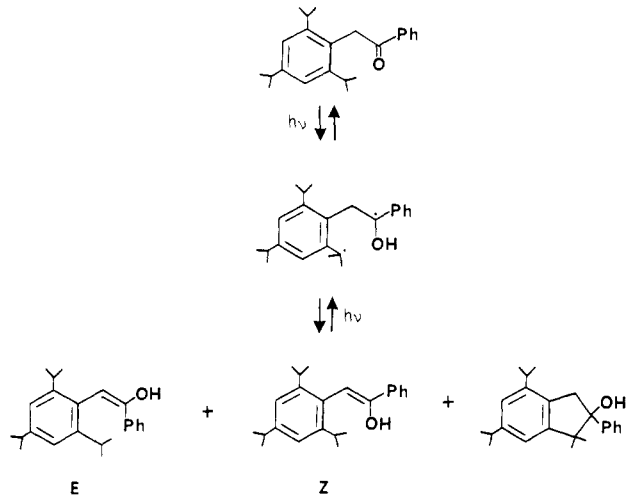
Scheme I




The sample was allowed to sit for 24 h, after which time the smaller vinyl signal had disappeared and been replaced by the ketone methylene signal at $\delta$ 4.6. The more stable enol did not disappear upon addition of 4 drops of acetic acid-$d_4$. The solution was then treated with 1 drop of HCl, neutralized with excess bicarbonate, evaporated, and redissolved in $CCl_4$. This treatment regenerated starting ketone quantitatively (Scheme I).

There are several intriguing aspects of these results. An explanation is required for the fact that the enol is a stable product at long-wavelength irradiation but not at shorter wavelengths. The solution containing only *Z* enol showed strong UV absorption below 330 nm, with $\lambda_{max}$ at 270 nm, and strong fluorescence, as expected for a stilbene derivative. A portion of this solution was irradiated for 2 h with the 300-nm lamps. This process produced a complicated NMR spectrum, which included the readily identifiable singlets at $\delta$ 4.6 and 5.93 for ketone and *E* enol. The *Z* enol obviously undergoes efficient photoisomerization to *E* enol and ketone, with indanol formation being slow and irreversible.

We originally assumed that indanol formation proceeds through 1,5-biradicals formed by triplet-state $\delta$-hydrogen abstraction.[2] We have verified the intermediacy of the expected 1,5-biradicals by laser flash spectroscopy.[8] Quenching studies indicate that triplet TipAP reacts in less than 1 ns. Since no physical decay of ketone triplets occurs so rapidly, the low quantum yield of cyclization must be due to predominant reversion of the biradical to starting ketone. In dioxane the quantum yield of enolization is very high. However, in hydrocarbon solvents the biradical apparently also returns directly to ketone. Such behavior has good precedent in the behavior of other 1,5-biradicals generated from ketones, in which hydrogen bonding to solvent impedes reketonization.[6,9]

We had considered the possibility of similar enol formation with the other $\alpha$-arylacetophenones that we studied[2] and therefore reinvestigated $\alpha$-mesitylacetophenone. Both capillary GC and NMR analysis of irradiated solutions revealed just indanol product, with no enol. This ketone cyclizes in a much higher quantum yield than TipAP, 0.54 with 0.5 M pyridine present. The 46% inefficiency represents biradical disproprotionation to both ketone and enol. The less hindered mesityl enol is expected to be much less stable with respect to the ketone;[4] any that may be formed apparently is too short lived to detect. The extent to which the increased steric hindrance in TipAP increases the enolization-cyclization ratio of the 1,5-biradical is remarkable, given what appears to be a not very ideal geometry for internal hydrogen transfer.

