EFFECT OF CYANIDE ON THE L-AMINO ACID OXIDASE REACTION*

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Cyanide had no effect on any of the processes in the catalytic mechanism of the L-amino acid oxidase reaction. However, the lifetime of the complex of reduced enzyme with product (E_rP) was greatly reduced by cyanide in stopped-flow turnover experiments at non-inhibitory levels of substrate, but unaffected by cyanide at inhibitory levels of substrate. These results were interpreted to mean that by trapping the free imino acid which accumulates transiently in the reaction cyanide lowers the concentration of the first product, P, which is released by the enzyme. By analogy with the known hydrolytic behavior of imines, it is concluded that P must be the imino acid which is known to be a product of the enzyme reaction. Since the interaction of P with E_r is freely reversible, the level of E_rP in turnover can be reduced either by trapping P with cyanide or by trapping E_r with substrate as E_rS.

The minimal kinetic scheme (1,2,3) for the oxidation of L-phenylalanine (S) by O_2 catalyzed by L-amino acid oxidase is the following (Equations 1-4)

$$E_o + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_oS \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_rP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E_r + P \qquad (1)$$

$$E_rP + O_2 \xrightarrow[H_2O_2]{k_5} E_oP \xrightarrow{k_6} E_o + P \qquad (2)$$

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$$E_r + O_2 \xrightarrow{k_4} E_o + H_2O_2 \tag{3}$$

$$E_r + S \overset{K_1}{\rightleftharpoons} E_rS + O_2 \xrightarrow{k_7} E_oS + H_2O_2 \tag{4}$$

It has been generally thought that, in analogy with the lactone released by glucose oxidase (4), the first free product, P, released by the amino acid oxidases is the imino acid, from which the α-keto acid is derived non-enzymatically via a carbinolamine. The best evidence for the existence of free imino acid has been the recovery of racemic amino acid after borohydride reduction (5), while transient pH changes during turnover have been interpreted as being due to imino acid accumulation (6).

In the course of a detailed kinetic study of the L-amino acid oxidase reaction, we found at pH values less than seven that the behavior of E_rP in stopped-flow turnover experiments monitored at 550 nm, when compared by inspection and computer simulation with the rate of O_2 depletion, was entirely consistent with its position in the kinetic scheme of Equations 1-4. However, at pH values greater than 7, the concentration of E_rP was unexpectedly high during turnover and even remained at a high level for several seconds after the O_2 had been depleted. We subsequently realized that this apparent anomoly is probably due to the transient accumulation of free imino acid (only the protonated form of which can be hydrated (7)), at a concentration approximating k_3/k_{-3}. The importance of the reversible interaction of P with E_r, which has not been recognized previously, is demonstrated in this communication by trapping the imino acid with cyanide. (7).

MATERIALS AND METHODS: L-Phenylalanine (Grade A) and lyophilized *Crotalus adamanteus* venom were obtained from Calbiochem. and the Ross Allen Reptile

Institute, respectively. The enzyme was purified by a modification of a published procedure (8) and showed a ratio A_{276}/A_{462} of 10.5 to 11.0. Turnover numbers were measured on the Yellow Springs O_2 electrode system, while stopped-flow spectrophotometric measurements were carried out on the Gibson-Durrum apparatus. Solutions were made anaerobic for reductive half-reaction (Equation 1) measurements with 1.0 mM D-glucose and 0.1 μM glucose oxidase. The enzyme concentration is expressed as μM FAD and all reactions were carried out at 25° and pH 8.1 in solutions containing 0.2 M KCl and 0.1 M Tris-HCl.

RESULTS AND DISCUSSION: It was first necessary to determine whether cyanide, in addition to trapping the imino acid, perturbed any of the steps in the catalytic mechanism.

Figure 1 shows that the cyanide has no effect on the reductive half-reaction (Equation 1) when this is monitored at 550 nm. The stopped-flow trace, which reflects the formation of E_rP from E_o and its subsequent decay to E_r, is a function of all the steps in Equation 1. Therefore, with the possible exception of k_{-3} (which exerts little control in this experiment because the level of P never exceeds the enzyme concentration) none of the rate constants in Equation 1 is affected by 0.25 M cyanide.

