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STUDIES ON THE KINETICS OF CYANOHYDRIN SYNTHESIS AND CLEAVAGE BY THE FLAVOENZYME OXYNITRILASE

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Summary

Almond oxynitrilase (D-α-hydroxynitrile lyase, EC 4.1.2.10) catalyzes the reversible condensation of HCN with aldehydes to form D-α-hydroxynitriles. Steady-state kinetic parameters for cleavage and synthesis of mandelonitrile and vanillin cyanohydrin were determined at pH 5.5 which is near the pH optimum of the enzyme. Benzaldehyde and vanillin act as competitive inhibitors of cyanohydrin cleavage while noncompetitive inhibition was observed for HCN. The results are consistent with an ordered uni bi mechanism in which aldehyde is the first substrate bound. Competitive inhibition of cyanohydrin cleavage was observed with various carboxylic acids, alcohols and inorganic anions. The effect of structure on the binding of these inhibitors suggests that the active site of oxynitrilase is located near a hydrophobic region and a positively charged group. Inhibitors which are reasonable analogues for cyanide anion, such as azide and thiocyanate, do not bind to the enzyme-aldehyde complex. This suggests that during cyanohydrin formation the species which binds to the enzyme-aldehyde complex is HCN rather than CN^-.

Introduction

Almond oxynitrilase (D-α-hydroxynitrile lyase, EC 4.1.2.10) is a glycoprotein, contains 1 mol of FAD per molecular weight of 75 000, and catalyzes the reversible condensation of HCN with a variety of aldehydes to form D-α-hydroxynitriles [1—5].

$$RCHO + HCN \rightleftharpoons RCH(OH)CN \quad (1)$$

The physiological substrate is D-mandelonitrile (R = phenyl) which is formed as

a product from the decomposition of the cyanogenic glycoside amygdalin.

Although oxynitrilase has been studied since 1908 [6] the steady-state kinetic mechanism has not previously been determined for the enzyme from almonds or other sources. In contrast, the kinetics of the nonenzymic formation and cleavage of cyanohydrins has been extensively studied [7—11]. In the presence of optically active cations asymmetric cyanohydrin synthesis is observed for the reaction of benzaldehyde with HCN in chloroform [11—13]. In these reactions stereospecific interaction of the optically active cation with benzaldehyde or cyanide may occur in such a manner that the attack of cyanide ion on one side of benzaldehyde is favored. Asymmetric synthesis is inhibited by optically inactive cations and protic solvents and has not been observed in aqueous solution.

In order to provide a basis for comparison of the enzymic and nonenzymic reactions we have investigated the mechanism of catalysis by oxynitrilase. Recent studies in our laboratory show that FAD is important as a structural component near the active site but does not function as a redox-active catalyst during turnover of the enzyme (see Note, p. 209). In this paper we report the effect of various parameters on the kinetics of the enzyme-catalyzed formation and decomposition of cyanohydrins. The results provide information regarding the nature of the active site and the steady-state kinetic mechanism of the enzyme. An initial report of these findings has been published elsewhere [14] along with preliminary studies on the role of the coenzyme in catalysis.

Experimental procedure

Materials

Oxynitrilase purified from sweet almonds was obtained as a gift from Dr. Armin Ramel. The enzyme preparation exhibited spectral properties ($\epsilon_{460} = 11.3 \cdot 10^3\ M^{-1} \cdot cm^{-1}$, $A_{390}/A_{460} = 1.09$, $A_{275}/A_{460} = 10.1$) similar to that previously reported by Becker and Pfeil [1]. Vanillin, obtained from Sigma, was recrystallized from ethanol/water before use. Benzaldehyde and potassium cyanide were purchased from Fisher Scientific Company. Benzaldehyde was purified before use by extracting an ether solution with 0.1 M sodium borate buffer, pH 9.0, which removed a benzoic acid contaminant. The ether layer was then washed with water, dried over anhydrous calcium chloride, and evaporated under a stream of argon gas at room temperature. The purified benzaldehyde was stored at 4°C under argon. Benzyl alcohol was obtained from Matheson Coleman and Bell. It was purified by treating an ether solution with charcoal followed by extraction with 2.0 M sodium bisulfite to remove a small benzaldehyde contaminant. The ether layer was then washed with water, dried over anhydrous calcium chloride and evaporated under nitrogen. Pentanoic, hexanoic and heptanoic acids were purchased from Aldrich and vacuum-distilled before use. All other chemicals and reagents were of the best commercially available grade and used as received.

