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Kinetic Isotope Effect of the L-Phenylalanine Oxidase from *Pseudomonas* sp. P-501

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***Pseudomonas* L-phenylalanine oxidase (deaminating and decarboxylating) mainly catalyzes oxygenation when L-phenylalanine is used as the substrate, but oxidation when L-methionine is used as the substrate. Using [C_α-H]-DL-methionine and [C_α-D]-DL-methionine as substrate, the reductive half reaction of FAD cofactor of enzyme has been studied by stopped-flow spectrophotometry. The rate of reduction of FAD cofactor has a kinetic isotope effect (KIE) of 5.4 and 4.1 in the absence and presence of 30% glycerol, respectively. The KIE is independent of temperature, but the rates of the reductive half reaction are dependent on temperature, indicating that thermally induced motion at the active site drives the H-transfer reaction by H-tunneling.**

Key words: flavoprotein, H-tunneling, kinetic isotope effect, reductive half reaction, stopped flow.

Abbreviations: DAO, D-amino acid oxidase; KIE, kinetic isotope effect; MADH, methylamine dehydrogenase; PAO, L-phenylalanine oxidase (deaminating and decarboxylating).

L-Phenylalanine oxidase [EC 1.13.12.9; PAO] from *Pseudomonas* sp. P-501 catalyzes both oxidative deamination and oxygenative decarboxylation (*1–3*). The enzyme is formed as an inactive proenzyme and activated by proteolysis (*4*), and it has 2 mol of noncovalent FAD and consists of 2 mol each of α and β subunits (*3*, *5*). On the basis of spectral and kinetic studies, its reactions with β-thienylalanine (oxygenase substrate) and L-Met (oxidase substrate) are explained by the same scheme (*6*). The scheme is essentially the same as that for D-amino acid oxidase (DAO) (*7*) and tyramine oxidase (8). One of the catalytically important species is the purple intermediate, which consists of the reduced enzyme and an imino acid derived from a substrate (*9*), by analogy with DAO (*10–12*) and lysine monooxygenase (*13*). The scheme can be written as two consecutive reactions:

$$E_{ox} + S \leftrightarrow E_{ox} \cdot S \rightarrow E_{red} \cdot P_{im} \rightarrow E_{red} + P \quad (1)$$

$$E_{red} \cdot P_{im} + O_2 \rightarrow E_{ox} + P \quad (2)$$

where E_{ox} and E_{red} represent the oxidized and reduced forms of the enzyme unit, respectivey, and P_{im} and P represent an imino acid derived from a substrate and a final product, respectively (*6*).

Since the pioneering work on hydrogen (in the form of proton, hydride or hydrogen) tunneling in biological systems near room temperature (*14*), evidence of hydrogen tunneling continues to accumulate (for recent review, see Ref. *15*). Among flavoenzymes, hydrogen tunneling has been reported for monoamine oxidase (*16*), glucose oxidase (*17*), sarcosine oxidase (*18*), trimethylamine dehydrogenase (*19*), morphine reductase (*20*), and pentaerythritol tetranitrate reductase (*20*), but not for amino acid oxidase. As described above, the catalytic reaction of PAO can be described by two reactions, the reductive half and the oxidative half reactions. Therefore, PAO is ideally suited to studies of H-transfer during substrate oxidation using the stopped-flow method. The kinetics of C-H bond cleavage is conveniently followed by the absorbance change at 465 nm, since H-transfer occurs concomitantly with the reduction of the bound FAD.

This work reports the deuterium isotope effect on the L-Phe oxidase reaction and on the reductive half reaction at various temperatures. The results are consistent with the C-H bond cleavage of substrate by quantum tunneling from the vibrational ground state of the reactive C-H bond of the substrate.

EXPERIMENTAL PROCEDURES

Materials—[C_α-H]-DL-Phe and [C_α-H]-DL-Met were from Wako Pure Chemical Industry Ltd., and [C_α-D]-DL-Phe and [C_α-D]-DL-Met were from C/D/N Isotopes Inc. The minimum content of D in the deuterium compounds is 98.9%. All other chemicals were obtained from Wako Chemicals. PAO was prepared from *Pseudomonas* sp. P-501 as described (*1*) with minor modification. The purified enzyme was found to be more than 95% pure by SDS-PAGE. Protein concentration was determined by measuring the absorbance at 280 nm using an absorbance coefficient of $A_{1\%/1cm}$ = 19.7 (*2*). The concentration of enzyme unit (*e*) was expressed in terms of the bound FAD, which was determined spectrophotometrically, using an extinction coefficient of 11.2 mM^{-1} cm^{-1} at 465 nm (*2*). Absorption spectra were measured in a double beam spectrophotometer, type V-520-SR or type V-560, from Japan Spectroscopic.

