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Heterogeneous subunit structures in the pyranose 2-oxidase homotetramer revealed by theoretical analysis of the rates of photoinduced electron transfer from a tryptophan to the excited flavin

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ABSTRACT

Pyranose 2-oxidase (P2O) from *Trametes multicolor* forms a homotetramer in which each of the subunits contains flavin adenine dinucleotide (FAD). The fluorescence of P2O decays with two lifetime components; a slow (358 ps) and a fast (~90 fs) decay. The lifetime of the fast component is emission-wavelength dependent and is ascribed to fast photoinduced electron transfer (ET) from Trp168 to the excited isoalloxazine (Iso*) in FAD. The donor–acceptor distances were ~0.7 nm. The extraordinary heterogeneous decays were analyzed with atomic coordinates obtained by a molecular dynamics simulation and the ET rate by Kakitani and Mataga. The emission-wavelength dependent decays in the fast component were elucidated by introducing emission-wavelength dependent standard free energy related to electron affinity of Iso*. Examination of all possible combinations of the four subunits revealed that the slow component was from subunit A and the fast component was from the other three subunits. Agreements between the observed and calculated decays were all excellent. The large difference in the fast and slow fluorescent lifetimes is ascribed to the difference in the standard free energy gap related to electron affinity of Iso*. The dependence of the logarithmic ET rates on the center-to-center distance displayed approximate linear functions (Dutton rule) when the rate was relatively slow and parabolic functions when the rate was ultrafast. The Dutton rule originated from the exponential term of the ET rate, not from the electronic coupling term.

1. Introduction

Pyranose 2-oxidase from *Trametes multicolor* (P2O) forms a homotetramer in which each subunit contains flavin adenine dinucleotide (FAD) as a coenzyme with a molecular weight (Mw) of 68 kDa [1]. In nature, P2O is distributed in the hyphal periplasmic space of wood-degrading basidiomycetes, [2] and catalyzes oxidative degradation of lignin to produce hydrogen peroxide (H_2O_2) [3]. Each FAD is covalently linked to the respective P2O subunit His167 residue via the flavin 8α-methyl group [4]. Several crystal structures of P2O have been determined, [5–7] and the reaction mechanisms of P2O have been investigated by transient kinetics, kinetic isotope effects and site-directed mutagenesis [8–11].

It was recently demonstrated that the ultrafast fluorescence dynamics of P2O displays two transient fluorescence spectra with different lifetimes of a mean of 88 fs and 358 ps in the wild type (WT) P2O [12]. The shorter lifetime fluorescence displayed a time-dependent spectra with a peak emission at ca. 540 nm, while the longer lifetime displayed a time-independent spectrum at ca. 510 nm. The ultrashort lifetimes were ascribed to the photoinduced electron transfer (ET) from Trp168 to the excited isoalloxazine (Iso*) [12]. The long lifetime emission peak was similar to that obtained by steady-state excitation, suggesting that the fluorescence spectrum under steady-state excitation is likely to be derived from the same source as the long lifetime fluorescence. The characteristics of the transient behaviour of the fluorescence from the WT P2O are similar to the H167A and T169S single substitution isoforms of P2O with and without an acetate ligand [13]. The conformational heterogeneities in the WT and point mutated P2Os

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were previously elucidated using the ET parameters contained in a theoretical ET rate given by Kakitani and Mataga (KM) theory, [14–17] and using the atomic coordinates with crystal structures [5–7]. The geometrical factors, such as the donor–acceptor distance and the H-bond distances, however, were found to be almost identical among the four subunits in the crystal structures. Hence, the heterogeneous conformations of P2O were elucidated in terms of the static dielectric constant of the entire protein.

It is very important to clarify whether the geometrical factors that are influential upon the ET rate are really uniform among the four subunits in solution. Structural heterogeneity among the four subunits of P2O was theoretically demonstrated in solution by molecular dynamics simulation (MDS) [18]. In the present work the experimental fluorescence dynamics of P2O were analyzed with MDS atomic coordinates and KM ET rate, and demonstrated subunit-based heterogeneity of the ET rates in the WT P2O.

2. Method of analyses

2.1. Molecular dynamics simulation

Details of MDS calculation were described in the previous work [18]. The initial WT P2O structure (pdb code: 2IGK) was retrieved from the Protein Data Bank [Research Collaboratory for Structural Bioinformatics (RCSB)]. Subsequently, all missing atoms were added using the Discovery Studio 2.0 software (Website, www.accelrys.com). Then, all missing hydrogen atoms were added using the LEaP module of the Amber version 10 suite package [19].

