Modeling an Elementary Step of the Enzyme Pyruvate Oxidase: Oxidation of a Thiamin Diphosphate-Bound Enamine Intermediate by a Flavin Analog

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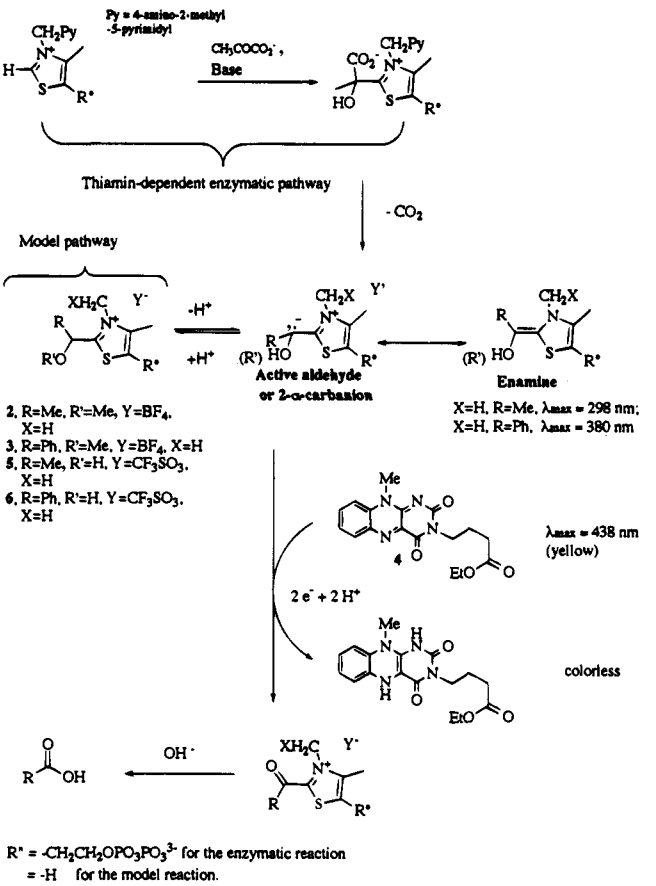
Subsequent to the first successful generation of enamines related to the central intermediate present on all thiamin diphosphate (ThDP)-dependent enzymes,[1] we determined its structure in solution, its mechanism of oxidation at an electrode,[2a] the pK's leading to it both in DMSO[2b] and in water,[2c] and the kinetics of proton transfer to and from the C2α position.[2c] Herein is reported the mechanism of oxidation of the enamine by a flavin adenine dinucleotide (FAD) analog, providing a model for the flavoenzyme pyruvate oxidase (POX), whose product is acetyl phosphate and CO_2.[3,4] Scheme 1 shows the consecutive functions of the two coenzymes in this reaction. While several models already exist for this reaction, none to date has clarified the mechanism of oxidation of the enamine by flavin.[5,6] The recent X-ray structure of POX[7] suggests that the enzyme mediates transfer of the electrons from ThDP to FAD over a distance of 9.5 Å.

The enamine was generated *in situ* by base extraction of the C2α proton from precursors of the active aldehyde **2** or **3** (Scheme 1),[1] so that progress of the oxidation step could be monitored directly without interference from steps prior to enamine formation, as in previous models. Isoalloxazine **4**[8] was selected as an FAD surrogate because of its high solubility in DMF.

Table 1 lists the results of the experiments. While anaerobic treatment of **2** or **3** with 3.0 equiv of $(TMS)_2NNa$ in dry DMSO resulted in the generation of the enamine (A_{298}, $\epsilon_1 = 7500$)[1] with a $t_{1/2}$ of 3 h, upon treatment of **2**-enamine with equiv of **4**, no decrease of A_{437} was observed in 1 h (entries A and B), indicating that no reduction of the isoalloxazine occurred. In DMSO, the redox potential of 3,10-dimethylisoalloxazine is −666 mV *vs.* SCE through one-electron oxidation, which is +497 mV larger than the one-electron redox potential of the enamine generated from **2**,[9] indicating that the oxidation of **2** by FAD is thermodynamically unfavorable. Similar conclusions were reached by others.[6c]

Scheme 1. Mechanism of POX and Model Reactions



R* = -$CH_2CH_2OPO_3PO_3^{3-}$ for the enzymatic reaction
= -H for the model reaction.