Methods

D,L-Vanillin cyanohydrin was prepared according to the method of Buck [15] and exhibited negligible absorption at 310 nm ($\epsilon \approx 200\ M^{-1} \cdot cm^{-1}$) as

compared with vanillin which exhibits an absorption maximum at this wavelength ($\epsilon = 9.12 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ at pH 5.5). D,L-Mandelonitrile was prepared by a modification of the procedure described by Becker et al. [16]. 200 ml of a 1.0 M KCN solution neutralized with glacial acetic acid to pH 5.4 was diluted with an equal volume of ethanol, mixed with 4.0 ml (4.2 g, 0.04 mol) of benzaldehyde, and stirred for 30 min at room temperature. Glacial acetic acid (20 ml) was then added and stirring was continued for an additional 10 min. The product was then extracted with chloroform. The chloroform layer was washed with water, dried over anhydrous sodium sulfate and evaporated under nitrogen at 30°C to yield a colorless oil. After washing with petroleum ether (40 ml, boiling point 38°—49°C) the oil solidified at 0°C to yield 2.0 g (38% yield) of colorless crystals. The crystals are stable for at least 6 months at —20°C as judged by the low absorption observed at 249 nm ($\epsilon \approx 200\ M^{-1} \cdot cm^{-1}$ at pH 5.0) and the appearance of benzaldehyde upon addition of KOH ($\Delta\epsilon_{249} = 13.2 \cdot 10^3\ M^{-1} \cdot cm^{-1}$).

Rates of cyanohydrin formation or decomposition were measured spectrophotometrically by monitoring the carbonyl absorption band of the free aldehyde using either a Gilford or a Beckman 25 spectrophotometer. All measurements involving benzaldehyde were made at its absorption maximum (249 nm). This was not possible in the case of vanillin ($\lambda_{max} = 310$ nm) owing to the relatively high substrate concentrations required in these studies and reaction rates were determined at one of two longer wavelengths. Kinetic studies involving vanillin as substrate or as product inhibitor were monitored at 359 nm ($\Delta\epsilon = 3.18 \cdot 10^2\ M^{-1} \cdot cm^{-1}$). Rates of vanillin cyanohydrin decomposition in the absence of added vanillin were determined at 339 nm ($\Delta\epsilon = 1.81 \cdot 10^3\ M^{-1} \cdot cm^{-1}$).

Preliminary studies were conducted to select buffers suitable for kinetic studies since various compounds, including anions, were found to inhibit oxynitrilase activity. With $2.1 \cdot 10^{-4}$ M D,L-mandelonitrile as substrate, no effect on enzyme activity was observed by varying the concentration of citrate buffer at pH 5.5 (5 mM—1.0 M) or at pH 6.5 (5 mM—0.1 M). Enzyme activity was also unchanged at various glutamate buffer concentrations (5 mM—0.08 M) in the pH range 4.0—5.5. Except where indicated, steady-state kinetic measurements were conducted in 0.1 M sodium citrate buffer, pH 5.5, which was also used for the standard assay of oxynitrilase activity with $2.1 \cdot 10^{-4}$ M mandelonitrile as substrate. Stock solutions of D,L-cyanohydrins were prepared freshly each day and exhibited negligible decomposition when maintained at 0°C. Initial rates of cyanohydrin decomposition were measured immediately after the simultaneous addition of small aliquots of enzyme and substrate to 1.0 ml of buffer at 25°C. Stock solutions of HCN at pH 5.5 used for kinetic studies were prepared by neutralizing KCN solutions with citric acid in an ice-salt bath and were standardized according to the procedure of Svirbely and Roth [7]. Initial rates of cyanohydrin formation were measured immediately after the simultaneous addition of small aliquots of enzyme and aldehyde to HCN-containing assay mixtures (1.0 ml) previously equilibrated to 25°C in stoppered cuvettes. Rates reported for enzymic reactions are corrected for the blank rate of cyanohydrin formation or decomposition measured under identical conditions. Least-squares analysis was performed on enzyme kinetic data in order to