SDS-PAGE—Gel electrophoresis was performed according to Laemmli (*21*). Protein samples were boiled

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for 5 min in 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol and 5% mercaptoethanol, then subjected to electrophoresis.

Assay of Enzyme Activity—The rate of overall reaction was estimated by measuring the consumption of oxygen dissolved in the reaction mixture with a Clark-type oxygen electrode (Yellow Springs Instruments, USA). The reaction mixture contained various concentrations of L-Phe in 20 mM potassium phosphate buffer (pH 8.0) at 25°C. The rate (v) was expressed as the mol oxygen consumed per second at a given concentration of enzyme. The maximum velocity (V) was obtained at the saturating concentration of L-Phe or L-Met. The apparent kinetic parameters, k_{cat} (V/e) and K_m, were calculated from the rates determined at various concentrations of L-Phe or L-Met. The concentration of molecular oxygen was assumed to be 253 μM at 25°C.

Flow Experiments—A flow apparatus equipped with a jacket for temperature control (Applied Photophysics, SX.17MV) was used to determine the rate of reduction of the FAD moiety of the enzyme, monitoring the absorbance change at 465 nm. The dead time of the apparatus was determined to be 2.3 ms by the method of Tonomura *et al*. (*22*).

Anaerobic reduction experiments were performed as follows. Solutions of the oxidized form of enzyme and amino acid substrates were introduced separately into reservoirs in the apparatus. To each solution, 10 nM glucose oxidase, 10 mM glucose and catalytic amounts of catalase were added. The solutions were kept for 1 h at a given temperature. Then the enzyme and substrate solutions were mixed in the flow apparatus, and the absorbance at 465 nm was monitored.

Stopped-flow traces for the reduction experiment were analyzed on the basis of the pseudo-first-order reaction, fitting Eq. 3 by use of the software provided with the apparatus.

$$A_{465} = C\exp(-k_{obs}\,t) + b \qquad (3)$$

where C is a constant related to the initial absorbance change, k_{obs} the observed rate constant for flavin reduction, and b an offset value to account for a non-zero base line.

RESULTS AND DISCUSSION

Early studies of H-tunneling in enzyme reactions were analyzed in terms of the Arrhenius plot (*e.g.*, *23*). But, in the methylamine dehydrogenase reaction, however, the substrate C-H bond cleavage was shown to occur by tunneling in terms of the Eyring plot (*24*). The latter seems to be better to apply and can be summarized as follows.

The temperature dependence for a unimolecular rate constant is given by Eq. 4.

$$k = \left(\frac{k_B}{h}\right)T\cdot\exp\left(-\frac{\Delta G^{\ddagger}}{RT}\right) = \left(\frac{k_B}{h}\right)T\cdot\exp\left(-\frac{\Delta H^{\ddagger}}{RT}\right)\cdot\exp\left(\frac{\Delta S^{\ddagger}}{R}\right) \qquad (4)$$

where k is the rate constant, T is the temperature in K, k_B is the Boltzman constant, h is Planck's constant, R is the gas constant, $\Delta G^{\ddagger}$ is the activation free energy, $\Delta H^{\ddagger}$ is the activation enthalpy, and $\Delta S^{\ddagger}$ the activation entropy. The temperature dependence of the rates of the methylamine dehydrogenase reaction is analyzed by the plot of Eq. 5 (Eyring plot).

$$\ln\left(\frac{k}{T}\right) = \ln\left(\frac{k_B}{h}\right) - \left(\frac{\Delta H^{\ddagger}}{RT}\right) + \left(\frac{\Delta S^{\ddagger}}{R}\right) \qquad (5)$$

The activation parameter $\Delta H^{\ddagger}$ is calculated from the slope of the plot. The criteria for the tunneling were that (i) $\Delta\Delta H^{\ddagger} = \Delta H^{\ddagger D} - \Delta H^{\ddagger H}$ is essentially zero, and (ii) A'^{H}/A'^{D} determined from the intercepts on the ordinate is similar to the KIE. A'^{H}/A'^{D} is a ratio corresponding to an Arrhenius pre-exponential factor ratio A^{H}/A^{D} (*24*). We used an Eyring plot (*23*) to see if H-transfer in the L-Phe oxidase reaction proceeds by quantum tunneling.