Apart from being another example of photogeneration of otherwise inaccessible enols,[10] these results put a new perspective on Hart and Giguere's results.[1] They suggested that the very low quantum yield rearrangement of the enol of $\alpha,\alpha$-dimesitylacetophenone to a mesityl vinyl ether proceeds from the ketone following enol to ketone rearrangement. Unfortunately, they did not consider the rapid and highly efficient indanol formation from mesitylacetophenone, which we had just published. Our present results indicate that their neglect of the cyclization probably does not detract from their conclusions. The increased steric hindrance afforded by a second mesityl group would not prevent triplet ketone from abstracting a $\delta$-hydrogen but might very well prevent the resultant biradical from undergoing any significant cyclization to indanol. A steady state favoring enol probably is established rapidly, with enol ether than forming slowly, probably from ketone as suggested.[1]

Finally, the significant difference in stability between *Z* and *E* enols warrants comment. It is not surprising that the more congested *E* isomer ketonizes relatively rapidly. Fuson long ago determined that the enol from TipAP is unstable with respect to the ketone.[4] However, the congestion about the $\alpha$ carbon afforded by the triisopropylphenyl group obviously makes protonation of the *Z* enol quite slow, a fact that is disguised by the thermodynamics and that makes such compounds likely candidates for kinetic studies of enol acid-base reactions.[11,12]

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## Thermodynamic Control of Electron Transfer of Flavoproteins by Substrate Binding[1]

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Flavoproteins are unique in their ability to transfer either a single electron or two electrons. In fact, the same enzyme can catalyze both modes of electron transfer.[2,3] The current hypothesis is that noncovalent binding forces from the apoprotein active site regulate the redox potentials of the flavin and control the flavoprotein reactivity.[4] However, the measured redox potentials of the flavoproteins are often inconsistent with the known catalytic mechanism, e.g., lactate oxidase, L-amino acid oxidase, and electron transferring flavoprotein.[5-7] We postulate these enzymes

(1) Abbreviations: DAAO, D-amino acid oxidase; LAAO, L-amino acid oxidase; $EFl_{ox}$, oxidized D-amino acid oxidase; $EFl^{-}$·, anion radical D-amino acid oxidase; $EFl_{red}H^{-}$, two-electron-reduced D-amino acid oxidase unprotonated; $EFl_{ox}$:Benz, benzoate-bound enzyme; $EFl^{-}$·:Benz, one-electron-reduced benzoate-bound enzyme; $EFl_{red}H^{-}$:Benz, two-electron-reduced benzoate-bound enzyme unprotonated; $Fl_{ox}$, oxidized flavin; $Fl^{-}$·, one-electron-reduced flavin; $Fl_{red}H^{-}$, two-electron-reduced flavin unprotonated; E, apoprotein. All potentials are reported vs. the standard hydrogen electrode.
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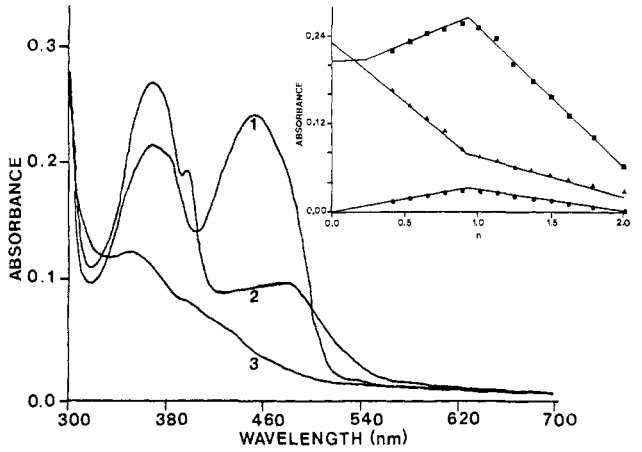


**Figure 1.** Coulometric titration of D-amino acid oxidase using 20.5 $\mu$M DAAO and 100 $\mu$M methyl viologen in 4.0 mL of 20 mM $Na_4P_2O_7$ (pH 8.5) at 18 °C. (1) Oxidized enzyme, (2) $n$ = 0.90 electron/mole, (3) $n$ = 1.80. Intermediate spectra have been omitted for clarity. Inset of Figure 1: plot of absorbance at 370 (■), 454 (▲), and 520 (●) nm vs. number of reducing equivalents added.