determine kinetic parameters.

The extinction coefficient of oxynitrilase at 460 nm was determined by the heat denaturation method previously described [17] except that the heating time was increased to 10 min. Calculations were made using a molar extinction coefficient of $11.3 \cdot 10^3$ at 450 nm for free FAD [18]. Turnover numbers were calculated based on enzyme concentration determined by the absorbance at 460 nm.

Results

The maximal velocity of the oxynitrilase reaction with mandelonitrile as substrate increases by less than a factor of 5 as the pH is increased from 4.0 to 6.5 and appears to reach a plateau between pH 5.5 and pH 6.0. The increase in maximal velocity is paralleled by a smaller (1.8-fold) increase in K_m values. In contrast the apparent rate constant for the initial nonenzymic decomposition of mandelonitrile is more than 300-fold larger at pH 6.5 than at pH 4.0 owing to a rate-limiting decomposition via the cyanohydrin anion (ϕ-CH(CN)OH $\rightleftharpoons$ ϕ-CH(CN)O$^-$ + H$^+$, pK = 10.73 [8]). (Above pH 7.0 the nonenzymic decomposition of mandelonitrile interferes significantly with measurements of initial velocities for the enzymic reaction.) The rate constant determined for cyanohydrin anion decomposition in the nonenzymic reaction (ϕCH(CN)O$^-$ $\rightarrow$ ϕCHO + CN$^-$, k = 792 min^{-1} [8]) is only 75-fold smaller than the fastest rate observed for the oxynitrilase reaction (59 300 min^{-1}). This suggests that a major shift in the effective pK of enzyme-bound versus free cyanohydrin could conceivably account for a significant portion of the large rate accelerations which are observed for the enzymic reaction at pH values considerably below the pK of the free cyanohydrin (e.g. 10^8-fold at pH 4.0).

Taking the magnitude of the blank correction factor into consideration, a pH value of 5.5 was judged as a near optimal condition for studying the enzymic reaction and used for all further studies. The steady-state kinetic parameters obtained for the synthesis and decomposition of mandelonitrile and vanillin cyanohydrin are summarized in Table I. A random uni-bi mechanism will not yield linear double reciprocal plots in the direction of α-hydroxynitrile synthesis unless one substrate is saturating. In principle this mechanism would appear inconsistent with the linear plots observed for the synthesis of mandelo-

TABLE I

STEADY-STATE KINETICS WITH NATIVE OXYNITRILASE

V_1 and V_2 are maximal turnover numbers in the direction of α-hydroxynitrile decomposition and synthesis, respectively. Reaction rates were measured at 25°C in citrate buffer, pH 5.5 (0.1 M for V_1, 0.17 M for V_2 with benzaldehyde, and 0.25 M for V_2 with vanillin).