Effect of Temperature on the Oxidation of the Deuterium-Labeled L-Phe and L-Met—As only DL-forms of [C_α-D]amino acids were available, we tested whether D-amino acids affect the overall reaction of the enzyme. The data showed that D-Phe and D-Met did not affect the oxidation of L-Phe and L-Met, respectively(data not shown).

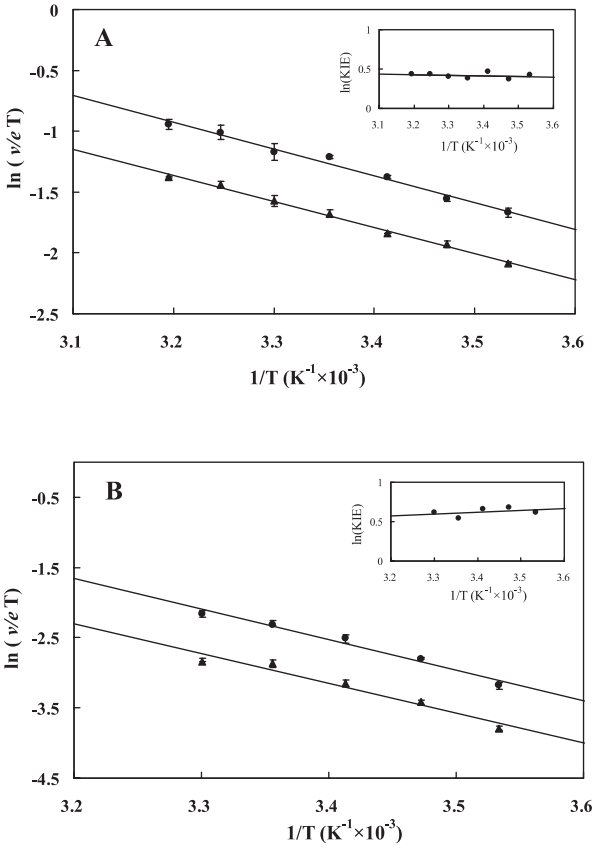

Fig. 1. **Temperature dependence for the rate of overall reaction (v/e) of PAO with L-Phe (A) and L-Met (B).** (A) [C_α-H]-DL-Phe (solid circles) or [C_α-D]-DL-Phe (solid triangles) was used as substrate at the concentration of 0.5 mM as L-form. $\Delta H^{\ddagger H}$ = 18.3 kJ/mol, $\Delta H^{\ddagger D}$ = 17.8 kJ/mol and A'^{H}/A'^{D} = 1.9 were obtained from the plot. Inset, plot of ln(KIE) *versus* 1/*T*. (B) [C_α-H]-DL-Met (solid circles) or [C_α-D]-DL-Met (solid triangles) was used as substrate at the concentration of 4.34 mM as L-form. $\Delta H^{\ddagger H}$ = 36.5 kJ/mol, $\Delta H^{\ddagger D}$ = 35.4 kJ/mol and A'^{H}/A'^{D} = 2.9 were obtained from the plot. Inset, plot of ln(KIE) *versus* 1/*T*.

Table 1. **Kinetic isotope effect and activation parameters for the reaction of PAO.**

Substrate	Reaction	30% glycerol	KIE	A'^{H}/A'^{D}	$\Delta H^{\ddagger}$ (kJ/mol)	
					C_α-^{1}H	C_α-^{2}H
Phe	Overall reaction	–	1.5 ± 0.1	1.9 ± 0.3	18.3 ± 1.1	17.8 ± 0.7
Met	Overall reaction	–	1.9 ± 0.3	2.9 ± 0.5	36.5 ± 2.6	35.4 ± 3.1
	Reductive half reaction	–	5.4 ± 0.3	5.2 ± 0.2	23.5 ± 1.3	23.7 ± 3.1
		+	4.1 ± 0.6	11.5 ± 0.5	33.8 ± 3.2	31.2 ± 3.4

The standard deviations of A'^{H}/A'^{D} and $\Delta H^{\ddagger}$ were determined by the method of Cornish-Bowden (*25*).