It had been reported that the rate of oxidation of mandelic acid methyl ester by isoalloxazine is much faster than that of 2-*O* -methylmandelic acid methyl ester,[10] suggesting that the enamine/2α-carbanion generated from **2** or **3** may not resemble the intermediate in the POX-catalyzed reaction. The reaction was therefore attempted with **5**,[11] bearing an unprotected OH group, previously assumed to decompose rapidly into aldehyde and thiazolium. The enamine generated from **5** by $(TMS)_2NNa$ decomposed with a $t_{1/2}$ of ca. 5 min, but no oxidation by **4** was apparent during this period (Table 1, C).[12] According to the literature, redox reactions of flavin are typically conducted in protic solvents.[13] On replacement of DMSO with 15% *t*-BuOH/DMF the reduction of **4** proceeded to completion within a few minutes (Table 1, D). Earlier, we concluded that the pK_a of **6** is 2 units lower than that of **5**,[2b] therefore the weaker base Et_3N could be used, to rule out possible radical reduction of **4** induced by strong bases, such as $(TMS)_2NNa$ or sodium *tert*-butoxide.[14] A 1000-fold excess of Et_3N was added anaerobically to a solution of **4** with excess **6** in anhydrous 15% *t*-BuOH/DMF, resulting in complete reduction of isoalloxazine in several minutes (Figure 1). The isoalloxazine peak could be quantitatively recovered by bubbling O_2 into the solution.

During optimization of redox conditions, neat DMF was found to be superior to a DMF/*t*-BuOH mixture (Table 1, H). Also, excess Et_3N could be replaced by a small excess of DBU. When 2.0 mM DBU in DMF was used, the concentration of

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Table 1. Oxidation of Enamine by Isoalloxazine[a]

entry	thiazolium	base[b] (no. of equiv)	solvent	reductn of isoalloxazine **4**
A	**2**	TMS amide (3)	DMSO	no reaction
B	**3**	TMS amide (3)	DMSO	no reaction
C	**5**	TMS amide (3)	DMSO	minor reduction
D	**5**	TMS amide (3)	15% *t*-BuOH/DMF	full reduction
E	**5**	Et_3N (1000)	15% *t*-BuOH/DMF	partial reduction
F	**6**	Et_3N (1000)	15% *t*-BuOH/DMF	full reduction
G	**6**	1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (3)	15% *t*-BuOH/DMF	full reduction
H	**6**	DBU (3)	DMF	full reduction

[a] The reactions were performed at 25 °C by mixing a 5-fold molar excess of thiazolium salts with isoalloxazine (0.08 mM) in 2 mL of solvent flushed with Ar. [b] To the mixture was added base (at the concentration indicated compared to that of the thiazolium salt) in one portion followed by vigorous shaking for 1 min.

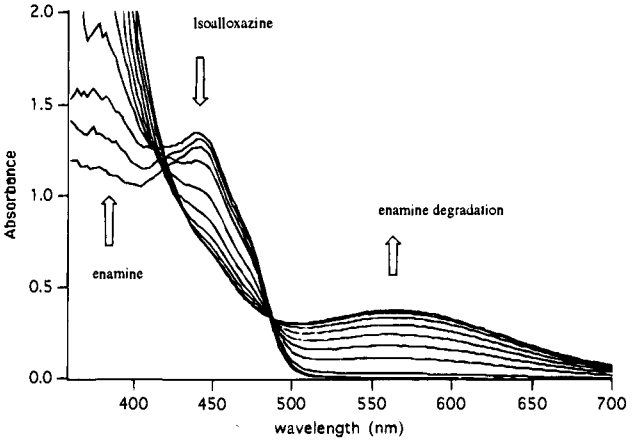

Figure 1. Repetitive-scan stopped-flow spectra recorded (Hi-Tech PQ-SF/SpectraScan instrument) at *ca.* 0.3 s intervals after mixing **4** (0.08 mM) and **6** (0.8 mM) in 15% *tert*-BuOH/DMF (0.25 mL) with Et_3N (0.1 mL) at 25 °C anaerobically.

enamine derived from 0.25 mM **6** reached 90% of its maximum value in less than 1 s and was stable for more than 10 s (Figure 2A). When isoalloxazine at 0.15 mM was present, the A_{449} first increased, then decreased (Figure 2B), enabling us to estimate a minimum value of k_{red}.[15]

