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HYDROLYSIS OF A THIOPEPTIDE BY CADMIUM CARBOXYPEPTIDASE A

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SUMMARY: Substitution of the active site zinc ion of carboxypeptidase A by cadmium yields an enzyme inactive towards ordinary peptide substrates. However, a substrate analog ($BzGlyNHCH_2CSPheOH$) containing a thioamide linkage at the scissile position is cleaved to the thioacid. The kinetic parameters and their pH dependencies are k_{cat}/K_m = 5.04 x 10^4 $min^{-1}M^{-1}$, decreasing with either acid or base (pK_{E1} = 5.64, pK_{E2} = 9.55), and k_{cat} = 1.02 x 10^2 min^{-1}, decreasing with acid (pK_{ES} = 6.61). The thiopeptide is less efficiently cleaved by native (zinc) carboxypeptidase A. This cadmium-sulfur synergism supports a mechanism wherein the substrate amide is activated by metal ion coordination to its (thio)carbonyl.

INTRODUCTION: The thioamide functional group, RC(=S)NHR, manifests many of the chemical properties of the biochemically ubiquitous carboxamide moiety. In addition to an obvious congeneric structural relationship, both kinds of amide have similar susceptibility to hydrolysis induced by acid or base, as has been demonstrated in a number of mechanistic investigations (1-4). In view of this close homology, it is surprising that enzymic hydrolysis of thiopeptides has received little scrutiny. Examination of thioamide substrate analogs for the common proteases might yield unique insight into the nature of chemical intermediates generated in the course of enzymic hydrolysis, as well as provide a basis for design of potentially bioactive substances. We have initiated an investigation of cleavage

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*EC 3.4.17.1; other abbreviations: MES, 2-(N-morpholino)ethanesulfonic acid; TRIS, tris(hydroxymethyl)aminomethane; AMPD, 2-amino-2-methyl-1,3-propanediol; CbzGlyGlytPheOH, substance 1.

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of the thioamide linkage by the metalloenzyme carboxypeptidase A (CpA*), employing 1 as substrate.

1: $C_6H_5CONHCH_2CONHCH_2CSNHCH(CH_2C_6H_5)CO_2H$

MATERIALS AND METHODS: Substrate. Benzoylglycylthioglycyl-L-phenylalanine (1), was synthesized by reaction between $BzGlyNHCH_2C(=S)SEt$ and $H_2NCH(CH_2C_6H_5)CO_2t$-Bu with subsequent acid treatment and isolation as the sodium salt. Details of preparation of intermediates will be provided elsewhere. Anal. Calcd for $C_{20}H_{20}N_3O_4SNa$: C,56.99; H,4.78; N,9.97; S,7.61. Found: C,57.37; H,5.07; N,9.88; S,7.29.

Cadmium Carboxypeptidase A. (5) Crystals of the zinc enzyme (Sigma Chemical Co., no. C0386) were suspended (5 mg/ml) in a 10.0 mM solution of 1,10-phenanthroline in 1.0 mM MES buffer (pH 7.0) at 25 °C for 1 hr, with four successive repetitions, followed by five 0.5 hr washings with 1.0 mM MES (pH 7.0). The crystalline apoenzyme was then treated with 1.0 mM MES containing 4.0 equiv. of $CdCl_2$ (or with $ZnSO_4$, to reconstitute the zinc enzyme as a control) at 25 °C with gentle stirring for 1 hr, followed by four washings with 1.0 mM MES (pH 7.0).

Kinetic Analysis. Cleavage of the thiopeptide bond was continuously monitored spectrophotometrically at 25.0 ± 0.1 °C by decrease of optical density at a suitable wavelength in the range 265 to 294 nm (region of significant thioamide absorbance). The difference between the initial absorbance of an unhydrolyzed thiopeptide solution and the final absorbance of the hydrolysis products is independent of pH, simplifying relative velocity measurements. Initial rates were ascertained graphically from the tangent to the absorption vs. time plot. Reaction solutions were buffered with MES (pH 5.20-7.07), TRIS (pH 7.63-8.09), or AMPD (pH 9.00-10.05) and were all 1.0 F in NaCl. Solutions contained additional cadmium ion (1.6-3.2 $\times 10^{-6}$ M at pH >6.61, 1.6 x 10^{-3} M at pH <6.61, in order to suppress dissociation of metalloenzyme). It was shown that cadmium ion at these concentrations is without effect upon the substrate in the absence of enzyme. Data was fitted by least squares, first to the Michaelis expression and then to an appropriate pH function.

Product Analysis. A preparative hydrolysis of 1 (150 mg) was carried out with a cadmium carboxypeptidase suspension (30 mg) in 6 ml of 1.0 mM MES, pH 7.0, at room temperature for 6.5 hr under a nitrogen atmosphere. After centrifugation and removal of the enzyme by filtration, acidification to a pH of 1 led to a precipitate of benzoylglycylthioglycine (BzGlyGlySH), identified by its NMR spectrum and elemental composition. Anal. Calcd for $C_{11}H_{12}N_2O_3S$: C,52.38; H,4.80; N,11.11; S,12.69. Found: C,52.62; H,5.09; N,11.19; S,11.32.

