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INACTIVATION OF ALANINE AMINOTRANSFERASE BY THE NEUROTOXIN β-CYANO-L-ALANINE

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SUMMARY: β-Cyano-L-alanine inactivates pig heart alanine aminotransferase. The nitrile and enzyme form a freely dissociable Michaelis complex which rearranges to a form of inactive enzyme. The inactivated enzyme slowly recovers activity at 25° in 100 mM phosphate buffer, pH 7.4. The observations are consistent with a mechanism of inactivation similar to that thought to apply to the suicide inactivator propargylglycine except that the putative covalent modification of the apoenzyme is relatively labile in the case of the nitrile.

INTRODUCTION: β-Cyano-L-alanine is a neurotoxic agent found in poisonous legumes responsible for outbreaks of human neurolathyrism (1). Its toxicity is mitigated by high doses of pyridoxal hydrochloride, and the compound elicits cystathioninuria but not certain other signs of hypovitaminosis B_6 in the rat (2, 3). A discriminate type of vitamin B_6 antagonism has thus been proposed as the basis of the toxicity (2, 3). It occurred to us that the mechanism of this vitamin B_6 antagonism may be the selective suicide inactivation of certain pyridoxal phosphate-dependent enzymes by cyanoalanine. This hypothesis was prompted by the discovery by Abeles and Walsh that the antibiotic C-propargyl-L-glycine is a suicide inactivator of liver γ-cystathionase and other B_6-dependent enzymes which abstract β-protons from their substrates (4, 5). Those enzymes curiously include pig heart alanine aminotransferase, which actively catalyzes exchange of the hydrogens at the β-position of amino acids with those of water although this activity has no apparent role in the catalysis of transamination (6, 7, 8). We report our findings with that enzyme in this communication.

MATERIALS AND METHODS: Pig heart alanine aminotransferase and rabbit muscle lactate dehydrogenase were obtained from the Sigma Chemical Company and dialyzed against buffer before use. β-Cyano-L-alanine and disodium NADH were Sigma products. Most kinetic assays were performed with a Gilford model 222 A spectrophotometer equipped with a model 6040A chart recorder. Rapid

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reactions included in Figure 2 were followed by means of a Gibson-Durrum stopped-flow apparatus.

One unit of aminotransferase was taken to be that which would convert 1.0 μmol of alanine to pyruvate per min at 25° in a solution containing 200 mM L-alanine, 20 mM α-ketoglutarate, 5.0 mM mercaptoethanol, 1.0 mM EDTA and 100 mM potassium phosphate, pH 7.4.

RESULTS: Progressive inhibition of alanine aminotransferase is conveniently demonstrated by the decay in the rate of pyruvate production from subsaturating levels of alanine when the enzyme is added to dehydrogenase-coupled assay solutions containing cyanoalanine. In Figure 1 we show that the initial velocity decays in a first-order exponential manner to a nonzero value. In Figure 2 it may be seen that the rate of decay saturates as the cyanoalanine concentration is increased, the half-maximal rate occurring at 67 mM while the apparent K_m value for L-alanine is 30 mM under these conditions. A maximal rate constant of 15 min^{-1} is asymptotically approached. However, the degree of inhibition, after this time-dependent process goes to completion, is half-

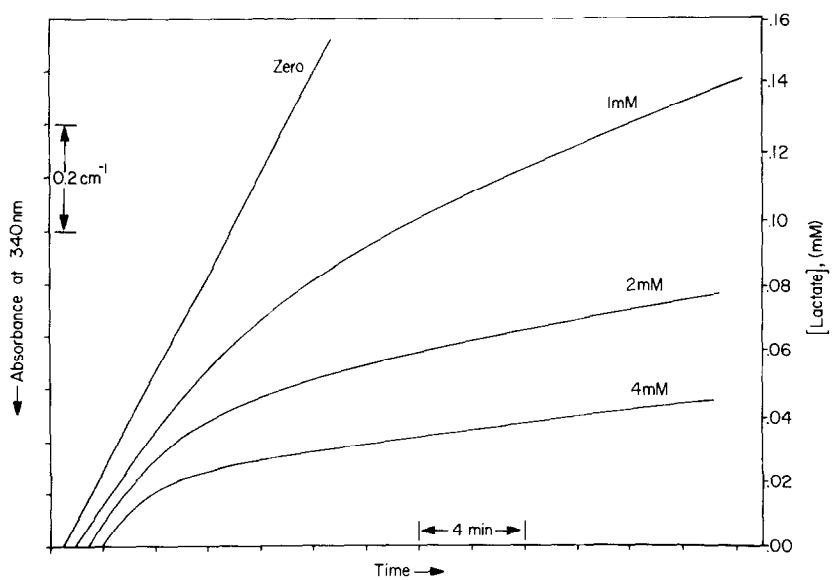
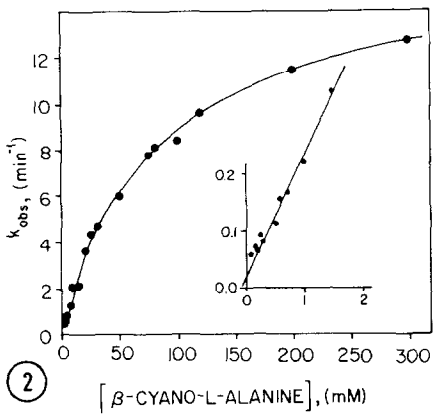


