Direct Evidence for Carbanions and Covalent N^5-Flavin-Carbanion Adducts as Catalytic Intermediates in the Oxidation of Nitroethane by d-Amino Acid Oxidase*

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SUMMARY

We have found that d-amino acid oxidase is rapidly and irreversibly inhibited by cyanide during oxidative turnover of the carbanion of nitroethane. The inhibited enzyme has an absorption spectrum which is characteristic of a covalent flavin-substrate adduct. The following summarizes the results and interpretation of experiments which establish the mechanism of cyanide inhibition as well as the chemical mechanism of oxidation of nitroalkanes by d-amino acid oxidase.

1. The kinetic mechanism of oxidation of the nitroethane carbanion (S-) to form acetaldehyde (P), hydrogen peroxide, and nitrite, was deduced from a combination of stopped flow and O_2-monitored kinetic measurements and is the following.

   \[ E_4 + S^- \overset{K_i}{\longrightarrow} E_5 \overset{k_1}{\longrightarrow} E_7 \overset{k_2}{\longrightarrow} E_8 + H_2O_2 \]

   \[ E_9 + O_2 \overset{k_1}{\longrightarrow} E_{10} \overset{k_2}{\longrightarrow} E_{11} + P \]

   Evaluation of all the rate and equilibrium constants showed that the major pathway for flavin oxidation is the oxidation of \( E_9P \) by O_2.

2. The rate of inhibition of the enzyme by cyanide is regulated by \( K_i \), and O_2 is not consumed during the inhibition process. Since the rate of cyanide inhibition increases as the O_2 concentration is raised, cyanide does not react with \( E_9P \) or with a species in rapid equilibrium with \( E_9P \).

Consequently cyanide must react rapidly with an intermediate, \( E_9X \), which is formed from \( E_9S \) in a reaction, or reactions, controlled by \( k_i \). This is depicted kinetically as follows:

\[ E_9S \overset{K_i}{\longrightarrow} E_9S + E_9P \]

\[ E_9P + O_2 \overset{k_1}{\longrightarrow} E_9P + O_2 \overset{k_2}{\longrightarrow} E_{11} + P \]

3. The inhibited enzyme (\( EI \)) contains, per FAD, 1 eq each of substrate and cyanide, but is lacking the nitro group.

4. Treatment of \( EZ \) with hot methanol produces a free flavin-substrate adduct in good yield and with no irreversible spectral or chemical modification. At 70°C and pH values greater than 8 the free flavin-substrate adduct releases cyanide and is readily converted (<10 min) under aerobic conditions to FAD, acetaldehyde, and, presumably, H_2O_2. Anaerobically, FADH_2 is produced. These solvent-catalyzed reactions of the adduct largely mimic the enzyme-catalyzed oxidation of the substrate.

5. The spectral and ionization properties, as well as the chemical reactivity, of the free flavin-substrate adduct closely resemble those of S-substituted dihydroflavins in general, and those of 5-cyanomethyl-1,5-dihydroflavin in particular. For these reasons we assign the structure 5-cyanoethyl-1,5-dihydro-FAD to the free flavin-substrate adduct.

The kinetic turnover mechanism, the locus of action of cyanide, and the structure of the free flavin-substrate adduct, taken together, enable us to propose a detailed chemical mechanism for the oxidation of nitroethane carbanion by d-amino acid oxidase.

\( E_9S \) is a noncovalent complex in which the substrate carbanion is sufficiently close to the flavin (presumably the N^5 position) to perturb the electronic properties of the latter. Attack of the carbanion at N^5 of the flavin is controlled by \( k_e \), and results in the formation of 5-nitroethyl-1,5-dihydro-FAD. Elimination of nitrite forms a highly reactive cationic imine (\( EX \)) at the N^4 flavin position to which solvent adds to
form a carbinolamine. Finally, FADH$_2$ is eliminated from the carbinolamine, leaving acetaldehyde ($P$) noncovalently bound to $E_r$. Cyanide attacks the cationic imine (EX) in competition with solvent to form 5-cyanoethyl-1,5-dihydro-FAD (EI). This adduct is not reactive with O$_2$ under conditions normally used to study the enzyme.

The molecular, as opposed to the kinetic, details of the oxidation-reduction processes catalyzed by flavoenzymes have remained elusive during the 40 years since the first flavoprotein, namely Old Yellow Enzyme, was discovered by Warburg and Christian (1). Given the three oxidation-reduction states of the flavin nucleus we must ask whether these states are interconverted through an orderly series of conventional chemical intermediates in which electron transfers are achieved by rearrangements of covalent adducts formed successively with the flavin nucleus by the reducing and oxidizing substrates, or whether the oxidation-reduction processes occur without the formation of covalent adducts. We address ourselves in this paper to the question of the chemical pathway by which enzyme-bound FAD is converted to FADH$_2$ by nitroethane at the active site of $\alpha$-amino acid oxidase.

A priori, there are three chemical mechanisms by which enzyme-bound FAD might be converted to FADH$_2$ by physiological substrates of simple flavoprotein oxidases (Equations 1, 2, and 3).

\[
\begin{align*}
\text{(E,S)} & \quad E - \text{FAD} \cdots [\text{C} - \text{NO}_2] \xrightarrow{k_r} E - \text{HFAD} \cdots [\text{C} - \text{NO}_2] \quad \text{fast} \\
\text{(EX)} & \quad E - \text{HFAD} \cdots [\text{C} - \text{OH}] \quad \text{fast} \quad E - \text{P} \\
\text{(EI)} & \quad E - \text{HFAD} \cdots [\text{C} - \text{CN}] \\
\end{align*}
\]

We discount homolytic mechanisms because of the consistent failure to detect free radical intermediates and the total lack of catalytic reactivity of the semiquinone state of the enzymes (3, 4). Equations 1 and 2 represent group transfer mechanisms in which covalent flavin-substrate adducts (shown in square brackets) mediate the transfer of reducing equivalents from substrate to flavin. In the first case (Equation 1) the adduct is bonded through carbon and is formed by nucleophile attack of an enzyme-generated substrate carbanion on the flavin nucleus. In the event that the lifetime of the carbanion is sufficiently short to escape experimental detection, proton removal and adduct formation will appear to be synchronous processes (dashed arrow in Equation 1). The adduct in the second case (Equation 2) is distinguished by nucleophilic attack of, and subsequent bonding through, the electronegative grouping $-\text{XH}$ (5). Equation 3 represents a heterolytic oxidation-reduction process in which some combination of protons and 2 electrons (a hydride, for example) is transferred without covalent adduct formation.

If a covalent substrate-flavin adduct, whose kinetic properties qualify it as an intermediate lying directly on the catalytic pathway, could be trapped as a stable and isolable entity, the mechanism of Equation 3 would be ruled out. Furthermore, the structure of the trapped adduct would determine the fundamental chemical features of flavoenzyme catalysis and would serve to distinguish between Equations 1 and 2. Unfortunately, neither of the postulated adducts (see Equations 1 and 2) regardless of the position on the flavin nucleus at which nucleophilic addition of the physiological substrate occurs, is likely to have chemical properties which would allow trapping by a chemical agent in a process competitive with enzymatic turnover.

Consequently, we have taken a novel approach involving the use of preformed carbanions of nitroalkanes as analogs of the postulated enzyme-bound carbanions derived from physiological substrates. Our intention, therefore, is to test specifically the validity of Equation 1 as a description of the chemical mechanism of flavoprotein oxidase catalysis. We were led to these experiments through the observation that $\alpha$-amino acid oxidase is slowly inactivated during the oxidative turnover of nitromethane (6). The spectrum of the inactivated enzyme showed that chemical modification of the flavin nucleus had occurred. We reasoned that a 2nd molecule of the nitromethane anion (which had been slowly formed by ionization of the neutral substrate originally present) had probably interacted with a flavin-substrate adduct to form a species incapable of reacting with O$_2$. Consequently, we searched for an inhibitory anion of simple structure and found that cyanide was an extremely effective inhibitor of nitroalkane oxidation. Equation 4 outlines our strategy and emphasizes the close relationship between the mechanism of oxidation of nitroalkanes and that of physiological substrates (Equation 1) if the latter must be converted to enzyme-bound carbanions before covalent adduct formation with the flavin nucleus.

It will be noted that, after attack of the nitroalkane carbanion on the flavin nucleus to form a covalent adduct, elimination of nitrite generates a highly reactive electrophilic center at which suitable nucleophiles such as cyanide (or a 2nd nitroalkane anion) can react and thereby interrupt the oxidation-reduction process by forming a stabilized flavin substrate adduct (EI). The three most familiar flavoprotein oxidases of this type, all of which are reactive with nitroalkanes (2), are $\alpha$-amino acid oxidase (EC 1.4.3.3) from hog kidney (with which this paper is chiefly concerned), glucose oxidase (EC 1.1.3.4) from certain moulds, and $\lambda$-amino acid oxidase (EC 1.4.3.2) from rattlesnake venom.
The latter adduct is formally identical with that which would result from the interaction of the enzyme-bound carbanion of a physiological substrate with the flavin nucleus (see Equation 1).

We shall show that the scheme of Equation 4 accurately describes the chemical mechanism of reduction of the flavin nucleus of d-amino acid oxidase by the carbanion of nitroethane. We shall also show, by examination of the spectral and chemical properties of the free flavin adduct resolved from the flavin-substrate adducts involving the N^2 position of enzyme-bound FAD.

