THE KINETICS OF IMINO ACID ACCUMULATION IN THE D-AMINO ACID OXIDASE REACTION*

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When the oxidation of D-phenylalanine by D-amino acid oxidase is measured in stopped-flow turnover experiments there is a lag in the formation of keto-phenylpyruvate which becomes progressively greater as the pH is raised from 6.5. Borohydride trapping experiments show that the transient accumulation of free imino acid largely accounts for the lag at pH 8.7. These results are consistent with the known hydrolytic behavior of imines.

We recently used cyanide to trap the imino acid released in the L-amino acid oxidase reaction and concluded that the free imino acid (see Equation 1) accumulates transiently in the reaction at pH values greater than 6.5 (1).

$$E_r\text{-Imine} \overset{E_r}{\rightleftharpoons} \text{Imine} \overset{H_2O}{\rightleftharpoons} \text{Carbinolamine} \rightleftharpoons \alpha\text{-Keto Acid} \qquad (1)$$

(Imine $\rightleftharpoons$ Enamine; α-Keto Acid $\rightleftharpoons$ Enol Acid)

Hafner and Wellner (2), on the other hand, while obtaining the most direct evidence yet for the existence of free imino acids as products of the amino acid oxidase reactions (by recovering racemic amino acid after borohydride

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treatment) suggested that the hydration of the imino acid produced in the oxidation of D-alanine by D-amino acid oxidase is rapid enough at pH 8.3 to cause the carbinolamine to be the transient product which accumulates significantly in the formation of pyruvate. Because the behavior of the imino acid after release by the enzyme should be identical in the L-and D-amino acid oxidase reactions we have compared the extent of imino acid accumulation with the magnitude of the lag in keto-phenylpyruvate production by D-amino acid oxidase. We find that most of the lag can be attributed to imino acid accumulation.

EXPERIMENTAL: Highly purified preparations of D-and L-amino acid oxidase were obtained, respectively, from hog kidney (3) and from Crotalus adamanteus (4). The sources of other materials were as follows: D-phenylalanine (shown to contain less than 0.03% L-isomer), Calbiochem; Sodium borohydride, Fisher; FAD, Sigma; Catalase, Boehringer Mannheim Corp.

All kinetic measurements were carried out at 25° with solutions containing 0.05M potassium pyrophosphate, pH 8.7, catalase (0.1 mg per ml), 20μM FAD and saturated with O_2 (1.2mM). O_2-monitored enzyme turnover was measured on the Yellow Springs instrument while stopped-flow phenylpyruvate-monitored turnover was carried out on the Gibson-Durrum apparatus at 330 nm with 2.0 cm pathlength.

The method for trapping and analyzing the imino acid as the racemic amino acid, was similar to that described by Hafner and Wellner (2) except that enzymatic analysis was employed. In the experiment of Figure 3, enzyme turnover was initiated by the addition of enzyme to 3.0 ml of solution, the composition of which is given in the legend of Figure 1. After 15 seconds 0.07M sodium borohydride was added. The enzyme reaction in 3 mls was quenched at the times indicated in Figure 3 by mixing with 0.2 ml of 12 N HCL. The pH was then adjusted to 8.7 with KOH and the concentration of L-phenylalanine (corresponding to one-half of the trapped imino acid) was estimated by the consumption of O_2 in the L-amino acid oxidase reaction using excess O_2.

The enamine (2-aminocinnamate) was prepared non-enzymatically by the interaction of 0.01M phenylpyruvate with 50% ammonium sulfate (w/v) at pH 8.1. After the absorbance increase at 300 nm had ceased, the solution was made 0.05 M in H_2O_2 in order to oxidatively decarboxylate unreacted phenylpyruvate. After eight minutes 0.01 mg/ml catalase was added to destroy excess H_2O_2 and the spectrum was recorded. The pH was first adjusted to 4 to form phenylpyruvate and then the solution was made 1.0 N in KOH. This forms the enolate, of known extinction coefficient (4), from the concentration of which the spectrum of the enamine (λ_{max} = 300 nm, ε_{300} = 2.25 X 10^4 M^{-1} cm^{-1}) can be calculated. The identity of this spectrum with that of the same enamine produced during the hydrolysis of glycyldehydro-phenylalanine (5,6) confirmed that the synthesis of 2-amino-cinnamate had been achieved.

