial interest.

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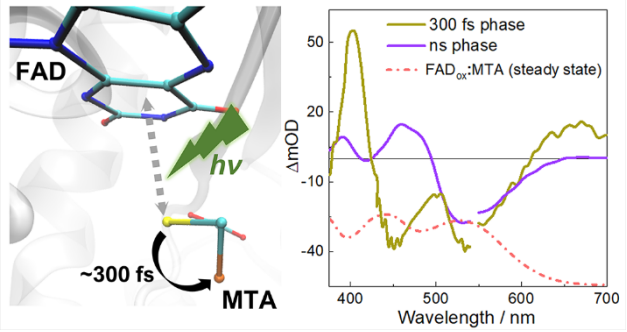

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