RESULTS: Apocarboxypeptidase A was prepared by removal of the active site zinc ion with the chelating agent 1,10-phenanthroline (5), and the metalloenzyme was then reconstituted with cadmium chloride. As

a control, the zinc enzyme was also reconstituted from the apoenzyme by an identical procedure. Enzymic activity was then assayed at pH 7.6 with the well-behaved carboxamide substrate carbobenzyloxy-glycylglycyl-L-phenylalanine (CbzGlyGlyPheOH) (6). For zinc carboxypeptidase A (Zn-CpA), kinetic parameters (k_{cat},K_m) were obtained from initial rate measurements at varying substrate concentrations, which are in close agreement with previously measured values (7). This ensures that the metal exchange procedure does not denature the enzyme. The identically prepared cadmium enzyme (Cd-CpA) was comparatively inactive against this carboxamide substrate, as previously reported (5). The relative activities under comparable conditions were in the ratio 11:1 (Zn:Cd). Since it was not feasible to distinguish between residual activity of Zn-CpA and true catalysis by Cd-CpA, this experiment establishes only a limiting value for the kinetic competency of the cadmium enzyme; its true activity may be much lower than 9% of that of the native zinc enzyme.

Table 1. Comparison of Kinetic Parameters for Zn- and Cd-Carboxypeptidase A.[a]

Zn-CpA, substrate = CbzGlyGlyPheOH
k_{cat}/K_m = 3.09 (±0.15) x 10^7 $min^{-1}M^{-1}$; pK_{E1} = 6.2 (±0.1), pK_{E2} = 9.0 (±0.1)[b]
k_{cat} = 1.06 (±0.04) x 10^4 min^{-1}, pK_{ES} = 6.0 (±0.1)[b]
(K_m = 3.4 x 10^{-4} M at pH = 7.6)
Zn-CpA, substrate = BzGlyGlytPheOH (I)[c]
k_{cat}/K_m = 7.4 (±1.1) x 10^2 $min^{-1}M^{-1}$.
k_{cat} = 3.3 (±0.7) min^{-1}
(K_m = 4.5 x 10^{-3}M at pH = 7.6)
Cd-CpA, substrate = BzGlyGlytPheOH (I)[c,d]
k_{cat}/K_m = 5.04 (±0.11) x 10^4 $min^{-1}M^{-1}$; pK_{E1} = 5.64 (±0.05), pK_{E2} = 9.55 (±0.05)
k_{cat} = 1.02 (±0.03) x 10^2 min^{-1}, pK_{ES} = 6.61 (±0.08)
(K_m = 2.1 x 10^{-3} M at pH = 7.6)

[a]Values given with standard error in parentheses. [b]Rate constants, our measurement; pK values taken from ref. 9. [c]Glyt = thioglycyl: according to Jones, W. L., Nestor, J. J., Jr., and du Vigneaud, V. (1973) J. Am. Chem. Soc. 95, 5677-5679. [d]Rate constants, limiting values from pH profiles.

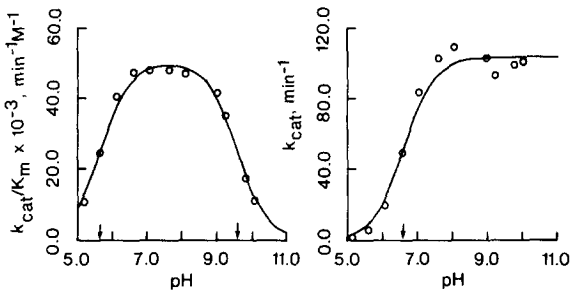


Fig. 1. Effect of pH upon kinetic parameters for cadmium carboxypeptidase A hydrolysis of 1. Concentrations: enzyme 8.56×10^{-7} to 1.73×10^{-6} M; substrate 3.14×10^{-5} to 1.94×10^{-2} M. Arrows indicate inflection points (pK).

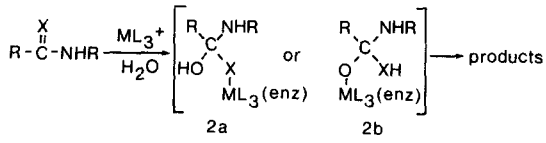
When the thioamide substrate analog 1 was subjected to the respective metalloenzymes, a striking reversal of relative activity was noted. Whereas the zinc enzyme was a quite inefficient catalyst for hydrolysis of this substrate, cadmium carboxypeptidase A cleaved the thioamide linkage at a respectable rate (Table 1). Since Zn-CpA is inactive, this must be true catalysis by Cd-CpA. A preparative reaction was carried out under the agency of the cadmium enzyme in order to confirm the identity of the products. Benzoylglycylthioglycine was isolated and characterized, demonstrating that C-N cleavage and not C-S cleavage occurs in the course of hydrolysis.