RESULTS AND DISCUSSION: Keto-phenylpyruvate can be conveniently monitored in stopped-flow turnover experiments through the weak $n \rightarrow \pi^*$ carbonyl transitions at 330 nm (ε_{330} = 65 M^{-1} cm^{-1}). Although the enzyme itself absorbs, 330 nm is an isobestic point for the kinetically significant enzyme complexes. Figure I shows that the rate of phenylpyruvate formation at pH 8.7 achieves

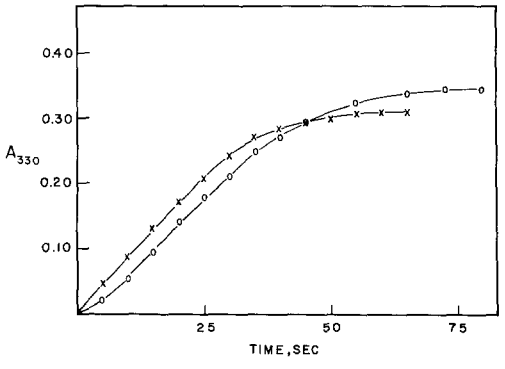

Figure 1. Comparison of keto-phenylpyruvate formation monitored at 330 nm in stopped-flow turnover experiment (-O-) with total product formation (-X-) calculated from O_2-monitored turnover and expressed in keto-phenylpyruvate absorbance units. The solutions contained (after mixing in the case of the stopped-flow experiment) 2.2 μM D-amino acid oxidase, 10 mM D-phenylalanine and 1.2 mM O_2. Other conditions given in Experimental.

a steady state value only after a pronounced lag. The lag (which is observed in the L-amino acid oxidase reaction (4)) is undetectable below pH 6.5, but becomes progressively longer at higher pH values. It is evident that a non-absorbing species, which could be the imine, enamine, carbinolamine or enol-phenylpyruvate (or a mixture of these) is accumulating transiently in the reaction. The concentration of the species can be obtained by comparing the experimental time course for the phenylpyruvate formation with that calculated for total product from the rate of O_2 depletion in a separate experiment and expressed in keto-phenylpyruvate absorbance units. This comparison is made in Figure 1 and, for the first 15 seconds of turnover, in Figure 2. Figure 2 shows that, at 15 seconds, when the rate of phenylpyruvate reaches a steady state value equal to the rate of total product formation, the amount of accumulated non-absorbing material (from the vertical distance, in keto-phenylpyruvate equivalents) is 0.32 mM. This corresponds to 145 turnovers of the enzyme.

If the non-absorbing species accumulating in the experiment of Figure 2 is the imino acid then trapping with borohydride should be initiated at 15 seconds into turnover. The amount of trapped imino acid, compared to that

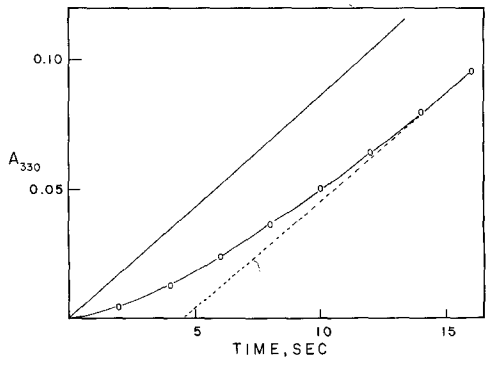

Figure 2. Expansion of first 15 seconds of the reaction of Figure 1. The vertical distance between the trace for total product (-X-) and the dashed line represents the accumulation of 0.32 mM nonabsorbing product. The reciprocal of the time at which the dashed line intersects the time axis, 0.22 $sec.^{-1}$, represents the first order rate constant for conversion of the nonabsorbing product to keto-phenylpyruvate.

