REDUCTION OF D-AMINO ACID OXIDASE BY β-CHLOROALANINE: ENHANCEMENT OF THE REDUCTION RATE BY CYANIDE*

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Using rapid reaction techniques, it is shown that there is no effect of cyanide on the first three phases of the anaerobic reaction of β-chloroalanine with the enzyme. However, the rate of appearance of free fully reduced enzyme, following the third phase, is increased 50-fold by cyanide and shows saturation kinetics with respect to cyanide. To account for this increased reductive rate in the presence of cyanide, it is proposed that cyanide attacks an enzyme-bound dehydroalanine formed in the elimination reaction.

Recently Walsh et al. (1) have shown that α-D-β-chloroalanine is a unique substrate of D-amino acid oxidase. The enzyme not only catalyzes the oxidation reaction found with unsubstituted amino acids but it also catalyzes the elimination of HCl. Since the distribution of oxidation and elimination products is dependent upon the oxygen concentration while the sum of the products is independent of oxygen, it is likely that a common intermediate is involved. Kinetic isotope effect measurements showed this common intermediate, which is probably a substrate α-carbanion, is formed by partially rate-limiting cleavage of the α-carbon-hydrogen bond. An additional striking feature of the chloroalanine reaction is the slowness at which the enzyme is converted to the free, fully reduced, form. Monitoring pyruvate formation under anaerobic conditions, Walsh et al. (1) measured a reductive constant of $1.05 \times 10^{-3} sec^{-1}$ which is sixteen times less than the value for D-alanine (2,3). This communication will be concerned with the overall rate of reduction of D-amino acid

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oxidase by DL-chloroalanine and will demonstrate that cyanide greatly enhances this rate. A suggestion of a possible mechanism for cyanide action will be presented.

EXPERIMENTAL: D-amino acid oxidase was purified and assayed as described by Yagi (4,5). The ratio A_{274}/A_{455} was 10.3 and the specific activity was 13 μMoles/min/mg (25°C). α-DL-β-chloroalanine was purchased from the Cyclo Chemical Company. Traces of serine were removed by a Dowex 50W-X2 cation exchange resin with a 0 to 4 N HCl linear gradient. The resulting product was crystallized as described by Walsh et al. (1). All other chemicals were reagent grade obtained from the Fisher Chemical Company. Solutions were made anaerobic with V^{+2}_{-} deoxygenated N_2 and the glucose-glucose oxidase system (6). Spectra were recorded on a Cary 15 Spectrophotometer. Stopped flow measurements were made on a Gibson-Durrum Spectrophotometer. Both were temperature regulated to 25°C. All reactions were performed in 0.1 M sodium pyrophosphate buffer with the pH adjusted to 8.3 with HCl.

RESULTS: The effect of cyanide on the static spectrum of a reaction mixture containing DL-chloroalanine and oxidized enzyme is demonstrated in Figure 1. After the anaerobic addition of oxidized enzyme to 40 mM chloroalanine, a spectrum with reduced 450 nm intensity but with 550 nm absorbance is formed. This spectrum, recorded after five minutes, changes slowly over an hour interval. However, if oxidized enzyme and 0.1 M KCN are mixed with 40 mM chloroalanine, the enzyme becomes completely reduced within five minutes. For comparison, a spectrum of reduced enzyme obtained with 40 mM DL-alanine is included. The two spectra are essentially identical. There is no detectable effect of 0.1 M KCN on the visible spectrum of either the oxidized or free reduced enzyme. At the end of each experiment, the enzyme exhibits complete oxidative activity as measured with D-alanine. Thus these results clearly demonstrate that cyanide enhances the rate of reduction of the enzyme by DL-chloroalanine.

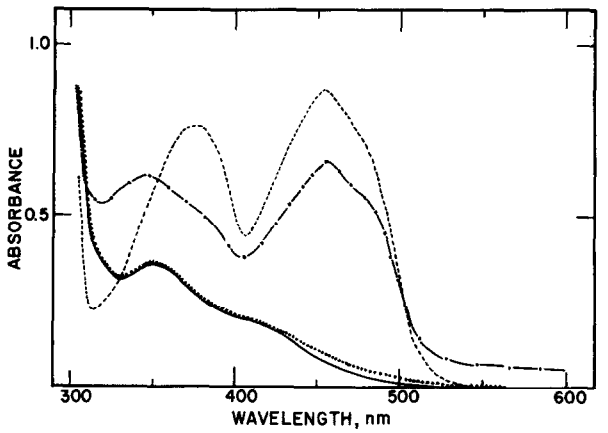

Figure 1. The effect of cyanide on the spectra of anaerobic reaction mixtures of chloroalanine and D-amino acid oxidase. After mixing with D-amino acid oxidase, the respective solutions contained either 0.1 M KCN (---), 40 mM α-DL-β-chloroalanine (-•-) or 40 mM α-DL-β-chloroalanine with 0.1 M KCN (···). For comparison, the spectrum of D-amino acid oxidase with 40 mM DL-alanine in the presence of 0.1 M KCN is included (——). Spectra were recorded five minutes after mixing. The final pH was 8.3.