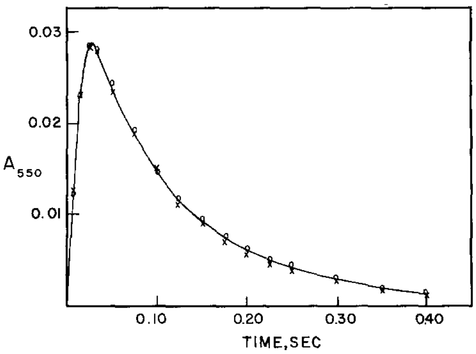

Figure 1. Stopped-flow anaerobic half reactions (see Equation 1) monitored at 550 nm showing the lack of effect of cyanide on the formation and disappearance of E_rP. After mixing, the solutions contained 6.1 μM L-amino acid oxidase, 1.0 mM L-phenylalanine and either no cyanide (-o-) or 0.25 M cyanide (-x-). The pH was 8.1 and the temperature was 25°.

The oxidative steps of Equations 3 and 4 were evaluated from pseudo-first order rate constants for O_2 depletion in O_2-monitored experiments with 0.05 μM enzyme. The rationale of such measurements is that at O_2 concentrations $\leq$ 15 μM, the enzyme exists in turnover almost entirely as E_r and E_rS (the ratio of these being determined by K_1 and the concentration of S) since $k_3 >> k_5\ [O_2]$ and all other steps are rapid compared with $k_4\ [O_2]$ and $k_7\ [O_2]$ (3). As S is increased from 1.0 mM to 75 mM, the rate of O_2 depletion decreases to a plateau value in a hyperbolic fashion, with K_1 corresponding to the value of S at half-maximal change. After dividing by the enzyme concentration, the limiting values of the pseudo-first order constant for O_2 depletion at low and high S correspond to k_4 and k_7, respectively. The value of k_4 obtained in this way agrees well with the value determined directly by mixing E_r with O_2 in the stopped-flow apparatus and monitoring the appearance of E_0 at 462 nm (Equation 3). The values of k_4, k_7 and K_1 ($2.2 \times 10^5\ M^{-1}\ sec^{-1}$, $5.4 \times 10^4\ M^{-1}\ sec^{-1}$ and 14 mM, respectively) were affected no more than 5% by 0.25 M cyanide.

Finally, the effect of cyanide on k_5 and k_6 was tested by measuring the turnover number under conditions (0.24 mM O_2, 1.0 and 75 mM S) where E_0 is regenerated largely through Equation 2. These processes were affected less than 5% by 0.25 M cyanide.

Turning now to the behavior of E_rP as monitored in stopped-flow turnover experiments at 550 nm, we note in Figure 2 that cyanide has a pronounced effect, resulting in a greatly shortened lifetime of E_rP. As we shall subsequently show, it is most probable that P represents the imino acid. In view of the evidence that none of the enzyme catalyzed processes is affected by cyanide, the results of Figure 2 must be due to the trapping of the imino acid, which, in the absence of cyanide, builds up transiently during turnover to levels approximating k_3/k_{-3}. The condition $k_{-3}\ [P][E_r] \rightarrow 0$ is approached at the highest cyanide concentration. The inset of Figure 2 shows the cyanide dependence of the first order decay of E_rP during the last five seconds of turnover, assuming that during this period the decay of