**EXPERIMENTAL PROCEDURE**

**Materials**

D-Amino acid oxidase was purified from hog kidney by the method described by Yagi et al. (7, 8). The benzamide-holoenzyme complex was stored at 5°. The holoenzyme was separated from benzamide by dissolving approximately 40 mg of enzyme in 1 ml of 0.1 M sodium pyrophosphate buffer, pH 8.3, and adding a few crystals of DL-alanine. This solution was then passed through a Sephadex G-25 column yielding benzamide-free holoenzyme. The absorbance ratio A_274/A_435 of the holoenzyme was 10.3. Assay of the enzyme under standard conditions (air-equilibrated solution at 25°) containing 10^{-5} M DL-alanine and 0.1 M sodium pyrophosphate, pH 8.3, gave a specific activity based on 425C = 23.0 (9) of 13.8 pmoles of product min^{-1} mg^{-1} while the turnover number, based on FAD content, was 11.3 s^{-1} (10).

Crystalline catalase and glucose oxidase were purchased from Sigma Chemical Co. Alcohol dehydrogenase was obtained from the Boehringer Mannheim Corp. These three enzymes were used without further purification.

Nitroethane was purchased from the Aldrich Chemical Co. Before use, it was redistilled and if not used immediately was stored at 5°. The anion of nitroethane was formed, unless otherwise stated, in 0.1 M sodium pyrophosphate, pH 8.3, at 25°. The solution was adjusted to the desired pH with HCl or KOH. All pH values were determined on a Radiometer 22 pH-meter with a GK-2322-C electrode.

The enzyme activity was monitored on the Yellow Springs oxygen electrode system with a solution initially air-equilibrated at 25°. The O_2 concentration in this solution was 0.24 m&l (11). The enzyme reaction was usually initiated by the addition of nitroethane anion to the O_2-equilibrated solution containing the enzyme. This order of addition was chosen to eliminate changes in the state of ionization of nitroethane anion as much as possible. Nitroethane anion had little effect on the O_2 electrode calibration.

Varying O_2 concentrations were obtained by the use of gas flow meters manufactured by Kontes Glass Co. Either pure O_2 or air was used in one valve, while N_2 was used in the other. The resulting mixture was bubbled through the assay solution for 3 to 5 min. The concentration of O_2 in the assay mixture was measured relative to an air-equilibrated solution.

All spectra and optical kinetics were measured either on the Cary 15 or Gibson-Durrum Stopped Flow Spectrophotometer. Both were temperature-regulated to 25 ± 0.2°. These experiments were arranged such that less than 10% of the nitroethane anion was lost due to protonation during the course of the experiment. The pH of the reaction mixture was measured at the end of each experiment.

Data from stopped flow experiments were plotted when applicable as first order rate constants over at least three half-lives. If the total absorbance change was less than 0.1, voltage changes were plotted directly. However, if the absorbance change was greater than 0.1, the voltage changes were first converted to absorbance. The anaerobic and oxidative half-reactions, as well as turnover, were monitored at several wave lengths.

Solutions were made anaerobic by slowly bubbling V^2+ deoxygenated N_2 through the solution for at least 5 min. This time was sufficient for complete anaerobiosis as judged by O_2 depletion measured on the O_2 electrode. For static experiments, Thunberg or glass-stoppered cuvettes were used.

In stopped flow experiments, solutions were made anaerobic with 10^{-5} m glucose oxidase and 10^{-4} m glucose.

Resolution of Enzyme—The apoenzyme of d-amino acid oxidase was prepared by dialysis against 1 m KBr as described by Massey and Curti (12). However, this method for resolution of holoenzyme was not successful when the enzyme was inactivated by cyanide and nitroethane. The flavin-substrate adduct was separated instead from the inactive holoenzyme in 0.1 M sodium pyrophosphate, pH 8.3, by the addition of boiling methanol to a final concentration of 80%. The protein was removed by centrifugation at 15,000 × g for 10 min at 5°. The flavin-substrate adduct remained in the supernatant solution with a yield of 80 to 100%. The methanol was removed by flash evaporation.

**Methods**

**Kinetic and Spectral Measurements**—All reactions were performed, unless otherwise stated, in 0.1 M sodium pyrophosphate, pH 8.3, at 25°. The solution was adjusted to the desired pH with HCl or KOH. All pH values were determined on a Radiometer 22 pH-meter with a GK-2322-C electrode.

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(without heating) and the resulting solid was dissolved in the desired buffer. Unless noted, the modified FAD prepared in this manner was used for further experiments on the same day.

Attempts at recovering active apoenzyme from the holoenzyme which had been inactivated by cyanide in the presence of nitroethane were unsuccessful.

Product Analysis—Acetaldehyde was assayed for as described by Bergmeyer (13). Hydrogen peroxide was determined by the difference in any of these assay methods.

We note (recognizing that the nitroalkanes are of the same oxidation state as amines and alcohols) that nitroalkane oxidation is formally analogous to the oxidative deamination of an amino acid normally catalyzed by this enzyme when the latter reaction is written to include nonenzymatic hydrolysis of the imine (15, 16) released by the enzyme (Equations 6 and 7).

\[
\begin{align*}
\text{H}-\text{C}^\text{NO}_2^- + \text{O}_2 + \text{OH}^- &\rightarrow \text{C} = \text{O} + \text{NO}_2^- + \text{H}_2\text{O}_2 \\
\text{H}-\text{C}^-\text{NH}_3^+ + \text{O}_2 + \text{OH}^- &\rightarrow \text{C} = \text{O} + \text{NH}_3 + \text{H}_2\text{O}_2
\end{align*}
\]

We conclude that 1 mole each of \(\text{H}_2\text{O}_2\), \(\text{NO}_2^-\), and \(\text{acetaldehyde}\) is produced for each nitroethane anion and \(\text{O}_2\) consumed. This result is identical with that found for the oxidation of nitromethane by \(\text{n-amino acid oxidase}\) (6) and simpler than the stoichiometry given by glucose oxidase (2). We note (recognizing that the nitroalkanes are of the same oxidation state as amines and alcohols) that nitroalkane oxidation is formally analogous to the oxidative deamination of an amino acid normally catalyzed by this enzyme when the latter reaction is written to include nonenzymatic hydrolysis of the imine (15, 16) released by the enzyme (Equations 6 and 7).

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\end{align*}
\]

Reactivity of Neutral and Anionic Nitroalkanes with Holo- and Apeoenzyme—At pH 8.1, with equilibrium mixtures of the neutral and anionic nitroalkanes, the order of reactivity (expressed as \(\phi_1\)), the apparent bimolecular constant for flavin reduction as given in Equation 8) is \(2\)-nitropropane > \(1\)-nitropropane > nitroethane > nitromethane. Table I summarizes the results of experiments which show that the apoenzyme is incapable of oxidizing nitroethane anion with greater than 0.2% of the efficiency of the holoenzyme. These results also emphasize how much more reactive the substrate becomes when proton abstraction is carried out before interaction of the substrate with the enzyme. The true difference in reactivity of the neutral and carbanionic nitroethane species with the holoenzyme (which in the case of glucose oxidase and nitromethane is a factor of \(10^6\) in favor of the carbanion (21)), would require a systematic kinetic analysis over a wide range of pH. No oxidation of nitroethane by free FAD in the dark has been detected, although photochemical oxidation does occur (6).

Most of the experiments involving the prior formation of the nitroethane anion were carried out within a few minutes of placing the anion in a solution at pH 8.3 and 25°. We found, using the tetroxotrimethane method (17), that the half-time for protonation of the carbanion under these particular conditions is 54 min. Consequently, very little neutral nitroethane was present in any of the experiments described here.

Steady State Turnover Kinetics—When initial rates of \(\text{O}_2\) consumption were plotted in double reciprocal form with nitroalkane anion and \(\text{O}_2\) as variable substrates, parallel line patterns resulted (see Fig. 1). The steady state rate equation describing these results is given by Equation 8.

\[
\frac{E_T}{v} = \frac{[\text{FAD}]}{[\text{OT}]} + \frac{[\text{FAD}]}{[\text{O}_2]} \tag{8}
\]

The values of the steady state coefficients \(\phi_{\text{FAD}}\), \(\phi_{\text{O}_2}\), and \(\phi_{\text{FAD}}\) at 25° and pH 8.3 (0.1 M sodium pyrophosphate) for nitroethane carbanion are given in Table II. The value of \(\phi_{\text{FAD}}\) is 200 times greater than the corresponding value (see Table II) found with nitromethane at pH 8.3 (6). As is generally the case in flavoprotein oxidase reactions (18, 19), the steady state coefficients are related in a simple fashion to the kinetic constants obtained by direct measurement in the stopped flow experiments to be described. Also shown in Table II are the steady state coefficients

![Fig. 1. \(O_2\)-monitored turnover data plotted in double reciprocal form according to Equation 8 in the text. Inset shows the ordinate intercepts plotted with \(O_2\) as variable substrate. The reactions were carried out in 0.1 M sodium pyrophosphate, pH 8.3, at 25°.](http://www.jbc.org/)

TABLE I

| Nitroethane       | Holoenzyme | Apeoenzyme |
|-------------------|------------|------------|
| Nitroethane carbanion | 0.47       | <0.001     |
| Neutral nitroethane | 0.006      | <0.001     |
for 2-nitropropane carbanion. They are very similar to those for nitroethane carbanion.