Aldehyde	V (min^{-1})		K_M (M)		
	V_1	V_2	RCH(OH)CN	RCHO	HCN
Benzaldehyde	37 800	102 000	$5.9 \cdot 10^{-4}$	$1.5 \cdot 10^{-4}$	$5.7 \cdot 10^{-2}$
Vanillin	8 000	28 500	$3.0 \cdot 10^{-2}$	$1.5 \cdot 10^{-2}$	1.1

nitrile and vanillin cyanohydrin. In practice, additional evidence is desirable since small deviations from linearity may be difficult to detect. Product inhibition with respect to α-hydroxynitrile substrate can provide such evidence and can also be used to distinguish between various other possible mechanisms. It is found that vanillin (K_i = 2.2 mM) and benzaldehyde (K_i = 0.12 mM) are competitive inhibitors. Noncompetitive inhibition is observed for HCN with either mandelonitrile [K_i = 18 mM (slope), 63 mM (intercept)] or vanillin cyanohydrin [K_i = 0.27 M (slope), 0.53 M (intercept)] as substrate. The observed inhibition patterns and linear secondary plots clearly exclude a random uni-bi mechanism. The noncompetitive product inhibition observed with HCN is inconsistent with a rapid equilibrium random uni-bi mechanism but could occur if HCN also forms a dead-end complex with E-RCH(CN)OH. (Dead-end complexes with other enzyme forms yield competitive inhibition.) This possibility requires the slope inhibition constant observed for HCN to be independent of the α-hydroxynitrile used as substrate and can be excluded by the different values observed with mandelonitrile (18 mM) and vanillin cyanohydrin (0.27 M). The results are consistent with an ordered uni-bi mechanism in which HCN is the first product released and provide strong evidence for this mechanism since other possibilities can be excluded.

$$\mathrm{E + RCH(CN)OH \rightleftharpoons E\text{-}RCH(CN)OH \rightleftharpoons E\begin{matrix}\diagup \mathrm{RCHO} \\ \diagdown \mathrm{HCN}\end{matrix} \rightleftharpoons E\text{-}RCHO + HCN \rightleftharpoons E + RCHO}$$

For this mechanism the aldehyde product inhibition constant corresponds to the dissociation constant for the enzyme-aldehyde complex which can also be calculated from the point of intersection of primary double reciprocal plots with aldehyde as the variable substrate. That the calculated dissociation constants for benzaldehyde and vanillin (0.13 mM and 2.3 mM, respectively) are nearly the same as the observed inhibition constants (0.12 mM and 2.2 mM, respectively) provides evidence for the internal consistency of the data. The overall dissociation constant for mandelonitrile (ϕCH(CN)OH ⇌ ϕCHO + HCN), calculated using steady-state kinetic parameters ($K_D = 9.6 \cdot 10^{-3}$ M), is similar to the value obtained for the nonenzymic reaction from direct equilibrium measurements at pH 5.5 ($K_D = 4.2 \cdot 10^{-3}$ M) [8]. Although the dissociation constant of vanillin cyanohydrin has not been directly determined the value obtained from steady-state kinetics ($K_D = 4.4 \cdot 10^{-2}$ M) is similar to the value observed directly with *p*-hydroxybenzaldehyde cyanohydrin ($K_D = 7.7 \cdot 10^{-2}$ M) [19]. The validity of this comparison is justified by previous studies which show that the stability of cyanohydrins formed with benzaldehyde derivatives is not appreciably affected by the introduction of a 3-methoxy substituent [19].

The effect of structure on the binding of various compounds which act as competitive inhibitors with mandelonitrile as substrate is summarized in Table II. Short-chain carboxylic acids and alcohols are weaker inhibitors as compared with the corresponding aromatic derivatives. As judged by comparisons with molecular models, maximal binding affinity is observed with aliphatic acids as the size of the inhibitor approximates the size of benzoate which is the most potent inhibitor among the compounds tested. These results and the observed