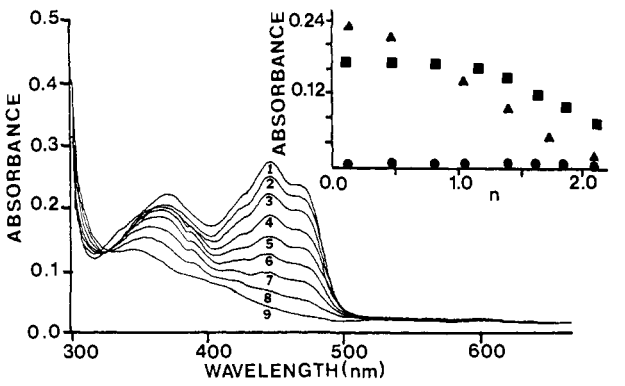


**Figure 2.** Coulometric titration of the benzoate complex of D-amino acid oxidase using 21.3 $\mu$M DAAO benzoate complex, 100 $\mu$M methyl viologen in 4.0 mL of 20 mM $Na_4P_2O_7$, and 0.2 mM sodium benzoate (pH 8.5) at 18 °C. (1) Oxidized enzyme, (2) $n$ = 0.35, (3) $n$ = 0.64, (4) $n$ = 0.93, (5) $n$ = 1.16, (6) $n$ = 1.40, (7) $n$ = 1.63, (8) $n$ = 1.86, (9) $n$ = 2.08. Intermediate spectra have been omitted for clarity. Inset of Figure 2: plot of absorbance at 370 (■), 454 (▲), and 520 (●) nm vs. number of reducing equivalents added.

are activated to perform their catalytic function and report here evidence that the mode of electron transfer (one electron vs. two electron) is regulated by binding of substrate or substrate analogue.

No radical was observed in the two-electron transfer known to occur during catalysis.[8] However, Massey and Palmer quantitatively produced the anion radicals (one electron reduced form) of DAAO and LAAO in 1966 using an artificial electron donor.[9] They were unable to determine the thermodynamic stability of these radicals because the technique to measure the redox potentials had not been developed. Now the methodology to determine the redox potentials of the flavoprotein oxidase enzymes is available.[10] Although no kinetic information can be acquired from this method, the data will enable the thermodynamic properties of DAAO to be addressed.

Figure 1 shows a coulometric titration of DAAO. The linearity and distinct breaks of the $A$ vs. $n$ plot indicate nearly quantitative formation and stabilization of the anion radical.[11] The 88% anion radical produced at $n$ = 1 shows the two electrons differ by a large amount of energy and are transferred sequentially in two separate one-electron steps. We measured the redox properties of DAAO using phenazine methosulfate, phenosafranine, 2-hydroxy-1,4-naphthoquinone, and pyocyanine as redox indicators[5] and obtained the following potentials:

$$EFl_{ox} + e^- = EFl^-\cdot \quad (1)$$

$$E^\circ_1{}' = -0.083 \text{ V}$$

$$EFl^-\cdot + e^- + H^+ = EFl_{red}H^- \quad (2)$$

$$E^\circ_2{}' = -0.208 \text{ V}$$

The 60-mV slopes of the Nernst plots signify single reversible electron transfers. The amount of radical formed in both the coulometric and potentiometric titrations agrees with the observed 125-mV separation.[12] Thus, both electrochemical experiments confirm the thermodynamic stability of the anion radical and indicate the electrons are transferred in separate one-electron steps.[13]

Figure 2 shows a coulometric titration of DAAO bound to a substrate analogue, benzoate. Only 5% of the anion radical was formed. We measured the redox potentials of benzoate-bound DAAO in the above mixture of redox indicators and obtained the following potentials:

$$EFl_{ox}\text{:Benz} + e^- = EFl^-\cdot\text{:Benz} \quad (3)$$

$$E^\circ_1{}' = -0.24 \text{ V}$$

$$EFl^-\cdot\text{:Benz} + e^- + H^+ = EFl_{red}H^-\text{:Benz} \quad (4)$$

$$E^\circ_2{}' = -0.13 \text{ V}$$

Therefore, benzoate binding shifts the potentials such that $E^\circ_1{}'$ is more negative than $E^\circ_2{}'$. The first electron is thermodynamically more difficult to transfer than the second electron, resulting in destabilization of the anion radical. This creates the conditions for two-electron transfer.

