EVIDENCE FOR A DIFFERENCE IN MECHANISM BETWEEN THE REACTIONS WITH CARBOXYPEPTIDASE A OF THE (-) AND (+)-ENANTIOMERS OF A THIOLESTER SUBSTRATE

J. Suh and E. T. Kaiser*

Department of Chemistry
University of Chicago
Chicago, Illinois 60637

Received April 14,1975

Summary: Cinnamoyl-carboxypeptidase A_γ has been isolated from the reaction of the enzyme with (+)-S-(trans-cinnamoyl)-α-mercapto-β-phenylpropionate. This is the first instance in which an acyl-enzyme has been detected directly during the esterase action of carboxypeptidase A. Also, the failure to detect such a species in the carboxypeptidase A-catalyzed hydrolysis of the (-)-enantiomer of the thiolester under similar conditions provides the first experimental demonstration that an enzyme can act on different enantiomers of a substrate through different mechanisms.

Recently, we reported[1] the pH dependencies of k_{cat}/K_m for the carboxypeptidase A_γ (CPA)-catalyzed hydrolysis of (-)- and (+)-S-(trans-cinnamoyl)-α-mercapto-β-phenylpropionate (I).** The bell-shaped pH profiles obtained were characterized by values of pK_{a_1} = 6.2 and pK_{a_2} = 9.0 in the case of (-)-I and pK_{a_1} = 8.2 and pK_{a_2} = 9.6 in that of (+)-I. At the time of the original report, no consideration was given to the possibility that acyl-enzymes might be detected directly in the course of thiolester hydrolysis. However, the observation that the pK_{a_1} value for the hydrolysis of the (+)-isomer was an unusually high one for an ester substrate,[3] together with model-building studies based on the X-ray data

---

*Supported in part by a grant from the National Institute of Arthritis, Metabolic and Digestive Diseases.

**From the comparison of the circular dichroism spectra of the thiolesters with that of O-(trans-cinnamoyl)-L-β-phenyllactate the L-configuration has been assigned to the (-)-thiolester and the D-configuration to the (+)-thiolester.[2]

of Lipscomb et al.,[4] suggested to us subsequently that one of the mechanisms which could account for the reaction of CPA with the compound might be that shown in Figure 1. According to this mechanism, in contrast to the way in which most substrates are thought to bind, the (+)-thiolester binds with its carboxylate group at the active site zinc ion rather than in contact with the guanidino group of Arg-145. The attacking nucleophile in the reaction of the (+)-thiolester is postulated to be the phenoxide form of Tyr-248, and the ionization of this residue is reflected in the value of pK_{a_1}. The ionization of the water bound to the zinc ion gives rise to the

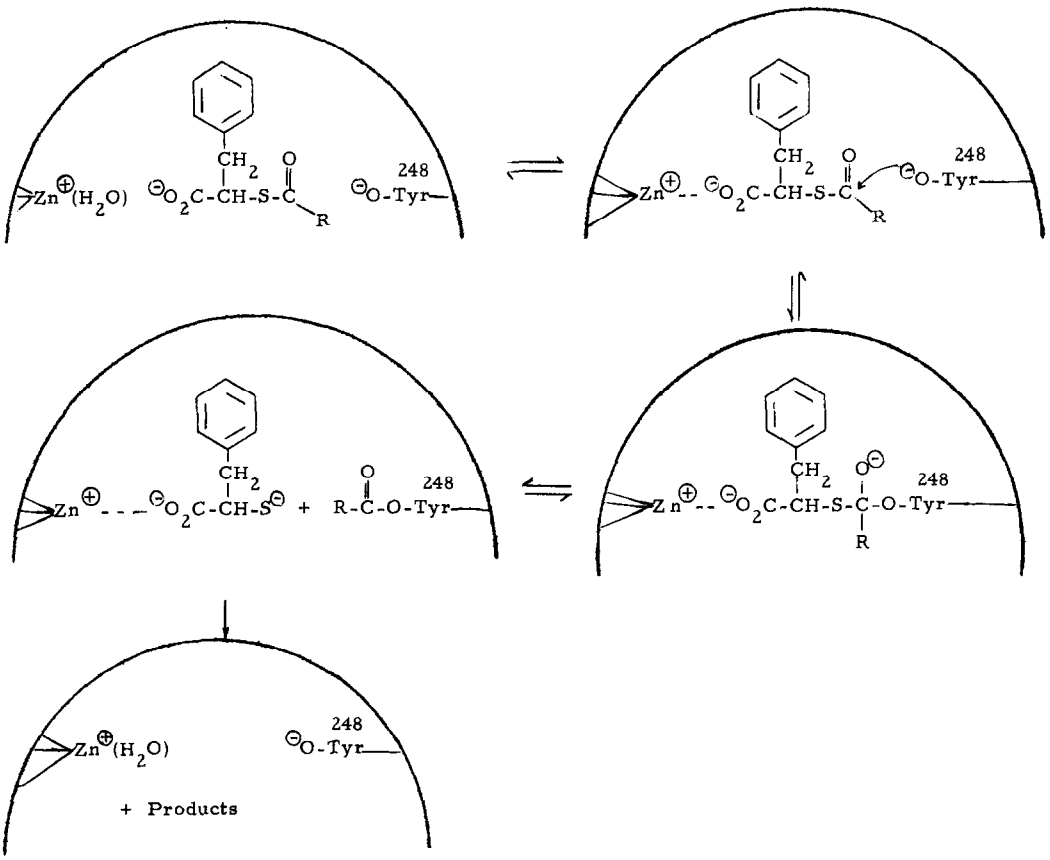

Figure 1. --Proposed mechanism for the reaction of (+)-I with carboxypeptidase.

value of pK_{a_2} which is seen. A major prediction of the mechanism of Figure 1 is that Tyr-248 should be cinnamoylated in the course of the reaction of (+)-I with CPA. Inspection of a model of CPA suggested that the cinnamoyl-enzyme linkage would be near the surface of the enzyme molecule and that it might not be difficult to detect this species.