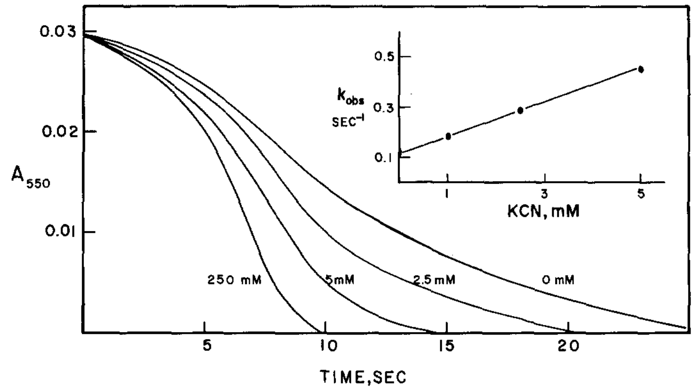

Figure 2. Stopped-flow turnover experiments at low substrate concentration showing the effect of cyanide on E_rP monitored at 550 nm. The cyanide concentration is indicated by each trace. After mixing, the solutions contained 6.1 μM L-amino acid oxidase, 0.24 mM O_2 and 1.0 mM L-phenylalanine. The pH was 8.1 and the temperature 25^{o}. The inset shows the dependence of the first order decay of E_rP during the last five seconds of turnover on the lower cyanide concentrations.

E_rP is controlled by free P and that this in turn is subject to simultaneous destruction by water and cyanide. At 0.25 M cyanide the rate of destruction of P is extrapolated to be 17 sec^{-1}. The possibility that cyanide operates directly on k_{-3} (which is the only rate constant for which strong evidence for cyanide insensitivity has not been obtained) is very unlikely in view of the fact that all other enzyme functions are unaffected by cyanide.

If, as we have argued, the effect of cyanide on the behavior of E_rP during turnover results from a decrease in the interaction of E_r with P through the trapping of P, then the trapping of E_r should have the same effect. High concentrations of S inhibit the enzyme reaction by trapping E_r as E_rS (Equation 4). Figure 3 shows that 0.25 M cyanide has very little effect on the behavior of E_rP in stopped-flow turnover experiments when the concentration of S exceeds K_1. It should also be noted that, following the spike of E_rP production and decay corresponding to the first turnover, the concentration of E_rP in subsequent turnovers is considerably less than that observed in Figure 2 with non-inhibitory levels of S in the absence of cyanide. These results are interpreted to

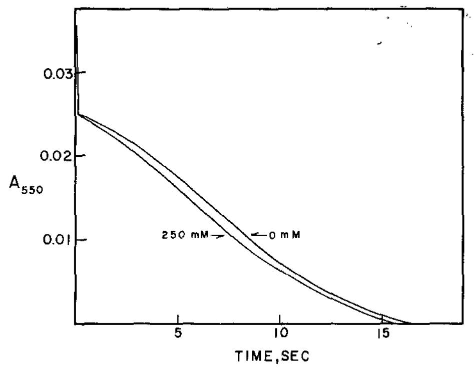

Figure 3. Stopped-flow turnover experiments at high substrate concentration (75 mM L-phenylalanine) showing the lack of effect of cyanide on E_rP monitored at 550 nm. The concentration of cyanide is indicated by each trace. All other conditions were identical to those given in Figure 2.

mean that the reaction and equilibration of S with E_r is now so effective that the process k_{-3} $[E_r]$ $[P]$ is no longer important in determining the level of E_rP. These experiments complement those of Figure 2, and substantiate the conclusion that the reversible interaction of P with E_r has to be taken into account if the turnover behavior of the enzyme under stopped-flow conditions is to be correctly interpreted.

We have assumed that the first product, P, released by the enzyme is the imino acid, which then accumulates transiently during turnover because, at pH 8.1, the rate of conversion to carbinolamine is small relative to the rate of formation of phenylpyruvate from the carbinolamine. This assumption is strongly justified by the fact that the attack and expulsion of water in the two step interconversion of other similar imine and carbonyl groups is generally rate-limiting above pH 5 (7). By the same token, the only other mechanism which would be consistent with the results of the cyanide trapping experiments, namely the release of the carbinolamine by the enzyme followed by a rapidly equilibrated side reaction with the imino acid, is ruled out. It is abundantly clear that P is not phenylpyruvate because the complex of this product with E_r does not absorb at 550 nm (9) and because the enzymatic origin of the free imino acid has been firmly established (5).

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