The force field parameters of FAD were taken from previous work [20]. The MD simulation was performed by using Amber10 and the amber03 force field was employed [19]. The geometrical crash of hydrogen atoms were removed by minimizing with 2000 steps of the steepest decent (SD) algorithm, followed by 3000 steps of conjugate gradient (CG) algorithm. The TIP3P water model was used as solvent. P2O protein was solvated by 63000 TIP3P water molecules, extending at the distance of 10 Å in each of the six directions: ($\pm x$, $\pm y$, and $\pm z$) around the P2O protein, result in the cubic box with the length of $147 \times 129 \times 140$ Å. Subsequently, appropriate counter ions were added into the simulation box to neutralize the charged residues. The secondary minimization of the whole system (protein, solvent and counter ion) was carried out with 2000 steps of SD and 3000 steps of CG minimization, respectively. The MD simulation was controlled under constant temperature and pressure ensemble (*NPT*) with a constant pressure of 1 atm and constant temperature of 298 K. The Berendsen thermostat was applied to control the temperature and the SHAKE algorithm [21] was employed to constrain all bonds involving hydrogen atoms throughout MD simulation. The long-range electrostatic interaction was described by a particle mesh Ewald approach with a spherical cutoff of 10.0 Å [22]. The MD simulations were calculated for 20 ns with a 0.002 ps time interval. The equilibration of the whole MD simulations system was observed by the global root of mean square deviation (RMSD). The MD simulations coordinates were collected from 15 ns to 20 ns of the production time with a time interval of 0.2 ps. A total of 25000 snapshots were used for the present analysis.

2.2. ET rate

The original Marcus theory [23,24] has been modified in various ways [14–17,25]. In the present analysis, KM theory [14–17] was used because it is applicable for both non-adiabatic and adiabatic ET processes, and has been found to give satisfactory results for both static ET analysis with fluorescence lifetimes [13,26–28] and dynamic ET analyses with fluorescence decays of flavoproteins [29–33]. The observed fluorescence decays in P2O were non-exponential, and expressed as two-exponential decay functions [12]. The fast component, with a lifetime of ~88 fs was emission-wavelength (λ) dependent, while the slow component, with a lifetime of 358 ps, was emission-wavelength independent. The emission-wavelength dependent ET rates for the fast component described by the KM model for P2O were expressed by Eq. (1).

$$k_{ij}^{f} = \frac{\nu_0^f}{1+\exp\left\{\beta^f\left(R_i - R_0^f\right)\right\}}\sqrt{\frac{k_B T}{4\pi\lambda_S^{if}}}\exp \left[-\frac{\left\{\Delta G_{fj}^0 - e^2/\varepsilon_{DA}^f R_i + \lambda_S^{if} + E_{Net}^{if}\right\}^2}{4\lambda_S^{if} k_B T}\right] \quad (1)$$

The P2O monomer contains 9 tryptophan (Trp) and 15 tyrosine (Tyr) residues. In the present work the ET rates only from Trp168 were taken into account among these aromatic amino acids, because the Trp168–Iso distance is within 0.8 nm in all subunits, while those in the other aromatic amino acids were longer than 1.2 nm (Table S1, Supporting information (SI)) [12]. In Eq. (1),k_{ij}^f is the ET rate of the fast component from Trp168 to the Iso* in subunit i at the emission wavelength j, ν_0^f is an adiabatic frequency and β^f is the ET process coefficient of the fast component. R_i and R_0^f are the Trp168–Iso distances in subunit i and its critical distance for the ET process of the fast component, respectively. Note that R_i is expressed as a center-to-center distance (R_c) rather than as an edge-to-edge (R_e) distance [13,26–33]. The ET process is adiabatic when $R_i \le R_0^f$, and non-adiabatic when $R_i > R_0^f$. The term $-e^2/\varepsilon_{DA}^f R_i$ in Eq. (1) is the electrostatic energy (ES) between the Iso anion and a donor cation (ESDA), where ε_{DA}^f is the static dielectric constant between Trp168 and Iso in the fast component. The terms k_B, T, and e are the Boltzmann constant, temperature and electron charge, respectively. E_{Net}^{if} is the net ES energy (Net*ES*) of Trp168 in subunit i for the fast component, which is described later. λ_S^{if} is the solvent reorganization energy [23,24] of the ET donor in subunit i for the fast component, and is expressed in Eq. (2).

$$\lambda_S^{if} = e^2\left(\frac{1}{2a_{Iso}} + \frac{1}{2a_{Trp}} - \frac{1}{R_i}\right)\left(\frac{1}{\varepsilon_\infty} - \frac{1}{\varepsilon_{DA}^f}\right), \quad (2)$$

where a_{Iso} and a_{Trp} are the radii of Iso and Trp and ε_∞ is optical dielectric constant. In this study the previously determined [13,26–33] values of Iso (a_{Iso}) and Trp (a_{Trp}) of 0.224 and 0.196 nm, respectively, were used, which assumes that they are spherical, whilst ε_∞ was set at 2.0.

The standard free energy gap for the fast component was expressed with the ionization potential of Trp (E_{IP}) by Eq. (3).

$$\Delta G_{fj}^0 = E_{IP} - G_{fj}^0 \quad (3)$$

Here G_{fj}^0 is the standard Gibbs energy related to the electron affinity of Iso* of the fast component at an emission wavelength j. The experimental value of E_{IP} for Trp is 7.2 eV [34]. It was assumed that the ET rate of the fast component was emission-wavelength dependent through G_{fj}^0, because G_{fj}^0 is related to the electronic energy of Iso*, and further it depends on hydrogen bonds (H-bonds) between Iso* and the surrounding amino acids [27,28].