Figure 2a shows the triphasic kinetics at 550 nm previously reported to occur in the reaction of chloroalanine with D-amino acid oxidase (7). These results are unchanged whether or not cyanide is present. However the disappearance of the third phase to give free reduced enzyme is shown in Figure 2b to be greatly enhanced by the presence of cyanide. Under identical conditions, there was no effect of cyanide upon the anaerobic formation and decay of the 550 nm species with DL-alanine as substrate. Thus the effect of cyanide is specific for the chloroalanine reaction and exhibits an effect only on the rate at which the third phase disappears to give free fully reduced enzyme.

The cyanide dependence of the rate constant for decay of the third phase is shown in Figure 3. With 40 mM DL-chloroalanine the decay of 550 nm absorbance is first order. A double reciprocal plot of k_{obs} versus cyanide concentration yields a straight line with a non-zero ordinate intercept. The constants describing this line are a first order rate constant k_1 of 0.05 sec^{-1} and a K_m of 25 mM. Since the first three phases are not affected by cyanide, spectral species having properties significantly different than those of the

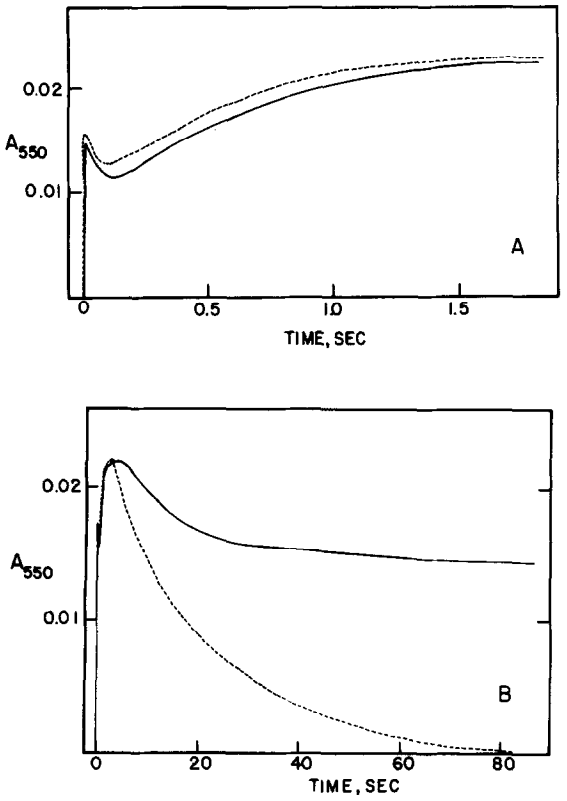

Figure 2. A) Stopped flow anaerobic reactions showing the lack of effect of cyanide on the first three kinetic phases at 550 nm. B) Identical experiments to those in A), except for a 40-fold longer time scale, showing the enhancement of the rate of 550 nm absorbance decrease caused by cyanide following attainment of the third phase. The spectrum of the enzyme at the end of these experiments corresponded to that of free, fully reduced enzyme (see Figure 1, traces —— and •••).

After mixing, the solutions contained 14 μN D-amino acid oxidase, 40 mM α-DL-β-chloroalanine and either no cyanide (——) or 0.1 M cyanide (---). The final pH was 8.3.

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first three phases do not accumulate. The interpretation of the double reciprocal plot we prefer is that the K_m approximates cyanide binding, and that cyanide binding results in only minor spectral changes. The term k_1 represents the addition of cyanide to an intermediate complex that is converted to E_r in a subsequent step that is rapid relative to k_1. The similarity of k_1 with the rate of disappearance of the 550 nm absorbing intermediate in the alanine reaction is probably fortuitous. In all of the above experiments, prior incubation