TABLE II

INHIBITION CONSTANTS FOR VARIOUS COMPETITIVE INHIBITORS OF OXYNITRILASE

Inhibitor	K_i (M)
Benzoate	$7.9 \cdot 10^{-6}$
Benzyl alcohol	$4.2 \cdot 10^{-5}$
Benzaldehyde	$1.2 \cdot 10^{-4}$
Mandelate	$2.3 \cdot 10^{-4}$
Glycolate	$6.4 \cdot 10^{-2}$
Acetate	$2.2 \cdot 10^{-2}$
Propanoate	$2.3 \cdot 10^{-3}$
Butanoate	$2.5 \cdot 10^{-4}$
Pentanoate	$9.1 \cdot 10^{-5}$
Hexanoate	$7.8 \cdot 10^{-5}$
Heptanoate	$1.2 \cdot 10^{-4}$
Azide	$7.9 \cdot 10^{-4}$
Thiocyanate	$1.8 \cdot 10^{-4}$
Ethanol *	0.18

* This concentration of ethanol causes 50% inhibition of activity in the standard mandelonitrile assay. Assuming that ethanol is a competitive inhibitor, as observed for benzyl alcohol, a value of 0.13 M is estimated for the inhibition constant.

inhibition with various inorganic anions (N_3^-, SCN^-, Cl^-, NO_3^-) suggest that the active site of oxynitrilase is located near a hydrophobic region and a group which is positively charged at pH 5.5.

While inhibition is observed with various monovalent anions, no inhibition was detected with citrate buffers at pH greater than or equal to 5.5, suggesting that divalent and trivalent anions did not bind to the enzyme. Consistent with this hypothesis inhibition was not observed at pH 5.5 with either 0.1 M sodium oxalate or with 0.1 M sodium pyrophosphate. That the monovalent citrate ion ($pK_{a2} = 4.75$) may act as an inhibitor is suggested by the observation that citrate buffers at pH values less than 5.5 did inhibit activity and were replaced by glutamate buffers for kinetic studies at low pH values.

Discussion

Values for kinetic parameters ($V = 131\,000$ min^{-1}, $K_{HCN} = 0.23$ M), calculated based on data reported by Becker and Pfeil for the synthesis of mandelonitrile with oxynitrilase [1], are similar to results shown in Table I. While a similar enzyme preparation was used, the observed similarity in results would appear inconsistent with the fact that the earlier studies were conducted under conditions (0.05 M sodium acetate buffer, pH 5.4, containing 50% (8.7 M) ethanol) sufficient to cause nearly complete inhibition of oxynitrilase activity in the standard mandelonitrile assay (see Table II). However, it is pertinent that the benzaldehyde concentrations (0.17—0.40 M) used by Becker and Pfeil represent an enormous excess as compared with the K_m value determined for benzaldehyde in this study ($1.5 \cdot 10^{-4}$ M). Using this value it can be shown that at 0.17 M benzaldehyde competitive inhibition with 0.05 M acetate would not be detectable while competitive inhibition, even with 8.7 M ethanol, would cause only a 5% decrease in activity.

The preceding discussion assumes that compounds which act as competitive inhibtors with respect to cyanohydrin as substrate will also exhibit competitive binding with aldehyde substrates but not with HCN. This is required by the ordered uni-bi mechanism proposed for oxynitrilase and has been verified by direct binding studies described elsewhere [14] which show that inhibitors such as azide or thiocyanate do not bind to the enzyme-aldehyde complex. Since azide and thiocyanate may be regarded as reasonable analogues for cyanide anion, these results suggest that during cyanohydrin formation the second substrate binds to the enzyme-aldehyde complex as HCN rather than as cyanide anion. Values obtained for V and K_m in the direction of cyanohydrin cleavage appear to reach a plateau around pH 6.0. A similar effect of pH on enzyme activity in the direction of cyanohydrin synthesis was reported by Becker and Pfeil [1]. In order to evaluate the hypothesis that the second substrate binds as HCN, it would be useful to extend these studies to include measurements at pH values near the pK_a of HCN. However, these studies are not feasible even with the relatively rapid spectrophotometric assay used in these studies since the rate of the nonenzymic reaction interferes with initial rate measurements above pH 7.0.

Note added in proof

Our studies on the role of FAD in oxynitrilase catalysis were published [Jorns, M.S. (1979) J. Biol. Chem., 254, 12145-12152] after this article was prepared for publication.

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