We should emphasize that concentrations of nitroethane anion exceeding about 5 mM inactivate the enzyme. This requires special care in the estimation of initial velocities. The inhibition, noted previously (6), is most probably due to the interaction of a second nitroethane anion with the enzyme in a process entirely analogous to the effects of cyanide to be described.

The supposition that nitroalkanes function as simple substrate analogs of d-amino acids was strongly reinforced by the finding that benzoate, a classical competitive inhibitor of the d-amino acid substrates, acts as a competitive inhibitor of nitroethane anion. The double reciprocal plots with nitroethane anion as variable substrate and at different levels of benzoate were accurately linear and gave a $K_i$ value of 3.0 µM. This value is in excellent agreement with the value of the dissociation constant (3 µM) determined for the binary enzyme-benzoate complex (20).

**Stopped Flow Anaerobic Half-reaction Measurements**—When enzyme and nitroethane anion were mixed anaerobically in the stopped flow apparatus the reduction of flavin, monitored at 463 nm, occurred in three phases. The last part of the second phase, together with the third phase, is shown in Fig. 2. The first phase was detected by comparison of absorbance amplitudes with those expected from static spectra and was too rapid to be resolved ($t_{1/2} < 3$ ms), while the second and third phases were easily resolved independently and from each other. The spectrum corresponding to the termination of each phase is shown in Fig. 3.

The species formed very rapidly in the first unresolved phase ($E_o S$) has a spectrum slightly less intense than that of $E_o$ at wave lengths less than 500 nm. Above 500 nm, however, $E_o S$ differs from $E_o$ in having weak absorption which extends to 800 nm. The flavin in $E_o S$ is clearly in the oxidized state. The spectrum of the species ($E_o P$) produced in the second phase is indicative of a reduced form of the enzyme, but is not identical with that of $E_o$. The third, very slow, phase does result in the formation of $E_o$, as is shown in Fig. 3 by comparison with $E_o$ produced by o-alanine.

The interpretation of these spectral changes in the anaerobic half-reaction is straightforward. Firstly, the initial and rapid (unresolved) burst in absorbance at 550 nm, which is due to the formation of $E_o S$ and which occurs within the deadtime of the apparatus, can be used to titrate $E_o S$. These data are given in Fig. 4. The equilibration of $E_o$ with $E_o S$ is very rapid compared with subsequent processes in the anaerobic half-reaction and the absorption difference between the termination of the second and third kinetic phases of the anaerobic reductive half-reaction is shown in Fig. 2. $E_o$ (---), fully reduced enzyme obtained with o-alanine under anaerobic conditions. $E_o$ (- - -), fully reduced enzyme obtained after the third phase of the anaerobic reductive half-reaction with nitroethane carbanion (see Fig. 2). All spectra were measured, or calculated from measurements, in 0.1 M sodium pyrophosphate, pH 8.3, at 25°, with 41 µM enzyme. Although not shown, the spectrum of $E_o S$ extends to 800 nm.

**TABLE II**

| Steady state coefficients | Rate constant (nitroethane carbanion) | Value (nitroethane carbanion) | Value (nitroethane) | Value (2-nitropropane carbanion) |
|---------------------------|---------------------------------------|-----------------------------|------------------|---------------------------------|
| $\phi_{t1}$              | $k_e$                                  | $2.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ | $2.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ | $2.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ |
| $\phi_{t2}$              | $k_e$                                  | $2.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ | $2.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ | $2.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ |

Fig. 2. Example of anaerobic reductive half-reaction monitored by transmittance increase at 463 nm in the stopped flow apparatus. Visible on this relatively long time scale is the final portion of the second kinetic phase ($t_{1/2} = 0.18$ s and easily resolved on faster sweep) corresponding to the conversion of $E_o$ to $E_o P$ and the third, very slow, phase corresponding to the conversion of $E_o P$ to $E_o + P$ (see Equation 9 and discussion in text). The reaction was carried out in anaerobic 0.1 M sodium pyrophosphate, pH 8.3, at 25°, by mixing enzyme (20.5 µM) with an equal volume of 50 mM nitroethane carbanion.

Fig. 3. Absorption spectra of enzyme intermediates. $E_o$, fully oxidized enzyme. $E_o S$, complex of $E_o$ with substrate carbanion, $E_o P$, calculated from stopped flow turnover experiments as given in the text. $E_o P$, spectrum obtained from the absorbance difference between the termination of the second and third kinetic phases of the anaerobic reductive half-reaction (see Fig. 2). $E_o$ (---), fully reduced enzyme obtained with o-alanine under anaerobic conditions. $E_o$ (- - -), fully reduced enzyme obtained after the third phase of the anaerobic reductive half-reaction with nitroethane carbanion (see Fig. 2). All spectra were measured, or calculated from measurements, in 0.1 M sodium pyrophosphate, pH 8.3, at 25°, with 41 µM enzyme. Although not shown, the spectrum of $E_o S$ extends to 800 nm.
FIG. 4. Double reciprocal plot of absorbance increments at 550 nm (due to E₆S, see Fig. 3), obtained by stopped flow measurements of the anaerobic reductive half-reaction, versus nitroethane carbanion concentration. Since the formation of E₆S from E₀ is too rapid to be observed, the absorbance of E₆S is obtained as the difference between the absorbance at the beginning of the reductive half-reaction (due to E₅D) and the absorbance at the end of the reaction (which is zero, because E₆ does not absorb above 530 nm). The reactions were carried out anaerobically in 0.1 M sodium pyrophosphate, pH 8.3, at 25°C, by mixing 41 μl enzyme with equal volumes of nitroethane carbanion solutions.

The fact that the values of the abscissa intercepts in Figs. 4 and 5 are identical confirms the fact that these intercepts represent −Kᵣ⁻¹. The value of k₁ exceeds the maximum turnover number (φₑ⁻¹) by a factor of 2.5. The conversion of E₀S to E₆P can not, therefore, be the only rate-controlling first order process in turnover. It is clear, however, that the first two processes of Equation 9 are obligatory steps in turnover because the slope of Fig. 5 (which, since E₀S is in rapid equilibrium with E₀ and S⁻, corresponds to k₋₁/kₚ₋₁) is equal to the substrate coefficient, φₑ, obtained from the steady state turnover experiments (Fig. 1 and Equation 8). Eₑ (E-FADH₂) was found in stopped flow experiments to be oxidized by O₂ with a bimolecular rate constant of 2.5 × 10⁴ M⁻¹ s⁻¹. However, the rate of formation of E₆ (k₉ = 0.01 s⁻¹) is 250 times smaller than the maximum turnover number and is much too slow for this species to be an obligatory catalytic intermediate. In this and all other respects, Equation 7 is formally analogous to the kinetic mechanism required for many physiological substrates of both the d- and L-amino acid oxidases (19, 21, 22). The analogy extends even to the fact that product dissociation from oxidized enzyme partially controls the maximum turnover velocity.

Stopped Flow Monitored Turnover Experiments—Typical stopped flow turnover traces monitored at 463 nm are shown in Fig. 6. There is an initial rapid absorbance decrease corresponding to formation of E₆P during the first half of the first turnover, followed by slower absorbance decrease as turnover proceeds and the O₂ is depleted. A small part of the slow absorbance decrease during turnover is again due to enzyme inactivation by a 2nd molecule of nitroethane anion. The half-time of the initial rapid absorbance decrease becomes smaller as the concentrations of the substrate and of O₂ are increased at fixed concentrations of O₂ and substrate, respectively. Such experiments also show that the amplitude of the initial rapid phase increases as the substrate concentration is raised (see Fig. 6) and decreases as the O₂ concentration is raised. We conclude that E₆P reacts with O₂ to form a species E₆O which absorbs at 463 nm. At this stage, therefore, our results from the half-reaction and turnover measurements are explained by the following scheme (Equation 10).
FIG. 7. Dependence of rate of inhibition ($k_{ob}$) of $\alpha$-amino acid oxidase on cyanide concentration during oxidative turnover of nitroethane carbanion. The first order rates of inhibition were calculated directly from $\Delta$-electrode traces which, over the time intervals involved, showed an approximately constant rate of $O_2$ consumption in the absence of cyanide. The experiments were carried out in 0.1 M sodium pyrophosphate, pH 8.3 and 25°C, with 0.24 mM $O_2$ and 1.0 mM nitroethane carbanion initially and 2.6 

In analogy with the $\alpha$-amino acid oxidase reaction (19), we assign to $k_1$ the value of $k_0^{-1}$ (namely $8.7 \times 10^8$ M$^{-1}$ s$^{-1}$) and compute $k_0$ (4.5 s$^{-1}$) from the relationship $\phi_0 = (k_1 + k_0)/k_0$. With this information we can determine the distribution of $E\alpha$, $E\alpha+S$, $E\alpha+P$, and $E\alpha$P during turnover and, as shown in Fig. 3, we are able to calculate the spectrum of $E\alpha$P. Because this spectrum is intermediate between those of oxidized and reduced enzyme and because acetaldehyde was found to have no effect on the spectrum of $E\alpha$, the species labeled $E\alpha$P may actually be a steady state mixture of two intermediates.