As in any complexation system the ratios of the binding constants of the electroactive species (flavin) determine the redox potentials of those species. The redox potentials above and the previously measured binding constants for oxidized flavin ($Fl_{ox}$) to apoprotein (E) and enzyme to benzoate enable the calculation of the binding constants of flavin to apoprotein. Benzoate binding causes the binding constants of $Fl_{ox}$,[14,15] $Fl^-\cdot$, and $Fl_{red}H^-$ to apoprotein to shift from $2.0 \times 10^6$, $3.0 \times 10^9$, and $8.0 \times 10^9$ $M^{-1}$ to $1.0 \times 10^{12}$, $1.9 \times 10^{13}$, and $1.7 \times 10^{15}$ $M^{-1}$, respectively. The binding of $Fl_{ox}$ to apoprotein in the presence of benzoate is increased by a factor of $10^6$ while the binding of $Fl^-\cdot$ to apoprotein differs by only $10^3$ and the binding $Fl_{red}H^-$ to apoprotein differs by $10^5$.

In the free enzyme, the low binding constant of $Fl_{ox}$ with respect to $Fl^-\cdot$ causes the first electron-transfer potential to occur 200 mV positive of the potential of free flavin (eq 1).[16] However, in the presence of benzoate, the binding constants of $Fl_{ox}$ and $Fl^-\cdot$ differ only by a factor of 10. Thus, benzoate binding causes the potential of the first electron to be shifted 160 mV negative with respect to DAAO (eq 3). Substrate analogue binding clearly regulates the potential of the first electron transfer by causing a huge difference in the binding of flavin to apoprotein. The two-electron reduced flavin is also influenced by benzoate in that $Fl_{red}H^-$ is bound to the apoprotein tighter than $Fl^-\cdot$. As a result, the second electron potential in the presence of benzoate is shifted positive by 70 mV with respect to free enzyme (eq 4).

The changes observed in the binding constants when benzoate binds DAAO are consistent with other evidence that flavin in the benzoate-bound enzyme is in a different environment than free

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enzyme. In support of this, benzoate-bound DAAO (Figure 2) has different spectral properties than oxidized free enzyme (Figure 1). The increase in resolution of the flavin spectra in the presence of benzoate indicates that the flavin is in a more hydrophobic environment.[17] In addition, the p$K$ of N(3) of the flavin shifts from 9.6 to 10.7 in the presence of benzoate.[15] Finally, circular dichroism suggests the spectral changes observed on binding of benzoate can be interpreted as a direct interaction between benzoate and the isoalloxazine ring and/or a local conformational change in the protein.[18]

The above data show the net effect of the substrate analogue binding is, in fact, to shift the free energy of the first and second electron transfers such that two-electron transfer is the thermodynamically favored process. DAAO is the first example of a general control mechanism applicable to flavoproteins.

**Acknowledgment.** Support of the research was provided by the National Institute of General Medical Sciences (GM 29344).