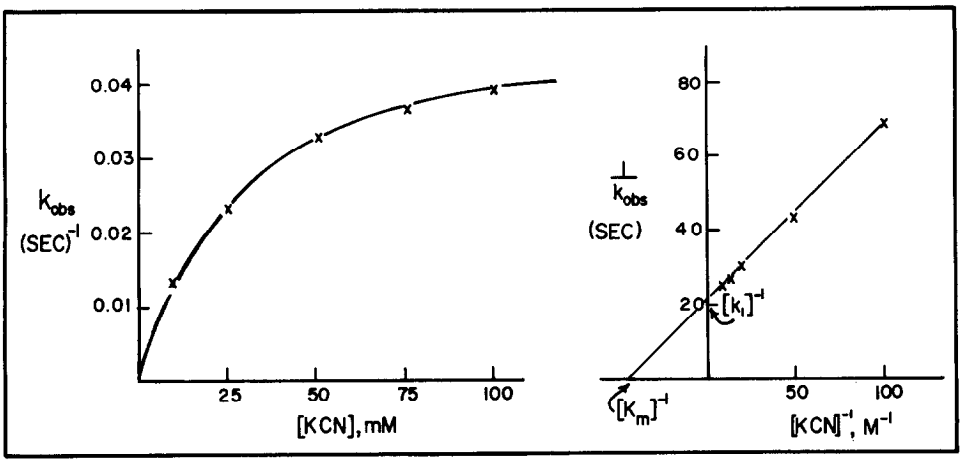

Figure 3. Data from stopped flow anaerobic reactions of Figure 2B (---) showing the dependence of the rate of 550 nm absorbance decay upon cyanide concentration. Values for K_m and k_1 were obtained from double reciprocal plots of KCN versus k_{obs}. Experimental details are the same as those given in Figure 2.

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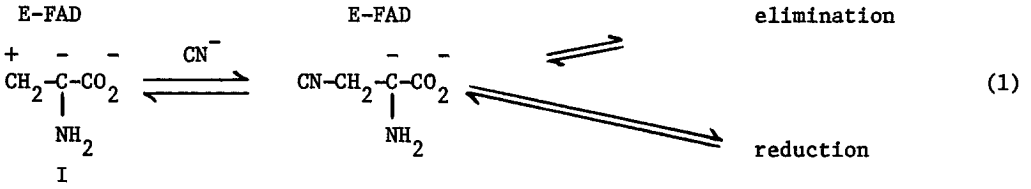
of either the enzyme or the substrate with cyanide before mixing enzyme with substrate yielded identical results.

DISCUSSION: There is no straightforward relationship of the three phases observed in the anaerobic reaction of chloroalanine with D-amino acid oxidase to the steady state parameters describing the elimination reaction (7). Thus the following discussion will be centered on a qualitative explanation for the increased overall rate of chloroalanine reduction of the enzyme by cyanide. First it should be noted that a similar effect of cyanide was observed on the L-amino acid oxidase reaction under (aerobic) turnover conditions with phenylalanine as substrate (8). This result was attributed to a decrease in imino acid concentration which, in turn, diminished the concentration of the 550 nm absorbing species which was identified as a non-covalent complex of E_r and imino acid. This interpretation is eliminated in the present experiments with chloroalanine since the spectrum of the species accumulated after 5 minutes in the absence of cyanide (see Figure 1) does not resemble the spectrum reported for

the complex between the imino acid derived from alanine and reduced D-amino acid oxidase (9).

Recently, it has been conclusively shown that carbanions are substrates for flavoprotein oxidases and that these are oxidized through covalent adduct formation at the N-5 position of the flavin nucleus (10). It was proposed that the holoenzyme generates and stabilizes an α-carbanion of the natural substrate which then attacks flavin at the N-5 position in an analogous fashion to yield oxidized product and reduced flavin through rearrangement of the N-5 flavin-substrate adduct (10). With chloroalanine the stabilized carbanion eliminates chloride to yield an enzyme-bound enamine as an alternative to reduction of the flavin (1). If the holoenzyme stabilizes an α-carbanion of the substrate then it may also be expected to give weight to the resonance structure of dehydroalanine (from the elmination reaction) in which the β-carbon has a positive charge associated with it (see structure I of Equation 1).



(1)

Thus, unlike free enamines in solution, the enzyme-bound enamine may be susceptible to nucleophilic attack by cyanide at the β-carbon to yield the α-carbanion of β-cyanoalanine as shown in Equation 1. Since the cyano group is a much poorer leaving group than chloride, this carbanion would not undergo the elimination reaction but would be forced into the reduction pathway. This could explain the increased rate of formation of free reduced enzyme caused by cyanide.

Our proposal predicts that enzyme reduction by chloroalanine in the presence of cyanide would lead to α-imino-β-cyanopropionate. This species, by analogy with previous studies (8), would in the presence of cyanide form 2,3-dicyanoalanine which, in turn, would decarboxylate to α-aminosuccinonitrile. Upon hydrolysis of this species, the expected product would be aspartate. Attempts to isolate radioactive aspartate when radioactive cyanide was used in

the enzyme reaction have so far been unsuccessful. This may be due to difficulty in the hydrolysis of α-aminosuccinonitrile as well as to a problem of detection since this product would form at concentrations stoichiometric with the enzyme. Further study of product identification is in progress.

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