Kinetics and Mechanism of Inhibition of Enzyme by Cyanide—Cyanide very rapidly inhibits the oxidation of the carbanions of nitromethane, nitroethane, and 2-nitropropane by $\alpha$-amino acid oxidase. The inhibition is complete and, for all practical purposes at 25°C (see later) irreversible. Moreover, the inhibited enzyme does not oxidize $\alpha$-alanine. The dependence of the rate of inhibition on the concentration of cyanide in the case of nitroethane carbanion is given in Fig. 7. The non-zero ordinate intercept is due to the relatively slow inhibition of the enzyme by a second nitroethane anion, as mentioned previously. These $O_2$-monitored experiments could not be carried out at cyanide concentrations greater than 1 mM because of the rapidity of inhibition. We shall subsequently show that the rate of inhibition reaches a limiting value at higher cyanide concentrations. The rate of enzyme inhibition by cyanide is also a function of the concentration of nitroethane anion and of $O_2$, as shown in Fig. 8, $A$ and $B$. The $K_m$ values for nitroethane anion and $O_2$ in Fig. 8, $A$ and $B$, are identical with the $K_m$ values determined for these substrates in steady state turnover experiments carried out under identical conditions (except for the absence of cyanide).

The effect of cyanide on the turnover pattern monitored at 455 nm in stopped flow experiments is very pronounced (Fig. 9). The first order absorbance decay caused by cyanide is saturable, as shown in Fig. 10, and the apparent $K_m$ for the effect of cyanide in these experiments is 18 mM. This value for $K_m$ explains why inhibition of the enzyme appears to be proportional to the cyanide concentration over the range shown in Fig. 7. The maximum rate of inhibition under these particular conditions is 0.83 s$^{-1}$. 

FIG. 8. $A$, dependence of rate of inhibition ($k_{obs}$) of $\alpha$-amino acid oxidase by 0.5 mM cyanide on the concentration of nitroethane carbanion. Other experimental conditions given in legend of Fig. 7. $B$, dependence of rate of inhibition ($k_{obs}$) of $\alpha$-amino acid oxidase by 0.5 mM cyanide on the concentration of $O_2$. Other experimental conditions given in legend of Fig. 7.

FIG. 9. Effect of 0.05 mM cyanide on oxidative turnover of 2 mM nitroethane carbanion monitored by stopped flow transmittance changes at 455 nm. The experimental conditions were the same as those given in the legend of Fig. 6. The rapid change caused by cyanide (when calculated as absorbance) is clearly first order when displayed on shorter time scales.
These results are highly significant for two reasons. Firstly (noting that the experimental conditions used in Figs. 7 and 10 were almost identical), the rates of inhibition from Fig. 7 accurately fit the initial portion of the curve obtained from the stopped flow turnover experiments of Fig. 10. We conclude, therefore, that the absorbance changes caused by CN\textsuperscript{-} at 455 nm under turnover conditions (Figs. 9 and 10) directly monitor the inhibition process. Secondly, the maximum rate of the 455-nm absorbance change in Fig. 10, namely 0.83 s\textsuperscript{-1}, is in excellent agreement with the value of 0.77 s\textsuperscript{-1} which is obtained for the rate of the anaerobic half-reaction under similar conditions (that is, 2 mM nitroethane anion as shown in Fig. 5). This point is further verified by the experiments of Fig. 11, which show that at high cyanide and saturating nitroethane anion the maximum rate of the 455-nm absorbance change is about 5 s\textsuperscript{-1}. This value, when corrected to infinite cyanide by noting that cyanide is present at a concentration of 2.8 \times K_{\text{CN}}^m, agrees almost precisely with the value of 6.2 s\textsuperscript{-1} determined for k\textsubscript{2} in anaerobic reductive half-reactions (see Fig. 5). These experiments, therefore, identify kinetically the locus of cyanide action as being within the sequence from E\textsubscript{5/2}S to E\textsubscript{P}, and will prove to have important predictive value when we describe the properties of the flavin-substrate which can be separated from the enzyme after cyanide treatment.

The kinetic experiments just described clearly suggest that inhibition by cyanide does not require the prior or simultaneous interaction of O\textsubscript{2} with the enzyme even though the experimental design thus far utilized requires O\textsubscript{2} to be present. This important conclusion was checked by a direct method in which the amount of O\textsubscript{2} consumed during inhibition was measured as a function of the cyanide concentration. Fig. 12 shows that the total amount of O\textsubscript{2} consumed during inhibition, when compared to the concentration of enzyme-bound FAD, approaches zero at high cyanide concentrations. Control experiments, also presented in Fig. 12, show that turnover with \textalpha\-alanine is negligibly affected by cyanide. These measurements completely substantiate the conclusion that cyanide interacts with a reduced, rather than oxidized, flavin species which is an obligatory catalytic intermediate during enzyme turnover with nitroethane anion. Furthermore, the enzyme resulting from treatment with 0.2 mM cyanide during nitroethane turnover in Fig. 12 had the spectrum characteristic of inhibited enzyme (see Fig. 14). The results of Fig. 12 are additionally useful because they establish, for the purpose of experiments to be discussed subsequently, that only about 0.06 of a complete turnover can occur in the presence of 0.2 mM cyanide.

From the data now at hand we can examine the reaction scheme of Equation 10, which was deduced entirely from the results of stopped flow half-reaction and turnover experiments in which cyanide was not present, to see whether this scheme satisfactorily accommodates the information from the cyanide inhibition experiments. Since cyanide was proved to interact with a catalytic species lying between E\textsubscript{5/2}S and E\textsubscript{P}, and since E\textsubscript{P} reacts with O\textsubscript{2}, the question as to whether both cyanide and O\textsubscript{2} react with E\textsubscript{P} can be tested experimentally. Fig. 8B clearly shows that O\textsubscript{2} increases, rather than decreases, the rate of inactivation by cyanide.
If cyanide reacted with $E_3P$, competition between $O_2$ and cyanide would result, rather than the synergistic effect actually observed in Fig. 8B. We must conclude, therefore, that cyanide in fact reacts with an intermediate $EX$ which is formed in a first order process, or processes (governed by $k_0$), from $E_oS$ and which is converted in turn by a first order process to $E_3P$. This is depicted in Equation 11. The chemical structures and spectral properties of the intermediates of the scheme of Equation 11, as well as a more complete interpretation of the kinetic data, will be treated under "Discussion."

\[
\begin{align*}
&\text{fast} \\
E_o + S &\xrightarrow{k_s} E_oS &\xrightarrow{k_{E_3P}} EX &\xrightarrow{k_E} E_3P \\
&\quad \downarrow k_t \quad \quad \downarrow k_{[CN^-]} \\
&\text{fast} \\
E_oP &\xrightarrow{k_{E_oP}} E_oS \\
&\quad \downarrow k_{[O_2]} \quad \quad \downarrow k_t \\
&\text{fast} \\
E_o &\xrightarrow{k_{E_oP}} E_oP \\
&\quad \downarrow k_{[O_2]} \\
&\end{align*}
\]

Equation 11

Although the kinetic data of the first and second phases of the anaerobic reductive half-reaction are completely unaffected by cyanide, a result which is now clearly understood from the finding that the rate of cyanide interaction with the enzyme is itself controlled by the rate of reduction of the enzyme, we noticed that the amplitude of the absorbance change corresponding to the second phase of the anaerobic half-reaction was increased in the presence of cyanide. This must result from the fact that $E_3P$ (the formation of which from $E_o$ is responsible for the second phase of the half-reaction in Fig. 2) has greater absorbance than $E_1$. This absorbance difference is maximal at 400 nm (compare Figs. 3 and 14) and Fig. 13 shows a plot of the amplitude of the second phase as a function of cyanide. The interaction of cyanide with the enzyme under these (anaerobic) conditions gives the same apparent $K_m$ value, namely 20 mM, as is observed in the kinetic studies of enzyme inhibition (see Fig. 10). This $K_m$ value, in terms of Equation 11, represents the ratio $k_{E_oP}:k_{E_3P}$. Furthermore, the absorbance increment at 400 nm caused by 0.09 mM cyanide (as well as the entire absorption spectrum) is exactly that predicted for the spectrum of $E_1$ shown in Fig. 14. This experiment confirms, therefore, that $E_1$ can indeed be formed under anaerobic conditions.

Studies of Cyanide-inactivated Enzyme—The absorption spectrum of $E_1$, the inhibited species of the enzyme resulting from cyanide treatment during turnover, is shown in Fig. 14. The spectrum is dominated at pH 8.3 by a peak at 332 nm ($\varepsilon_{322} = 5.4 \times 10^3$ M$^{-1}$ cm$^{-1}$) and is totally different from the spectrum of any enzyme species detected either statically or kinetically with nitroethane anion in the absence of cyanide (provided that appreciable inhibition by nitroethane has not occurred) or with physiological substrates in the presence or absence of cyanide. The spectrum of $E_1$ does, however, resemble the spectrum of the inhibited enzyme recovered after exposure to high concentrations of nitromethane (6).