Therefore, we used DL-[C_α-H]Phe and DL-[C_α-D]-Phe, and DL-[C_α-H]Met and DL-[C_α-D]-Met to study the primary deuterium isotope effect on the L-Phe oxidase reaction. In the following, the concentrations of amino acids are expressed as those of L-amino acid, although we actually used DL-amino acid. A preliminary experiment showed that the kinetic parameters obtained at 25°C in 0.1 M potassium phosphate buffer (pH 7.0) were as follows. The apparent V/e was 82.2 s^{-1} and 54.8 s^{-1} for L-[C_α-^{1}H]Phe H]Phe and L-[C_α-D]Phe, respectively, and the apparent K_m was 14.7 μM for L-[C_α-^{1}H]Phe, and 13.5 μM for L-[C_α-D]Phe. The kinetic parameters for Met under the same conditions were determined. The apparent V/e was 49.3 s^{-1} and 26.4 s^{-1} for L-[C_α-H]Met and L-[C_α-D]Met, respectively, and an apparent K_m was 390 μM for L-[α-H]Met, and 440 μM for L-[α-D]Met.

The temperature dependence of the overall rate was determined with [C_α-H]-L-amino acid and [C_α-D]-L-amino acid, and analyzed by Eyring plot (Fig. 1). The kinetic parameters obtained are shown in Table 1. Using $\Delta H^{\ddagger}$ values, we can calculate $\Delta\Delta H^{\ddagger}$ ($=\Delta H^{\ddagger H} - \Delta H^{\ddagger D}$). For L-Phe as substrate, $\Delta\Delta H^{\ddagger}$ and KIE were 0.5 kJ/mol and 1.5, respectively, and A'^{H}/A'^{D} was 1.9. For L-Met as substrate, $\Delta\Delta H^{\ddagger}$ and KIE were 1.1 kJ/mol and 1.9, respectively, and A'^{H}/A'^{D} was 2.7. The low $\Delta\Delta H^{\ddagger}$ and the similar value of A'^{H}/A'^{D} with the KIE values are compatible with the above criteria of quantum tunneling (*24*). But the rates of overall reaction are composed of those of oxidative and reductive (C-H bond cleavage) half reactions. Therefore, the above data are not conclusive but suggest that the quantum tunneling contributes to the C-H bond cleavage reaction. To clarify the point, we determined the rates of the C-H bond cleavage reaction directly.

Kinetics of the Reductive Half Reaction of Enzyme— When the enzyme was mixed with excess amounts of L-Phe or L-Met, the enzyme rapidly changed from yellow to purple (Fig. 2). This color change corresponds to the C_α-H bond cleavage (*6*, *9*). As expected from our previous kinetic results (V/e = 1,850 s^{-1} in Ref. *6*), the reductive half reaction with L-Phe was complete during the dead time (2.3 ms) of the mixing apparatus. Therefore, we used L-Met as substrate, since L-Met is less active.

Figure 3 shows the time course of the absorbance change of enzyme at 465 nm after mixing with 8 mM L-Met in 50 mM potassium phosphate buffer, pH 7.0 and at 10°C. The absorbance decrease was found to be monophasic, with a single rate constant obtained from fits of the data to Eq. 3. Similar experiments were performed at different concentrations of L-Met. The kinetic parameter, $k_{red/max}$ and the dissociation constant of the enzyme-substrate complex (K_d) were determined to be respectively 420 s^{-1} and 420 μM for L-[C_α-H]Met, and 108 s^{-1} and 360 μM for L-[C_α-D]Met.