Figure 1. Time-dependent inhibition of alanine aminotransferase by β-cyanoalanine. The production of pyruvate from alanine is followed by the coupling of the reaction to that of a large excess of lactate dehydrogenase. The decrease in absorbance of NADH is given as a function of time. The reaction solutions initially contain the indicated concentration of β-cyano-L-alanine as well as 3.0 mM L-alanine, 20 mM α-ketoglutarate, 0.2 mM NADH, excess lactate dehydrogenase, 5.0 mM mercaptoethanol, 1.0 mM EDTA, 100 mM potassium phosphate, pH 7.4, at 25°. The reaction is initiated by the addition of 0.15 unit of aminotransferase.



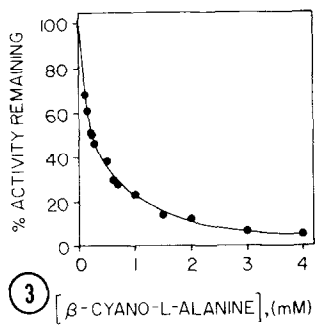


Figure 2. Apparent pseudofirst-order rate constant, k_{obs}, for decay of transamination velocity as a function of cyanoalanine concentration. The k_{obs} was calculated from semilogarithmic replots of curves such as those shown in Figure 1. The conditions are given in that figure legend except that the enzyme concentration varied from 0.018 to 1.3 unit ml^{-1}. The inset illustrates that the hyperbola does not intercept the origin.

Figure 3. Residual transaminase activity observed after the time-dependent inhibition at a given cyanoalanine concentration has gone to completion. The asymptotic velocity observed in curves such as those shown in Figure 1 was divided by the velocity observed in the absence of cyanoalanine. The conditions are given in that figure legend except that the enzyme concentration varied from 0.018 to 0.18 unit ml^{-1}.

maximal at only 0.2 mM β-cyano-L-alanine (Figure 3). Those findings can be best explained as follows. Cyanoalanine rapidly forms a readily dissociable complex at the active site. This complex is relatively slowly converted to a complex in which the enzyme may be regarded to be inactivated. The inactivated enzyme is slowly reactivated either by strict reversal of the inactivating process or by another reaction.

$$I + E_{act} \overset{K}{\rightleftharpoons} I\cdots E_{act} \xrightarrow{k_{inact}} E_{inact} \xrightarrow{k_{react}} E_{act} \qquad [1]$$

It is informative that the hyperbola shown in Figure 2 does not intercept the origin. The apparent decay rate constant, k_{obs}, may be extrapolated to a nonzero value of 0.018 min^{-1} at a cyanoalanine concentration of zero. The explanation is that k_{obs} is the sum of those apparent rate constants describing the inactivating and reactivating events (Equation 3).

$$E_{act} \underset{k_{react}}{\overset{\frac{[I]}{[I]+K}\,k_{inact}}{\rightleftharpoons}} E_{inact} \qquad [2]$$

$$k_{obs} = \frac{1}{\tau} = \frac{[I]}{[I] + K} k_{inact} + k_{react} \qquad [3]$$

The nonzero y-intercept thus provides the value for k_{react}.

Because the reactivation occurs slowly, it is also possible to follow the inactivation of the enzyme by incubating it with cyanoalanine and then testing the incubated solution for transaminase activity. Aliquots are withdrawn periodically and diluted into an assay solution containing a saturating level of alanine (Figure 4). The velocity then measured is nearly constant for several minutes, although the expected gradual acceleration is observed. It is also shown in Figure 4 that the activity lost upon incubation with cyanoalanine may be slowly recovered upon dilution of the inactivated enzyme solution. Reactivation also occurs during dialysis against phosphate buffer.