The derivative of FAD which is trapped by cyanide can be resolved in good yield (between 80 and 100%) from the apoenzyme by precipitation of the protein with hot 50% methanol (see "Methods"). It is interesting to note that the conventional KBr procedure developed for the resolution of F-FAD (12) fails to resolve $E_1$, suggesting, as will be subsequently confirmed, that the flavin in $E_1$ has a reduced, rather than oxidized, conformation. After the methanol is removed by flash evaporation, the absorption spectrum of the free flavin adduct closely resembles that of the inactivated holoenzyme when measured in the same

![Graph](http://www.jbc.org/)
solvent. As Fig. 14 shows, the 332-nm peak of the inactivated holoenzyme is blue-shifted by only 12 nm after the flavin adduct is separated from the apoenzyme. The spectrum of \( EI \) (\( \lambda_{\text{max}} = 332 \text{ nm} \)) could be recovered by combining the resolved flavin adduct with apoenzyme which had been prepared from native holoenzyme by the KBr procedure. This shows that no irreversible changes (covalent or otherwise) occur in the flavin adduct during resolution of the inhibited holoenzyme by hot methanol.

We next used \(^{14}\text{CN}^-\) in order to establish the amount of cyanide contained in the flavin-substrate adduct. This was accomplished by first separating the \(^{14}\text{C}\)-labeled inhibited enzyme from free \(^{14}\text{CN}^-\) on Sephadex G-25 as shown in Fig. 15. On the basis of \( \epsilon_{\lambda} = 5.4 \times 10^3 \text{ M}^{-1} \text{cm}^{-1} \) for \( EI \) (see Fig. 14), the ratio of \(^{14}\text{CN}^-\) to flavin in the pooled fractions containing \( EI \) (the first peak in Fig. 15) was 0.81. Samples of \(^{14}\text{CN}^-\)-labeled holoenzyme which had been separated by gel filtration were then resolved by hot 80\% methanol into flavin adduct and apoenzyme (which precipitated) and the supernatant solution containing the free flavin adduct was taken to dryness (without heating) by flash evaporation. The residue was dissolved in 70\% methanol and part of this solution was cycled twice more through the flash evaporation procedure. The ratios of \(^{14}\text{C} \) to flavin in these examples, namely 0.92 and 0.90, established that the specific radioactivity of the flavin adduct is reasonably constant and that the free flavin adduct contains 1 eq of cyanide. Next, a sample of the flavin adduct in 0.1 M sodium pyrophosphate, pH 7.9, which had undergone three cycles of flash evaporation, was heated at 70\° C for 10 min. This procedure, as we shall show, converts the free adduct to FAD and acetaldehyde. Part of the heated solution was analyzed for the ratio of \(^{14}\text{C}:\text{FAD} \), which was found to be 1.02. The increase in this ratio, compared to the values obtained before heating, is probably due to incomplete conversion to FAD (see "Discussion"). The remainder of the heated solution was made 80\% in methanol and subjected to two cycles of flash evaporation (with heating). Control experiments showed that between 80 and 90\% of free \(^{14}\text{CN}^-\) would be lost from the solution of adduct during flash evaporation under these conditions. The ratio of \(^{14}\text{C}:\text{FAD} \) in the solutions of adduct which had been heated and then subjected to flash evaporation was found to be 0.40 and 0.37, indicating that heating the flavin adduct at 70\° C for 10 min removes most of the cyanide. No intensive efforts were made to see whether this ratio approached zero after very long times of flash evaporation. These experiments do suggest, however, that a small fraction of the adduct is converted in a reaction with a relatively low activation energy to a species from which cyanide can not be released on heating (see "Discussion"). Such a species would explain why the ratio \( A_{320}/A_{440} \) in the FAD spectrum (see Fig. 18) is about 20\% too large (23). The results of the experiments involving \(^{14}\text{CN}^-\) are given in Table III. In summary, we conclude that both the inhibited enzyme (\( EI \)) and the free flavin adduct contain 1 eq of cyanide and that the free adduct, when heated to 70\° C for 10 min at pH 7.9, produces FAD and free cyanide in good yield.

The number of substrate equivalents bonded to the flavin in the holoenzyme intermediate \( (EX, \text{see Equation 11}) \) with which cyanide rapidly reacts was determined by measuring the catalytic activity remaining at saturating cyanide as the enzyme was titrated with nitroethane anion. Essentially no turnover occurs under these conditions (see Fig. 12). These results are graphed in Fig. 16 and show that the formation of fully inhibited enzyme requires the addition of 1 eq of nitroethane anion per FAD. Therefore, \( EI \) must contain one substrate equivalent per flavin.

It was next of interest to show whether or not \( EI \) contained the nitro group. This was carried out by inactivating the enzyme with saturating cyanide in the presence of nitroethane anion and then analyzing for the presence of NO\(_2^-\) after gel filtration on Sephadex G-25. \( Fig. 17 \) shows that each enzyme-bound FAD equivalent forms 1 eq of free NO\(_2^-\) before or during inactivation. Again, comparison of the experimental conditions of \( Fig. 17 \) and those of \( Fig. 12 \), with particular emphasis on the saturating cyanide concentrations employed, rules out the possibility that the NO\(_2^-\) detected in \( Fig. 17 \) resulted from substrate turnover. As an additional check, however, the consumption of \( O_2 \) in the experiments of \( Fig. 17 \) was measured and found to be negligible.

*Studies of Isolated Flavin Adduct—*The isolated flavin adduct, which we have shown contains 1 eq each of substrate and cyanide, but is lacking nitrite, is readily oxidized to FAD by heating at 70\° C for 10 min at pH 8.3 in the presence of \( O_2 \) (see \( Fig. 18 \)). Free cyanide is released during this reaction, as was shown in Table III. The yield of FAD in this reaction is about 70\%, whether the calculation is based on the amount of \(^{14}\text{CN}^-\) released or on the value of \( \epsilon_{320} \) (namely \( 6.3 \times 10^4 \text{ M}^{-1} \text{cm}^{-1} \)) for the free flavin adduct at pH 8.3. When the adduct is heated at 70\° C at pH 8.3 in the absence of \( O_2 \), the characteristic spectrum of the adduct is replaced by a spectrum which is very similar to that of FADH\(_2\) (see \( Fig. 18 \)). When \( O_2 \) was admitted to the solution of the adduct which had been heated anaerobically at 70\° C FAD was formed extremely rapidly (see \( Fig. 18 \)), as would be expected if FADH\(_2\) had been formed. The FAD formed in these reactions was identified not only by its spectral properties but also by its unique ability to activate the apoenzyme of \( \alpha \)-amino acid oxidase to the same extent as authentic FAD. The trace of FAD evident
TABLE III

Experiments with $^{14}$CN$^-$ showing that EI and the flavin-substrate adduct each contain 1 eq of cyanide per flavin and that most of this cyanide is released upon heating the adduct aerobically at pH 8.3 and 70° for 10 min. The starting material for these experiments was the $^{14}$CN$^-$-labeled EI which was obtained as described in the legend of Fig. 15.

| Experiment | Volume | $A_{280}$ | $A_{450}$ | Total radioactivity | Cyanide per flavin |
|------------|--------|-----------|-----------|--------------------|-------------------|
| 1. Pooled fractions of $^{14}$CN$^-$-labeled EI from Sephadex G-25 Column (see Fig. 15) | 9.2 | 0.280 | 8.81 | $10^4$ | 0.81$^c$ |
| 2. EI from Experiment 1 resolved by hot 80% methanol. Adduct concentrated by flash evaporation (without heating) and dissolved in 70% ethanol | 10.0 | 0.255 | 9.22 | $10^4$ | 0.92$^b$ |
| 3. Adduct from Experiment 2 concentrated by flash evaporation (without heating) and dissolved in 70% ethanol | 10.0 | 0.255 | 8.97 | $10^4$ | 0.90$^b$ |
| 4. Adduct from Experiment 3 flash-evaporated (without heating), dissolved in 70% methanol and flash evaporated again (without heating). Adduct then dissolved in 0.1 M sodium pyrophosphate, pH 8.3, and heated aerobically at 70° for 10 min | 3.0 | 1.05 | 7.05 | $10^4$ | 1.02$^c$ |
| 5. Solution from Experiment 4 made 80% in methanol, flash-evaporated (with heating) and redissolved in 80% methanol. Control experiments showed that between 80 and 90% of free $^{14}$CN$^-$ would be lost from the solution of adduct under these conditions | 10.0 | 0.272 | 2.38 | $10^3$ | 0.40$^c$ |
| 6. Solution from Experiment 5 flash-evaporated (with heating) and redissolved in 80% methanol | 10.0 | 0.262 | 2.08 | $10^3$ | 0.37$^c$ |

$^a$ Obtained by comparing absorbance of EI at 332 nm ($A_{332} = 5.4 \times 10^3$ m$^{-1}$ cm$^{-1}$) with the specific radioactivity of the $^{14}$CN$^-$ (2.48 $\times 10^4$ dpm per pmole).

$^b$ Obtained by comparing absorbance of adduct at 320 nm ($A_{320} = 6.3 \times 10^3$ m$^{-1}$ cm$^{-1}$) with specific radioactivity.

$^c$ Obtained by comparing absorbance of the FAD at 450 nm ($A_{450} = 11.3 \times 10^3$ m$^{-1}$ cm$^{-1}$) with specific radioactivity.