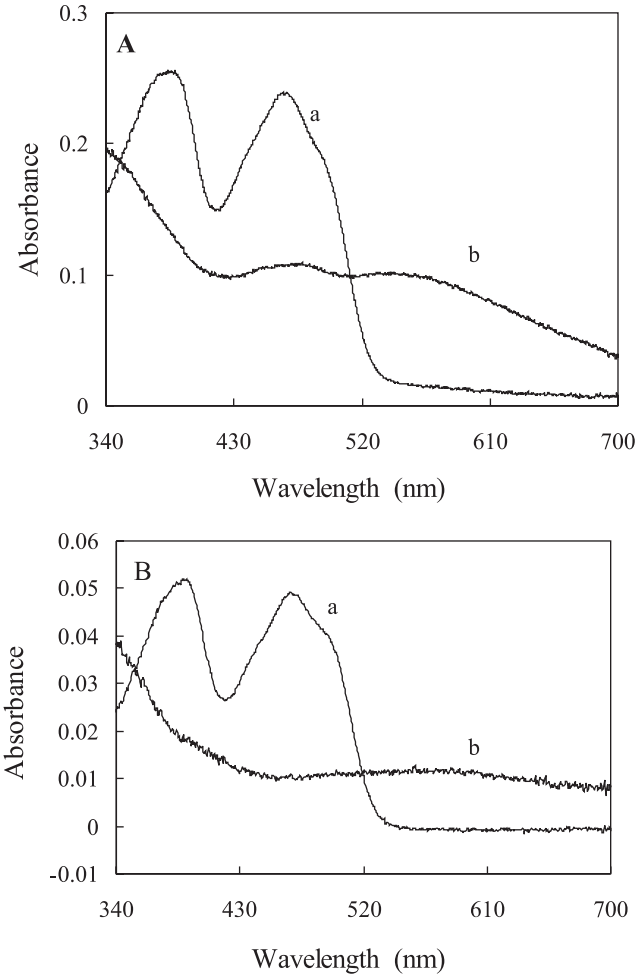

Fig. 2. **Spectral changes of PAO with sufficient amounts of L-Phe (A) and L-Met (B) in 20 mM potassium phosphate (pH 8.0) under aerobic conditions.** Before addition (a), immediately (b) after the addition of L-Phe or L-Met.

Based on the results, we determined the first-order rate constant ($k_{red/max}$) of reduction at 8 mM L-Met (higher than 10 × K_d) and at different temperatures.

Analysis by Eq. 5 of the rates determined for the two substrates revealed that the difference in the enthalpy of activation for protium versus deuterium transfer ($\Delta\Delta H^{\ddagger}$ was –0.2 kJ/mol) is essentially zero (Fig. 4A) and that the KIE is independent of temperature (Fig. 4A, inset). Moreover, the A'^{H}/A'^{D} value (5.2) determined from the intercepts of the plots was similar to the KIE (5.4, Table 1). These results agree with the criteria of activationless

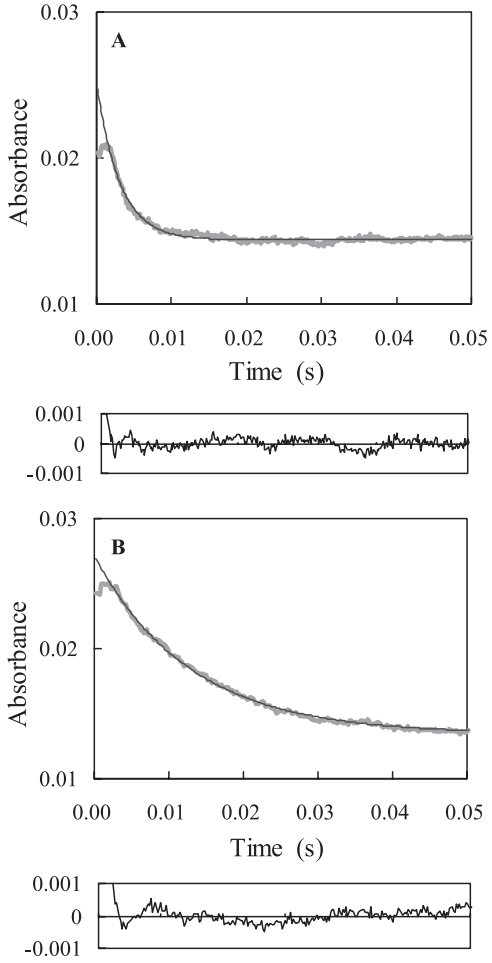

Fig. 3. **Time course of anaerobic reduction of PAO.** Absorbance changes of enzyme in 50 mM potassium phosphate buffer, pH 7.0 were monitored at 465 nm with a stopped-flow spectrophotometer at 10°C. The concentrations of PAO and Met after mixing were 2.6 μM and 8 mM, respectively. A, [C_α-H]-DL-Met. B, [C_α-D]-DL-Met. The thick line represents the data points to the sum of the five measurements and the solid thin line is the fit to the data points by Eq. 3.