We conclude that (1) the enamine, not **5**, is responsible for reduction of isoalloxazine, because base is required in this reaction; (2) the second-order rate constant k_{red} was calculated for the first time as $\geq(6.1 \pm 0.5) \times 10^3\ M^{-1}\ s^{-1}$,[15] *ca.* 750-fold larger than the value we estimate from published data (8.8 $M^{-1}\ s^{-1}$);[6a] (3) a stepwise transfer of electrons, which does not need a proton source, is compatible with the observations, consistent with an electron channel suggested by the X-ray results; (4) the major product of oxidation starting with **6** (and subsequent to alkaline hydrolysis) is benzoic acid according to GC–MS, confirming the intermediacy of 2-benzoylthiazolium ion, which is readily hydrolyzed to benzoic acid and free thiazolium; (5) isoalloxazine was reduced to dihydroisoalloxazine, and the broad band at 560 nm was confirmed to result from degradation of the enamine, rather than from the blue semiquinone;[16] (6) the results also support the claim by Diederich and co-workers that the redox potential of the active aldehyde must be ≤ -500 mV[6c] (more accurately, it should be ≤ -666 mV in DMF).

The ratio of the reported first-order rate constant for the oxidation of enamine by activated POX ($k \geq 413\ s^{-1}$)[17] to the second-order rate constant obtained in our intermolecular model

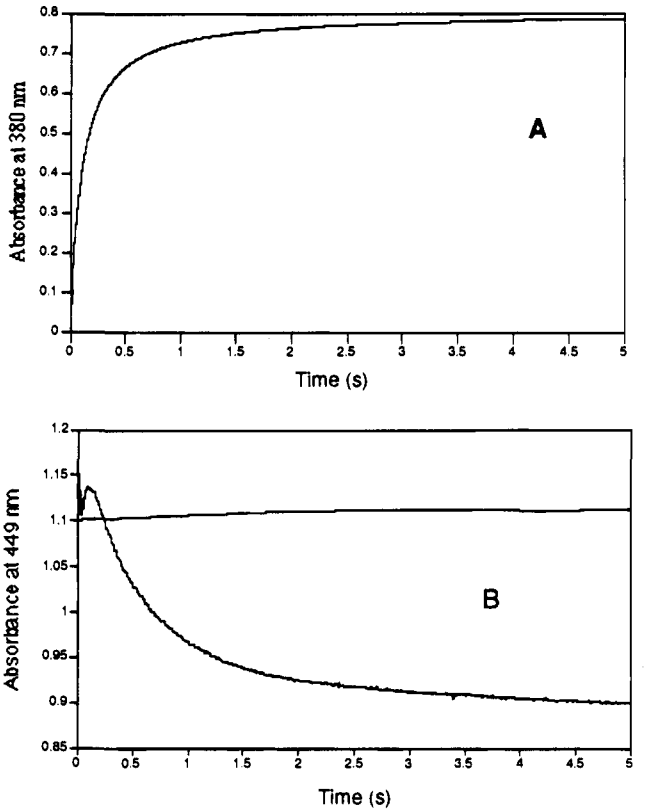

Figure 2. (A) Transient absorbance at 380 nm corresponding to enamine buildup generated from **6** with DBU in the absence of oxidation. (B) Transient absorbance at 449 nm corresponding to the reduction of isoalloxazine **4** by the enamine derived from **6**; the line at 1.1 absorbance indicates the background at this wavelength in the absence of **6**. See conditions in the text.

is ca. 0.1 M and could be viewed as the effective molarity of FAD bound to POX that would make the model and enzymatic rates comparable.[18] The small magnitude of this ratio suggests that the major function of the protein in POX is to bring the FAD and ThDP domains together, but due to the facility of reduction, close proximity of the two coenzymes is not essential, consistent with the X-ray results. These model studies are consistent with a mechanism for POX that proceeds via rate-limiting enamine formation followed by very rapid two-electron oxidation.

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(15) The maximum enamine concentration of 0.08 mM, generated from thiazolium **5** (0.50 mM) by treatment with DBU (2.0 mM) in a control experiment, was assumed to be maintained during the course of reduction of isoalloxazine (0.15 mM). The maximum slope of decrease of A_{449} was calculated to be $-0.512 \pm 0.045\ A\ s^{-1}$, equal to $(-d[\text{isoalloxazine}]/dt)$. As the initial rate $= k_{red}$ [isoalloxazine][enamine], $k_{red} \geq 6100 \pm 530\ M^{-1}\ s^{-1}$ could be estimated. The initial increase of A_{449} was shown to be due to a mixing artifact.

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(18) This comparison is made with the caveat that the rate constant measured on POX refers to a methyl substituent at the C2α position, our model data to a phenyl.