Fig. 16. Determination of number of substrate equivalents bound per flavin in EI. These experiments were based on the fact that at high cyanide concentrations the enzyme is stoichiometrically inhibited by substrate because all but 5% of turnover is prevented (see Fig. 12). Enzyme (0.064 mM) was incubated with nitroethane carbanion (at concentrations giving the ratio of substrate to enzyme-bound FAD indicated on the abscissa) and 0.2 mM cyanide in 0.1 M sodium pyrophosphate, pH 8.3, at 25°. The percentage of activity remaining was determined by the standard assay with n-alanine (see "Methods"). The observed abscissa intercept of 1.1 is higher than the expected value of 1.0 because of the small amount of turnover which occurs under these conditions (see Fig. 19).

Fig. 17. Demonstration that EI does not contain nitrite. The figure shows the separation of EI (first peak) from nitrite (second peak) on Sephadex G-25. EI (X---X) was measured at 330 nm while nitrite (O---O) was measured at 524 nm according to the method of Griess-Ilosvay (see "Methods"). EI was formed by incubating 0.112 mM n-alanine oxidase with 0.2 mM KCN and 1.0 mM nitroethane carbanion (total volume 3.75 ml) in 0.1 M sodium pyrophosphate, pH 8.3, at 25°. The O$_2$ consumed during inactivation corresponded to 0.109 eq of enzyme-bound flavin (compare with Fig. 12). After the products were separated on Sephadex G-25 (25 X 1 cm column) as shown in the figure, 0.42 pmole of EI and 0.46 pmole of nitrite were detected. After correction for the small amount of nitrite produced during turnover, this result shows that 0.98 eq of nitrite was released for each equivalent of EI produced by cyanide treatment.

in the spectrum obtained by heating under anaerobic conditions (Fig. 18) does not arise from the leakage of O$_2$ because the ratio of $A_{445}$ after heating aerobically, to $A_{445}$ after heating anaerobically and admitting O$_2$, was the same in two experiments in which the adduct concentrations differed by a factor of 2.

We find that the aerobic conversion of the cyanide-containing flavin adduct to FAD is extremely sensitive to pH and temperature. Less than 1% reaction occurred at 25° and pH 8.3 after
solution. FLD was also obtained in similar yield by admitting nm is used in Fig. 19 as the basis for a spectrophotometric titration of various well characterized flavin derivatives (25-28). Fig. 19 shows the absorption spectra of the adduct using, as a basis for comparison, the unique spectral and turnover reaction. We have noted, however, that after storage, it is no longer possible to obtain a good yield of FAD upon the addition of acetaldehyde to be 37.5% too low, owing to the reaction of cyanide release, the reaction of the adduct at 260 nm, corresponding to a pKₐ value of 6.4.

10 min, while only about 40% conversion took place when the adduct was heated to 70°C for 10 min at pH 5.2.

Some of the adduct solution which had been heated at 70°C in the presence of O₂ until the formation of FAD had ceased was examined for the presence of acetaldehyde by measuring the consumption of NADH in the alcohol dehydrogenase reaction at pH 6.0. When 0.09 mM adduct was heated aerobically, 0.034 mM acetaldehyde and 0.068 mM FAD were detected. Control experiments showed that the amount of free cyanide released from the adduct during heating would cause the observed yield of acetaldehyde to be 37.5% too low, owing to the reaction of cyanide with acetaldehyde. When this correction factor was applied, the ratio of acetaldehyde to FAD detected after heating the adduct became 0.80. No acetaldehyde was detected before the adduct was heated. Although measurements of H₂O₂ formation which would be difficult) were not attempted, we can conclude with some confidence that, after cyanide release, the reactions undergone by the freshly prepared adduct at 70°C give rise to exactly the same products as are observed in the enzymatic turnover reaction. We have noted, however, that after storage of the adduct at 5°C for 8 days in the presence of O₂ and at pH 8.3, it is no longer possible to obtain a good yield of FAD upon heating at 70°C.

We have investigated the structure of the substrate-flavin adduct using, as a basis for comparison, the unique spectral and ionization properties of various well characterized flavin derivatives (25-28). Fig. 19 shows the absorption spectra of the adduct at pH values of 9.75 and 2.25. The shift in λₘₙₐₓ (320 → 330 nm) should be noted. The large absorbance change at 260 nm is used in Fig. 19 as the basis for a spectrophotometric titration, resulting in a pKₐ value of 6.4. When the flavin-substrate adduct was subjected to 6 N HCl the solution became purple, having absorption bands at 525, 390, 355, and 308 nm. This must represent oxidation of the adduct cation to give a mixture of products (including a free radical species which was detected by ESR measurements). Oxidation of the cationic adduct appears to be very facile, since attempts to obtain the spectrum at 10°C, through which N₂ was bubbled, were unsuccessful. Addition of nitrite to the acid solution slowly generated the pale yellow flavoquinone chloride (FAD-HCl) which, upon neutralization with KOH, was deprotonated to form neutral flavoquinone (FAD).

**Discussion**

Our experiments enable us to propose a detailed chemical mechanism for a flavoprotein oxidase reaction. In order to facilitate the presentation and discussion of this mechanism, we shall first bring together the major facts which have resulted from our spectrophotokinetic and chemical studies of the oxidation of nitroethane anion by d-amino acid oxidase. These facts are as follows:

1. E₀ equilibrates rapidly with S- (Kₛ = 14 mM) to form E₀S, the spectrum of which is similar to that of E₀ except for weak absorbance at long wave lengths.

2. E₀S is converted to E₃P at a maximum rate of 6.2 s⁻¹.

3. Release of acetaldehyde (P) from E₃P is not an obligatory process in turnover.

4. E₃P reacts with O₂ to form E₅P with a bimolecular rate constant of about 10⁴ M⁻¹ s⁻¹.

5. Acetaldehyde (P) is released from E₅P at a rate of 4.5 s⁻¹ to regenerate E₀.

6. The enzyme reacts rapidly with cyanide during turnover to give a spectrophotometrically distinct species, E₁, which is incapable of reaction with either S⁻ or O₂. The rate of formation of E₁ is identical both with the rate of inactivation of the enzyme and with the rate of conversion of E₀S to its immediate product. The inactivation of the enzyme by cyanide is therefore regulated by kₛ (6.2 s⁻¹), representing the first order breakdown of E₀S during catalysis.

7. Inactivation of the enzyme by cyanide is not an O₂-requiring process. Increasing concentrations of either S⁻ or O₂ increase the rate of inactivation by cyanide. Cyanide must react, therefore, with a species EX which lies between E₀S and E₃P.

8. Nitrite is eliminated either prior to, or concomitant with, the attack of cyanide on EX.

9. The inactivated species E₁ contains, per flavin, 1 eq each of substrate (lacking the nitro group) and cyanide and has an
absorption spectrum at pH 8.3 which is characterized in particular by a maximum at 332 nm ($\epsilon_{332} = 5.4 \times 10^4$ M$^{-1}$ cm$^{-1}$).

10. The species $EI$ can be resolved in high yield into apoenzyme and a cyanide-containing covalent flavin-substrate adduct. Resolution is accompanied by a 12-nm blue shift at pH 8.3 in the absorption maximum of the adduct ($\epsilon_{320} = 6.3 \times 10^3$ M$^{-1}$ cm$^{-1}$).

11. The absorption maximum of the free flavin-substrate adduct undergoes the shift 320 $\rightarrow$ 330 nm as the solvent is changed from pH 9.75 to 2.52. Protonation of the anionic flavin-substrate adduct (corresponding to the change in absorption maximum from 320 to 330 nm) occurs with a $pK$ value of 6.4.

12. The free flavin-substrate adduct generates, in the presence of $O_2$, the normal products of the enzyme-catalyzed reaction, namely, approximately 1 mole each of FAD and acetaldehyde (and presumably $H_2O$), in a highly temperature- and pH-sensitive reaction.

13. In the absence of $O_2$, the free flavin-substrate adduct generates $FADH_2$.

14. In the presence of 6 N HCl and nitrite, the free flavin-substrate adduct produces $FADH^+$.

These facts are logically accommodated by the following chemical mechanism (Scheme 1).

The basic framework of Scheme 1 results from the interpretation of the stopped flow spectrophotometric measurements. The position of $ES$ is rigorously determined from the correlation of the effects of cyanide with the kinetic results. However, as in all kinetic studies, only those intermediates whose breakdown is wholly or partially rate-limiting in the reaction sequence under study will influence the rate measurements. Consequently, we have added certain intermediates, which, by well known chemical processes, serve to connect logically the other species for which direct spectrophotometric and chemical evidence exists. Our discussion of chemical mechanism is predicated throughout on the evidence obtained from studies of the isolated flavin-substrate adduct which, as we shall show, implicates covalent interaction of the substrate at the N$^2$ position of the flavin nucleus.

$ES$ is visualized as a noncovalent complex ($K_1 = 14$ mM, see Fig. 4) which is in rapid equilibrium with $ES$ and $S^-$ and in which the substrate carbanion is bound at the substrate site very close to the N-5 position of the flavin nucleus. The spectral properties of $ES$, particularly the long wave length absorption (see Fig. 3), must reflect electronic interaction between the donor carbanion and the acceptor flavin. We have measured by flow methods a spectrum very similar to that of $ES$ when D-amino acid oxidase interacts with $\beta$-chloroalanine (29).