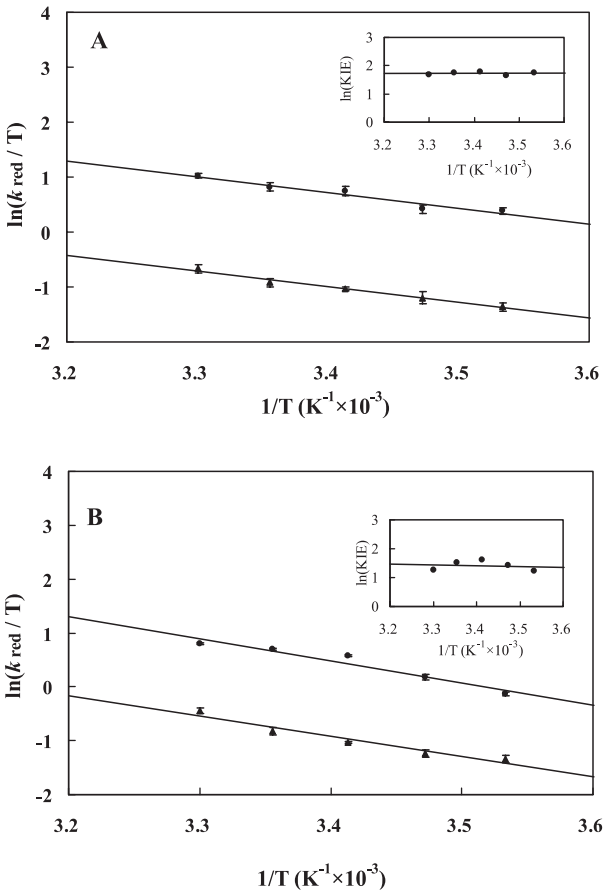

Fig. 4 **Temperature dependence on the rate (k_{red}) of the reductive half reaction of PAO with L-Met.** (A) [C_α-H]-DL-Met (solid circles) or [C_α-D]-DL-Met (solid triangles) was used as substrate in the absence of glycerol. $\Delta H^{\ddagger H}$ = 23.5 kJ/mol, $\Delta H^{\ddagger D}$ = 23.7 kJ/mol and A'^{H}/A'^{D} = 5.2 were obtained from the plot. Inset, plot of ln(KIE) versus 1/*T*. (B) [C_α-H]-DL-Met (solid circles) or [C_α-D]-DL-Met (solid triangles) was used as substrate in the presence of 30% glycerol. $\Delta H^{\ddagger H}$ = 33.8 kJ/mol, $\Delta H^{\ddagger D}$ = 31.2 kJ/mol and A'^{H}/A'^{D} = 11.5 were obtained from the plot. The concentration of [C_α-H]-DL-Met or [C_α-D]-DL-Met was 8 mM as L form. Inset, plot of ln(KIE) versus 1/*T*.

H-tunneling from the substrate ground state. However, the reaction rates are dependent on the temperature (Fig. 4A), as observed with other enzymes (*e.g. 16–20*, *23*), and the activation enthalpy determined was $\Delta H^{\ddagger H}$ = 23.5 kJ/mol and $\Delta H^{\ddagger D}$ = 23.7 kJ/mol. This apparent paradox can be explained theoretically (*24*), as in methylamine dehydrogenase (MADH, *26*). That is, a fluctuating, temperature-dependent, potential energy surface resulting from fluctuations in the PAO protein drives H-abstraction by quantum tunneling. Fluctuations of substrate L-Met do not contribute to the H-transfer reaction, since there is no difference in activation enthalpy between protium and deuterium transfer.

Direct demonstration of the fluctuations in the PAO protein compatible with H-transfer is difficult. An indirect experimental approach to protein fluctuation in H-tunneling was reported for MADH (*26*). The rate of the reductive half reaction of MADH was measured at various temperatures in the presence and absence of 30% glycerol, and KIE and the activation enthalpy were each found to be similar irrespective of the presence or absence of 30% glycerol. From the data, Basran *et al.* (*26*) proposed that quantum transfer, but not nuclear reorganization, is rate-limiting. We performed similar experiments to those for MADH (Fig. 4B). As shown in Table 1, KIE was independent of temperature, but decreased slightly with 30% glycerol and the activation enthalpy increased approx. 10 kJ/mol, corresponding to a bond energy of 1-2 hydrogen bonds. The result is slightly different from those with MADH (*26*).

The effect of solvent viscosity on protein dynamics has been reported (*e.g.*, *27–30*). The active site of proteins, such as myoglobin (*27*), sulfite oxidase (*28*), and horseradish peroxidase (*29*), has dynamic motion, and the motion decreases with increasing solvent viscosity. Molecular dynamic simulations of factor Xa indicated that the solvent viscosity affects the motion of core of the factor (*30*). Our results lead us to propose that the active site residue(s) in $E_{ox} \cdot S$ complex of PAO are in dynamic motion and reorganize temperature-dependently the conformation

of the active site to be compatible with H-transfer. In the presence of 30% glycerol, fluctuations (dynamic motions) of the PAO active site must be hindered, and the higher activation energies must consequently be required to induce an active site conformation compatible with H-tunneling as compared with a conformation without glycerol. Thus, less fluctuation attenuates the H-tunneling and leads to decreased KIE.

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