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## Relationship between Effective Nucleophilic Catalysis in the Hydrolysis of Esters with Poor Leaving Groups and the Lifetime of the Tetrahedral Intermediate

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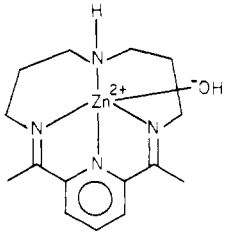
Many enzymes (chymotrypsin,[1] carboxypeptidase,[2] carbonic anhydrase[3]) catalyze the hydrolysis of esters with good leaving groups as well as esters with poor leaving groups. However, simple hydrolytic catalysts are often tested only on esters with good leaving groups. Simple catalysts that are almost as active as hydroxide in cleaving esters with good leaving groups can be millions of times less active than hydroxide in cleaving esters with poor leaving groups.[4]

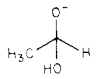
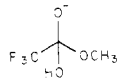
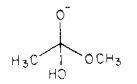
In this paper we report the catalytic hydrolysis of methyl trifluoroacetate. In a typical run, hydrolysis of 1 $\mu$L of the substrate in 10 mL of water at pH 8.0 and 25.0 °C was monitored to completion by the pH stat method. The reaction was monitored in the presence and absence of varying amounts of the zinc hydroxide (ZOH) prepared according to known procedures.[5] As




ZOH

a control, initial hydrolysis of 0.2 mL of methyl acetate was monitored by the method described above in the presence and absence of ZOH. In the presence of 50 mg of ZOH, the rate of hydrolysis of methyl trifluoracetate is increased sixfold whereas for the hydrolysis of methyl acetate there is no observable increase in the rate. No rate enhancement is observed with 10 mM borate buffer or imidazole buffer. Therefore, any rate increase by general base catalysis must be small.[4] For all of the above reactions, the kinetic data were corrected for a small background rate (rate without substrate).

**Table I.** Catalytic Efficiency of ZOH and the Lifetime ($1/k$) of the Intermediate

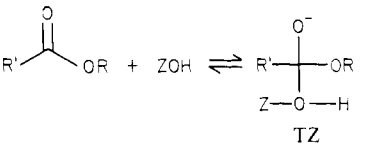
| intermediate structure | $k_{cat}/k_{OH}$ | lifetime of C-O bond, s |
|---|---|---|
|  | $6 \times 10^{-2}$ [a] | $10^{4}$ [b] |
|  | $3 \times 10^{-3}$ [a] | $10^{-3}$ [c] |
|  | $6 \times 10^{-3}$ | $10^{-1}$ [d] |
|  | $<10^{-5}$ | $10^{-6}$ [d] |

[a] Reference 9. [b] Sirs, J. A. *Trans. Faraday Soc.* **1958**, *54*, 201. [c] Reference 13. [d] Guthrie, J. P.; Cullimore, P. A. *Can. J. Chem.* **1980**, *58*, 1281-1294.

In general, nucleophiles do not catalyze the hydrolysis of esters efficiently if the basicity of the nucleophile is significantly less than that of the leaving group.[4] If the base strength of the nucleophile is significantly greater than the base strength of the leaving group, transacylation to the nucleophile is fast but the subsequent hydrolysis is slow.[6] Nucleophilic catalysis for hydrolysis of amides or esters involving strongly basic leaving groups and weakly basic nucleophiles had not been observed except when the nucleophile is bound to the substrate.[7,8]

The conjugate acid of ZOH ($ZOH_2$) has a p$K_a$ of 8.6 and the metal hydroxide has been shown to catalyze the hydration of acetaldehyde and carbon dioxide, and the hydrolysis of propionic anhydride by a nucleophilic mechanism.[9,10]

Equation 1 shows the addition of ZOH to an ester. The adduct




TZ (1)

(TZ) has three bonds that can be cleaved rapidly around a single oxygen. In general, adducts between esters with poor leaving group and weakly basic nucleophiles revert back to starting material rapidly.[4] Reversion of TZ to starting material can be significantly reduced if the metal-oxygen bond or the hydrogen-oxygen bond is cleaved more rapidly than the carbon-oxygen (metal hydrate oxygen) bond. The lifetimes ($1/k$) of the three bonds can be estimated since metal hydrate exchange rates,[11] proton transfer rates,[12] and lifetimes of tetrahedral intermediates[13] can be measured or estimated.

The lifetime of the C-O bond in TZ is more difficult to estimate than the lifetime of the C-O bond in T (eq 2). The lifetime of

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