The next intermediate for which kinetic and spectral evidence exists is $EIP$, which is formed from $ES$ at a limiting rate of 6.2 s$^{-1}$ (see Figs. 2 and 5). Because of the resemblance of the spectrum of $EIP$ to that of $ES$ (see Fig. 3) we tentatively identify $EIP$ as a noncovalent complex in which acetaldehyde resides close enough to the enzyme-bound $FADH_2$ to slightly perturb the electronic properties of the latter. The simplest hypothesis accounting for the initiation of covalent catalysis, considering the results of this paper and the lack of any experimental evidence to the contrary, is that of nucleophilic attack of the substrate carbanion within $ES$ on N-5 of the flavin nucleus, to give 5-nitroethyl-1,5-dihydroflavin. Although the latter intermediate is not observed spectrophotometrically, its spectrum is expected to resemble that of an enzyme-bound 5-substituted 1,5-dihydroflavin (such as $EI$ shown in Fig. 14) and to be quite different from that of $ES$. We assign $k_2$ (with a value of 6.2 s$^{-1}$) as representing the attack of the substrate carbanion at the N-5 flavin position. This is an interesting result, since we have been unable to detect any nonphotochemical reaction of nitroalkanes (equilibrated at pH 8.3) with free FAD. The enzyme, therefore, catalyzes the attack of the bound substrate carbanion at the N-5 position of the adjacent FAD, presumably by assisting the orientation of the carbanion for nucleophilic attack and perhaps by assisting electron redistribution of the flavin nucleus through

3 The interaction of $\beta$-chloroalanine with D-amino acid oxidase in stopped flow anaerobic reactions monitored at 560 nm occurs in four phases (29, 30). The first phase is rapid and is associated with a 3-fold kinetic isotope effect when $\alpha$-deuterated $\beta$-chloroalanine is used (30). The spectrum corresponding to the termination of the first phase (which is almost identical with the spectrum observed after 5 min, as shown in Fig. 1 of Reference 29) closely resembles the spectrum of $ES$ obtained with nitroethane carbanion (see Fig. 3, this paper). It seems likely, therefore, that the species formed in the first phase of the $\beta$-chloroalanine reaction is the enzyme-bound substrate $\alpha$-carbanion.
protonation of N-1. Evidence presented subsequently suggests that the N-1 position of the enzyme-bound flavin-substrate ad-duct is unusually basic. The chemical events following the formation of 5-nitroethyl-1,5-dihydroflavin can be deduced by considering the action of cyanide on the enzyme during turnover. We note that nitrite is eliminated either prior to, or simultaneously with, the attack of cyanide to form EI (see Fig. 17) and that O₂ reacts with E,P. The location of EX, the intermediate which is rapidly attacked by cyanide, can be determined as follows. Firstly, EX is not E₅S, because the rate of cyanide attack on EX is controlled by k₅ which has already been assigned as the attack of the substrate carbanion on flavin within E₅S. Consequently, EX must lie beyond E₅S. Secondly, no O₂ is consumed during rapid inactivation at high cyanide concentrations (see Fig. 12). Therefore EX can not be an intermediate derived from E₅P after the re- action of the latter with O₂. The possible location of EX is now limited to the reaction sequence spanned by 5-nitroethyl-1,5-dihydroflavin and E₅P, and including these species. E₅P is elimi- nated as a candidate for EX by the important finding (Fig. 8B) that the interactions of O₂ and cyanide with the enzyme in turn do not result in competitive behavior between these ligands. Such behavior (i.e., a monotonic decrease in k₅obs when plotted according to Fig. 8B) would be clearly expected if O₂ and cyanide were both reacting with E₅P. In fact, the reverse is true, since the effect of increasing the concentration of either nitroethane anion (Fig. 8A) or O₂ (Fig. 8B) is to increase the rate of inactivation (and hence the rate of formation of EI from EX) of the enzyme. Candidates for EX are now limited to 5-nitroethyl-1,5-dihydroflavin and the precursor of E₅P. The reaction of CN⁻ with either of these species would lead to the results of Fig. 8, A and B. However, direct E₅F⁺ displacement of nitrite from the 5-nitroethyldihydroflavin adduct by cyanide seems very unlikely on chemical grounds, since this process would have to proceed at a rate greatly exceeding 6.2 s⁻¹. Therefore we propose a well known type of chemical process, namely the elimination of NO₂⁻ from 5-nitroethyl-1,5-dihydroflavin. The assignment of the resulting cationic imine as EX explains why 1 eq of free nitrite is released during cyanide inhibition and, most important-ly, it provides the highly electrophilic center with which cyanide must react.

Identification of EX as a cationic imine at the N-5 position of the enzyme-bound flavin rationalizes not only the high reactivity of cyanide with the enzyme during turnover, but also the chemical pathway by which E₅S reaches E₅P during turnover in the absence of cyanide. By nucleophilic attack on the cationic imine (in analogy with, and competitive with, the attack of cyanide when the latter is present), H₂O or, less likely (31) OH⁻, forms a carbinolamine at the N₅ position from which dihydroflavin is eliminated to give acetaldehyde attached noncovalently to the fully reduced enzyme (E₅P). In order to explain why E₅O, E₅S, and E₅P are the only species detected spectrophotometrically in the stopped flow reductive half-reactions in the absence of cyanide we must conclude that nitrite elimination, carbinolamine formation, and subsequent elimination of dihydroflavin from the carbinolamine are all extremely rapid compared to the rate (6.2 s⁻¹ corresponding to k₅) of carbanion attack on flavin in the complex E₅S. Similarly, the rate of cyanide attack (corresponding to k₅obs(CN⁻), see Equation 11) on the cationic imine must greatly exceed 6.2 s⁻¹ in order that k₅ regulate the rate of formation of EI. The cationic imine would be expected to have a distinctive spectrum but because of its extreme reactivity in aqueous solution the spectra of compounds like this have not been detected

in model systems (25, 26). It is of interest, however, that anaerobic half-reaction measurements of the interaction of glu- cose oxidase with nitroethane anion revealed a spectrum cor- responding to a well known tautomer of the cationic imine, namely 5-ethyl flavoquinonium ion.⁴ It appears very likely therefore that the mechanism of Scheme I, at least for the se- quence E₅O → EX, applies also to the glucose oxidase reaction. The reductive half-reaction in the α-amino acid oxidase mechan- ism is completed by the dissociation of acetaldehyde (P) from E₅P to give enzyme-bound 1,5-dihydroflavin which is indistin- guishable from that produced by D-alanine (see Fig. 3). The latter is oxidized rapidly by O₂ with a bimolecular constant k₄ = 2.5 x 10⁴ M⁻¹ s⁻¹ to regenerate E₅O. However, the rate of disso- ciation of acetaldehyde from E₅P (₄₅₀ = 69 s, see Fig. 5) is competitive with the oxidation of E₅P by O₂ only at O₂ concentra- tions of the order of 1 μM. In routine turnover experiments, therefore, the dissociation of E₅P to give E₅ is not an obligatory process in the catalytic mechanism.

The deduction that EX lies directly on the catalytic path- way between E₅S and E₅P is obvious of great importance and warrants further scrutiny. Given the results of the flow measurements of the anaerobic half-reaction and the fact that O₂ is not required for CN⁻ inhibition, a possible alternative to Scheme I and Equation 11 is that given by Equation 12.

|  |  |  |
|---|---|---|
|  |  |  |

Here we might assume that the noncovalently bound acetal- dehyde in E₅P interacts accidentally with reduced flavin in a rapid equilibrium process (in order that the formation of EI be governed by the breakdown of E₅S) to form the cationic imine (EX). If this were so, the fact that EX can be trapped by CN⁻ as EI could not be used as evidence that covalent ad- duct formation between the substrate carbanion and the N-5 position of the flavin is an obligatory event in catalysis. How- ever, there is a great deal of evidence which is inconsistent with the scheme of Equation 12. Firstly, Equation 12 is kinetically equivalent to the case of direct attack of CN⁻ (as well as O₂) on E₅P and predicts that the rate of inhibition by CN⁻ should be decreased as the O₂ concentration is raised. As we have already emphasized, this is precisely opposite to the experimental results (see Fig. 8B). Secondly, we note from Fig. 13 that about one-third of the enzyme is converted to EI under anaerobic conditions in the presence of, for example, 10 mM CN⁻. Equation 12 (and the kinetically equivalent scheme in which both CN⁻ and O₂ react with E₅P) on the other hand would predict that at least 97% conversion to EI should occur un- der these conditions, because E₅P will be partitioned into EI and E₅ in the ratio of the rate of inactivation under these conditions (0.3 s⁻¹) to the competing rate of dissociation of E₅P (0.01 s⁻¹). Thirdly, we have treated E₅ (formed by re- action with 1.0 mM D-alanine) with acetaldehyde and CN⁻ under a variety of conditions and have failed to detect inactiva-
tion of the enzyme. Although the last mentioned experiment has certain defects (including the problems of rapid reaction of acetaldehyde and CN\(^{-}\), the uncertainty of whether significant amounts of \(E_P\) were formed and the validity of the spectroscopic assignment of \(E_P\) as \(E_1 = \text{CH}_3\text{CHO}\)) we can state that, without exception, all the experimental evidence supports Equation 11 whereas Equation 12 (as well as kinetically equivalent schemes) is clearly inconsistent with the results of several unambiguous experiments. It must be pointed out, however, that our experiments would not differentiate the scheme of Equation 11 from one in which \(EX\) is a rapidly equilibrating side product of the immediate product of \(ES\). This is an unfortunate dilemma inherent to all kinetic analyses. However, such a mechanism is deficient in two respects. Firstly, it is unreasonable to suppose that all covalent interactions of substrate with the flavin occur as uncatalyzed and fortuitous side reactions because free FAD is completely unreactive with the nitroalkane carbanions in the dark (6). Secondly, if it is supposed that \(N^\text{a}\) adducts are obligatory intermediates, but that the cationic imine \((EX)\) happens to be a rapidly equilibrating side product, then it is not obvious on chemical grounds why substrate oxidation should be so facile.