expected if all the accumulated non-absorbing material is imino acid, and if enzyme turnover is entirely unaffected by borohydride, is shown in Figure 3. The amount of imino acid accumulated during the first 15 seconds of turnover, obtained by extrapolation of the curve to its value at 15 seconds, is 0.22 mM. Thus the important conclusion from this experiment is that most of the non-absorbing product which accumulates during the first 15 seconds of turnover, before borohydride addition, is the free imino acid. As expected, the amount of accumulated imino acid varied with the enzyme concentration. It is not surprising that the additional imino acid produced and trapped after borohydride addition at 15 seconds is considerably less than predicted, since borohydride is likely to seriously affect enzyme turnover in two ways. Firstly, borohydride does not inactivate the enzyme, but forms a novel form of reduced enzyme which can be oxidized by O_2 (7). Therefore, by acting as an alternative reducing substrate, borohydride will inhibit the formation of imino acid from D-phenylalanine. Secondly, the O_2 could have been depleted very rapidly, compared to the experiment of Figure 1, because of enzyme-mediated O_2 reduction by borohydride. This effect would additionally inhibit imino acid formation. The finding that the amount of trapped imino acid did not

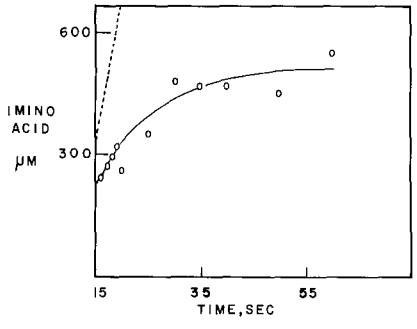

Figure 3. Comparison of the amount of imino acid trapped by borohydride addition after 15 seconds of turnover (-O-) with the amount predicted (-----) from the experiments of Figures 1 and 2. Except for the addition of 0.07 M borohydride at 15 seconds, the experimental conditions are identical to those described for Figure 1. The amount of imino acid accumulated before borohydride addition is the value (0.22 mM) obtained by extrapolation to 15 seconds. The dashed line is constructed on the assumption that borohydride does not affect enzyme turnover (see text).

vary with the borohydride concentration in the range from 0.03 to 0.25 M establishes that the trapping procedure is very rapid compared to both imino acid hydrolysis and the sampling frequency in Figure 3. Since Hafner and Wellner (2) found that borohydride reduction was 36 times faster than hydrolysis, and since the lag of Figure 2 is reciprocally related to the first order rate constant for hydrolysis of the imino acid (see legend of Figure 2), it can be calculated that the half-time for reduction of the imino acid is in fact no more than 0.1 seconds.

The enamine, enol-phenylpyruvate and hydrated keto-phenylpyruvate can all be eliminated as candidates for the non-absorbing material which accumulates during the first 15 seconds of turnover. Firstly, the enamine (ε_{330} = 5 X 10^3 M^{-1} cm^{-1}, see Experimental) absorbs much more than keto-phenylpyruvate at 330 nm and would therefore not cause a lag in absorbance. Secondly, the conversion of enol-to keto-phenylpyruvate is general base-catalyzed (with a Brønsted β value of 0.37) whereas the duration of the lag in Figure 2 is insensitive at constant pH to the type and concentration of buffer (4). Finally, the dehydration of hydrated keto-phenylpyruvate is in general much too slow to contribute to the observed lag (4).

In conclusion, therefore, we can state that imino acid accumulation largely accounts for the lag in keto-phenylpyruvate formation observed at pH 8.7, with perhaps a small contribution from the carbinolamine. This conclusion, together with the fact that the lag becomes progressively greater as the pH is raised from 6.5, is entirely consistent with the behavior of analagous imine-carbonyl interconversions in which the hydration (and its reversal) of the imine becomes rate-limiting at pH values greater than 5, because only the protonated form of the imine is hydrated (8). Our results also agree with the interpretation that transient pH changes during turnover result from imino acid accumulation (9).

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