We have found that the rate and extent of inactivation is independent of up to 50 mM mercaptoethanol, thus ruling out the inactivation of the enzyme by an electrophilic product which diffuses out of the active site

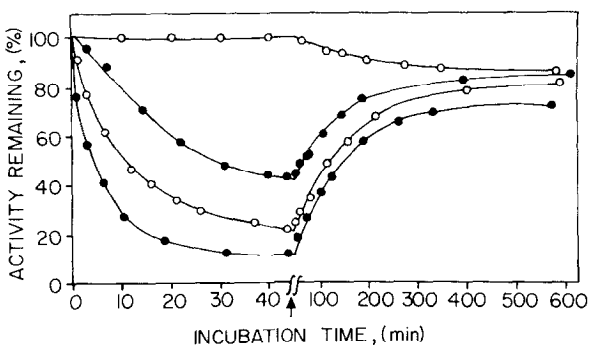


Figure 4. Inactivation of alanine aminotransferase during its incubation with β-cyanoalanine, and reactivation upon dilution of the inactivation solution. The enzyme (4.0 unit ml^{-1}) was incubated with β-cyano-L-alanine (0, 0.25, 0.50 and 1.0 mM) in a solution also containing 2.0 mM α-ketoglutarate, 5.0 mM mercaptoethanol, 1.0 mM EDTA, 100 mM potassium phosphate, pH 7.4, at 25°. Aliquots (5.0 μl) were withdrawn at the indicated times and tested for transaminase activity in an assay solution with a final volume of 1.0 ml. After 45 min (indicated by the arrow), the incubation solution was diluted 200-fold into a solution containing 200 mM L-alanine, 0.2 mM NADH, excess lactate dehydrogenase, 5.0 mM mercaptoethanol, 1.0 mM EDTA, 100 mM potassium phosphate, pH 7.4 at 25°. At the indicated times, transaminase activity was then assayed upon the addition of 20 mM potassium α-ketoglutarate. Note that the time scale of the figure is compressed by a factor of ten after 45 min.

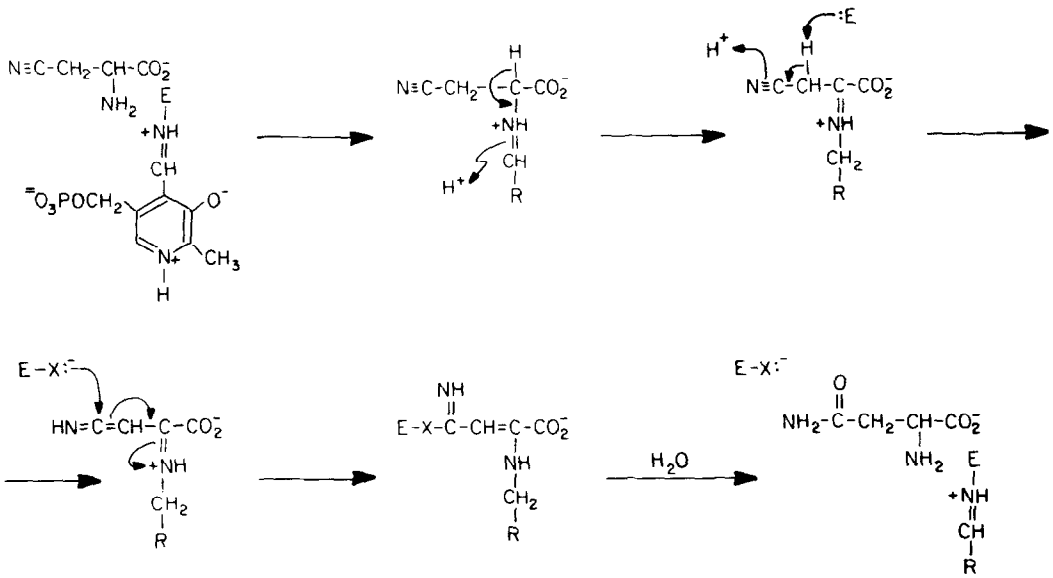


Figure 5. Probable mechanism by which cyanoalanine inactivates alanine aminotransferase (4, 9). The nucleophilic active-site residue which is modified may be the conjugate base of the general acid which protonates the nitrogen of the cyano group. The enzyme-inhibitor adduct may also hydrolyze (last step) to give α-ketosuccinamate, aspartate, or oxalacetate (14). Alternatively, reactivation of the enzyme may proceed with regeneration of the cyano group (14).

before the inactivation reaction subsequently occurs (9). When studied by the method described in Figure 4, the rate and extent of inactivation is also independent of the presence of α-ketoglutarate. Thus, transamination of cyanoalanine to cyanopyruvate does not occur at a rate comparable to the rate of inactivation.

DISCUSSION: The most straightforward explanation of our data is that cyanoalanine inactivates the enzyme by a mechanism essentially identical to that thought to apply to propargylglycine (Figure 5). The alkyne reacts via an allene to form a stable adduct whereas the nitrile reacts via a ketenimine to form a detectably labile adduct. Model reactions for the proposed mechanism include the facile addition of thiols to malononitrile to form labile thioimidate esters (10). Covalent but labile modification of the apoenzyme may also be the basis for the potent but readily reversible inhibition of γ-cystathionase (11, 12), aspartate

β-decarboxylase (13) and other enzymes by cyanoalanine. However, several mechanisms, including some not involving covalent binding to the apo-enzyme, are also conceivable so that further work is underway in order to clarify this phenomenon.

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