Since a connection between the nature of the active site metal ion and thioamide reactivity promises to be mechanistically significant, a study of pH-rate profiles was undertaken. In Fig. 1 are given the pH dependencies of k_{cat} and k_{cat}/K_m. The latter is the second-order rate constant for enzymic hydrolysis at high dilution. A typical bell-shaped curve is found; the line shown is a least squares fit to the equation $k_{cat}/K_m = (k_{cat}/K_m)_{lim}/[(1 + H/K_1) \cdot (1 + K_2/H)]$. The maximum rate, 5.04×10^4 $min^{-1}M^{-1}$, is found at neutral pH. The bracketing pK values of 5.64 and 9.55 must correspond to ionization constants of the catalyst (Cd-CpA), and

may be taken to indicate the presence of two functional groups on the free enzyme, whose protonation state is critical to the hydrolytic reaction (8). The first order rate constant for the enzyme-substrate complex (k_{cat}) has a sigmoidal pH dependency with a maximum velocity of 1.02×10^2 min^{-1} and a single apparent pK of 6.61. The fitted equation in this case was $k_{cat} = (k_{cat})_{lim}/(1 + H/K)$. While the source of these pH-induced inflections in the Michaelis parameters is subject to interpretation, it is pertinent to note that the pattern of pH dependencies is exactly the same as found for ordinary peptide substrates with Zn-CpA (9). In the latter case greater rates and somewhat different pK values have been reported. (Table 1). However, the similarities are sufficient to suggest that a common mechanism operates in both cases.

DISCUSSION: The most striking feature of our data is the correlation of activity between the nature of the active site metal ion and the type of acyl moiety present in the substrate. Although carboxypeptidase A has been exhaustively studied, the mechanism by which it cleaves carboxamide linkages is still not resolved (10). The most widely held view, based on crystallographic evidence (11), is that the active site zinc ion interacts with the substrate carbonyl, and polarizes it by Lewis acid coordination to oxygen, rendering the scissile amide linkage susceptible to nucleophilic addition. However, the possibility that a mechanism more closely aligned with that ascribed to carbonic anhydrase, in which the metal ion instead coordinates with and activates a nucleophilic hydroxide ion, cannot be excluded on previous evidence (12-14). We suggest that our results support the former mechanism.

With reference to Scheme 1, the distinction between the two proposals lies in the nature of the stabilization by the metal ion of the tetrahedral adduct (or, more precisely, the transition state



Scheme 1. (X = O,S; M = Zn,Cd).

for its formation or decomposition). The experimental observation that Cd facilitates thioamide hydrolysis (X = S) more than Zn, whereas for carboxamide hydrolysis (X = O) the opposite is true, is more in accord with intermediate structure 2a, based upon the known affinities between metal ions and chalcogenide ligands. For intermediate 2b (corresponding to the carbonic anhydrase mechanism) there is no such obvious rationalization of the cadmium-sulfur synergism.

This conclusion is of course contingent upon the supposition that both classes of substrate react by the same mechanism. This assumption finds support in the pH-rate profiles. Unambiguous interpretation of the pH dependencies of Michaelis parameters is in general not feasible (15). However, it is a fact that the thioamide-Cd-CpA system follows the same pattern with respect to pH variation as does the native (zinc) enzyme-substrate system. This is not true for commonly studied carboxylate ester substrates of CpA, for example (16). Comparison of the pK values occurring in k_{cat}/K_m reveals a somewhat greater separation for Cd-CpA than for Zn-CpA (Table 1). Previous mechanistic investigation leads to provisional assignments of these inflections; the lower pK corresponds to ionization of a water molecule coordinated to the active site metal (10,17,18) and the higher pK is due to the phenolic hydroxyl of active site residue Tyr 248 (10). Since these groups are potentially in

contact with one another (and with the carboxylate side chain of Glu 270) in the free enzyme (11), the ionizations are probably co-operative in nature (19). The single apparent pK in the k_{cat} profile most plausibly arises from a change in rate determining step within the enzyme-substrate complex, rather than from protonation of a specific functional group at that pH (10).

A mechanism corresponding to the foregoing assignments has been outlined in previous articles (10,20). Our interpretation of the intermediate steps in the hydrolysis of amide substrates by CpA is different in detail from that of other investigators of this enzyme. In brief, we suggest that a preponderence of evidence points to a reverse protonation mechanism, in which the phenolate of active site residue Tyr 248 functions as a general base, catalyzing addition of water to the metal-coordinated scissile amide linkage, yielding intermediate 2a. It is this step which is manifested in the pH-dependent range for k_{cat}, with a subsequent step being slower at high pH. We anticipate that further examination of thioamide hydrolyses will clarify the mechanism of carboxypeptidase A.

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