The makeup of the coefficients of the general steady state rate law (Equation 6) can now be stated as follows (Equation 13).

\[
\frac{E_P}{v} = \frac{k_f + \frac{k_se}{k_b} + \frac{k_{se}}{k_b}S + \frac{1}{k_b[O_2]}}{1 + \frac{1}{k_b[O_2]}}
\] (13)

It has been our general experience that the composition of the steady state coefficients for flavoprotein oxidation reactions can be routinely identified by the kind of correlation of steady state and rapid flow measurements described here. It is not possible, of course, to deduce the composition of the coefficients from steady state measurements alone.

The trapping and isolation of the covalent flavin-substrate adduct, together with the precision with which the locus of action of cyanide has been determined, are the pivotal results of this paper. Fortunately, there exists a large body of information concerning the physical properties and chemical reactivity of a wide variety of substituted flavins (24-28). This knowledge is in turn soundly based on structural determinations by x-ray crystallographic methods (32). We shall now summarize the evidence which points to the structure of our isolated cyanide-containing flavin-substrate adduct as being 5-cyanoethoxy-1,5-dihydroFAD.

Firstly, the hydroquinone structure of FADH\(_2\) is characterized by a \(pK_a\) value of 6.2 owing to the ionization of \(N^1\) (28, 27). The remaining three \(pK_a\) values governing the interconversions of the anionic, neutral, and cationic states of \(\text{FADH}_2\) and FAD are far removed from 6.2. The \(pK_a\) value of 6.4 obtained for ionization of the neutral flavin-substrate adduct (see Fig. 19) is therefore diagnostic of substitution at the \(N^1\) position, since substitution at any other position in the pyranine or pyrimidine rings would seriously perturb the molecular structure determining the basicity of \(N^1\). Thus 4a-flavin derivatives do not ionize in the \(pK_a\) range from 2 to 11 (25, 28, 33). 5-Alkylated 1,5-dihydroflavins have the expected \(pK_a\) values in the range from 6 to 7 (25, 27, 28). In particular, 5-cyanoethoxy-1,5-dihydroFAD has a \(pK_a\) value of 6.4 ± 0.2. Secondly, the \(\lambda_{\text{max}}\) and extinction coefficient values of 5-substituted 1,5-dihydroflavins differentiate these derivatives from all others. Without exception, the anionic 5-substituted derivatives have \(\lambda_{\text{max}}\) values between 300 and 355 nm with extinction coefficients in the range from 5 to 8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} (25, 28). The corresponding values for the flavin-substrate adduct (see Fig. 18) fall well within these ranges. In particular, the anionic states of 5-isopropyl-1,5-dihydroflavin (28) and 5-cyanoethyl-1,5-dihydroflavin have \(\lambda_{\text{max}}\) values of 325 and 326 nm, respectively. These are very similar to our value of 320 nm (see Fig. 18). Further, the protonation of \(N^1\) in 5-substituted 1,5-dihydroflavins (corresponding to the \(pK_a\) value of 6.4 in Fig. 19) causes a characteristic \(\lambda_{\text{max}}\) shift to longer wave lengths (25, 28). This shift is 10 nm in our case (Fig. 19) and is 15 and 16 nm, respectively, for 5-isopropyl, and 5-cyanoethyl dihydroflavin. Thirdly, the chemical reactivity of our adduct closely resembles that of 5-cyanoethyl dihydroflavin in particular, and is similar to that of 5-substituted dihydroflavins in general (25, 28). Thus, addition of \(\text{NO}_2^-\) to the flavin-substrate cationic adduct in 6 \(\times\) HCl causes oxidation to \(\text{FAD}^+\) and is observed with many \(N^1\) derivatives (25, 28). However, the cationic adduct is more easily oxidized than many cationic 5-alkyl dihydroflavins, since a mixture of oxidized products (including a flavin radical species) resulted from exposure of the adduct in 6 \(\times\) HCl to \(O_2\). This unusual behavior has also been seen with 5-cyanoethyl dihydroflavin. 5-Cyanoethyl-1,5-dihydroflavin has also been found to undergo facile oxidation when in the anionic state. Thus in dry organic solvents it is unreactive toward \(O_2\) while in water at \(pH\) 8 and in the presence of \(O_2\) the free oxidized flavin is produced in good yield. The behavior is identical with that which we have observed for the substrate-flavin adduct. In summary, therefore, comparison of the physical and chemical properties of the flavin-substrate adduct with those of known flavin derivatives (particularly 5-cyanoethyl-1,5-dihydroflavin) shows that the flavin adduct trapped by cyanide during turnover of nitroethane by \(N\)-amino acid oxidase is most probably 5-cyanoethyl-1,5-dihydro-FAD as is shown in Scheme 1.

We have observed that the isolated cyanoethyl dihydroflavin adduct, when heated to 70° at basic \(pH\) values, generates the same reaction products as those observed in the reaction directed by the enzyme. It seems likely that the mechanism of the solvent catalyzed oxidation of the free flavin adduct mimics, at greatly reduced efficiency, the enzyme-catalyzed pathway \(EX \rightarrow E,P \rightarrow E_1 \rightarrow E_2\). In particular, it will be noted that in the absence of \(O_2\) a major product is \(\text{FADH}_2\) (see Fig. 18). This is exactly analogous to the formation of \(E,P\) in the enzymatic mechanism (see Scheme 1) and suggests that heating of the free adduct is required for cyanide elimination, after which hydration of the cationic imine and subsequent elimination of \(\text{FADH}_2\), to form acetaldehyde, proceed rapidly. In the presence of \(O_2\), \(\text{FAD}\) and \(H_2O_2\) are formed extremely rapidly from \(\text{FADH}_2\) with a bimolecular rate constant of about \(10^6 \text{ M}^{-1} \text{ s}^{-1}\) (34). The chemical feature which permits the (anaerobic) oxidation-reduction process in the case of the carbaminoamine adduct is the electron-releasing property of the hydroyxy group. This is two bonds removed from \(N-5\) of the flavin nucleus and allows the elimination of \(\text{FADH}_2\) from the methyl-1,5-dihydro(iso)alloxazine and kindly communicated to us the spectral, ionization, and chemical properties of this compound.
carbinolamine. The fact that this feature is entirely missing in 5-eyanoethyl-1,5-dihydro-FAD probably explains why this compound is relatively inert to oxidation by O₂ at 25°, both on and off the enzyme. The marked pH dependence of adduct oxidation at 70° is probably explained by the ionization of N⁶. The anionic adduct would be expected to expel the negative cyanide ion much more effectively than the neutral adduct. The fact that the pKₐ value of the N⁶ proton may be appreciably higher when the adduct is bound to the enzyme (which is suggested by the similarity in the λₑₓₐₚ values for E₁ and for the neutral adduct as shown in Figs. 14 and 19, respectively) would also tend to stabilize the enzyme-bound cyanomethyl adduct toward oxidation.

Equation 14 accounts for at least 70% of the reaction undergone by the freshly prepared adduct at 70° and pH 8.3. There appear to be at least two other reactions taking place at pH 8.3. Firstly, traces of FAD are formed in the absence of O₂ (see Fig. 18). This could occur by elimination of the resonance-stabilized carbanion of CH₂CN. Secondly, storage of the adduct for 8 days at 5° and pH 8.3 in the presence of O₂ yields only about 40% of the theoretical amount of FAD. Heating at 70° had little effect on the spectrum which, apart from a peak at around 459 nm corresponding to FAD, showed a broad band in the 330 to 350 nm region. It is possible that the latter absorption is due to the pseudo base derived from the addition of OH⁻ to C⁵ of the oxidized adduct (29).

It has now been clearly established that flavoproteins as well as free flavins (35-37) can act as efficient (nonphotocatalytic) catalysts of carbanion oxidation. ⁶ It seems likely that the enzyme from Neurospora which was found by H. N. Little (38) to catalyze nitroalkane oxidation was in fact a flavoprotein oxidase.

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6 It seems likely that the enzyme from Neurospora which was found by H. N. Little (38) to catalyze nitroalkane oxidation was in fact a flavoprotein oxidase.

7 Calculations of super-delocalizability, frontier orbital density, and total electron density by Dr. Pill Soon Song (Texas Tech University, private communication) all show that nucleophiles such as carbanions will add to the N⁶ position of the flavin nucleus in preference to the C⁵ or C⁶α positions.
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Direct Evidence for Carbanions and Covalent N$^5$-Flavin-Carbanion Adducts as Catalytic Intermediates in the Oxidation of Nitroethane by d-Amino Acid Oxidase

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