We now wish to report the successful isolation of cinnamoyl-CPA from the reaction of (+)-I with CPA. The CPA-catalyzed hydrolysis of either (-)-I or (+)-I was followed at 320 nm or 340 nm. The concentrations and conditions employed are summarized in Table I. After no further absorbance change occurred, the reaction mixture (3.2 ml) was gel-filtered through a Sephadex G-25 (fine) column (void volume 11 ml). To 1 ml of the resultant protein solution from the reaction with (+)-I which showed enhanced absorption relative to native CPA from 250 to 340 nm (most accurately measured at 310-340 nm), 100 μl of a 1 M hydroxylamine solution of appropriate pH was added. Hydroxaminolysis to give cinnamohydroxamic acid was followed at 320 nm. When reaction appeared to be complete, sufficient 5 N NaOH (25 to 50 μl) was added to give a solution, the spectrum of which did not change appreciably over the wavelength range 310 nm to 400 nm on further addition of base. After correction for the small absorbance due to CPA, the absorbance readings measured on such alkaline solutions in the case of (+)-I agreed with the relative molar extinction coefficients of cinnamohydroxamate under the same conditions. Thus, they not only afforded the identification but also the quantitation of the cinnamohydroxamate formed, as illustrated in Table 1. No evidence for cinnamohydroxamate formation was obtained in the experiments performed on (-)-I.

These observations indicate that under the conditions used a cinnamoyl-enzyme can be isolated from the reaction of (+)-I with CPA but not from that of

TABLE I

Conditions for the Reactions of (+)-I and (-)-I with CPA and Yields in the Hydroxaminolysis Reactions

Substrate	Incubation period	S_o(M)	E_o(M)	pH	NaCl(M)	Tris(M)	MES[c](M)	Cinnamo-hydroxamate isolated (M)
(+)-I	40 min[b]	3.5×10^{-5}	6.06×10^{-5}	8.63	0.47	0.0094	-	1.41×10^{-5}
(+)-I	50 min[b]	3.5×10^{-5}	6.06×10^{-5}	8.63	0.47	0.0094	-	1.54×10^{-5}
(+)-I[a]	3 hr[b]	1.45×10^{-4}	9.71×10^{-5}	7.50	0.48	-	0.019	3.24×10^{-5}
(-)-I	3 min	3.47×10^{-5}	6.41×10^{-5}	7.50	0.50	0.0099	-	None

[a]The protein isolated by gel filtration after the incubation period was over showed a λ_{max} of 280 nm. Under the same conditions CPA, N-acetyl-O-cinnamoyltyrosinamide and cinnamate had λ_{max} values of 278 nm, 285 nm, and 270 nm, respectively.

[b]The absorbance change at 320 nm or 340 nm during the incubation period was less than that expected for the full conversion of (+)-I to cinnamate and α-mercapto-β-phenylpropionate (MP). This was due to the fact that cinnamoyl-CPA, a rather stable species, was the product and that strong inhibition of CPA by MP prevented complete reaction.

[c]2-(N-morpholino)ethanesulfonic acid.

(-)-I. Reaction of the cinnamoyl-enzyme with hydroxylamine gives rise to a substantial yield of cinnamohydroxamate. The only source other than cinnamoyl-CPA for the cinnamohydroxamate produced in our experiments might be unhydrolyzed substrate incompletely separated from the protein by gel filtration. This possibility was excluded by the fact that even if the entire cinnamohydroxamate formed were postulated to be produced from unreacted substrate bound to CPA, the bound substrate calculated to be present would account for less than 20% of the enhanced absorption seen at 325-340 nm for the gel-filtered protein after the reaction of CPA with (+)-I.

As discussed earlier in this article, a likely site for the cinnamoylation of CPA by its reaction with (+)-I is the hydroxyl of Tyr-248. Rate data for the hydroxyaminolysis of the gel-filtered solutions containing the cinnamoyl-enzyme species are compared in Table II to results measured with phenyl cinnamate. Although electronic effects would be expected to favor the hydroxaminolysis of phenyl cinnamate relative to cinnamoyl-Tyr slightly, the somewhat higher rate of reaction (3 to 4-fold) actually seen with the latter species may be related to the unusually low pK_a for the ionization of Tyr-248. In this connection the report that hydroxaminolysis of two acetylated Tyr residues in the vicinity of the active site of CPA which had been polyacetylated by reaction with acetylimidazole occurs at an accelerated rate relative to that of other acetyl-Tyr residues in the chemically modified enzyme[5] is worth noting. Further work in our laboratory to more firmly identify the site of cinnamoylation of CPA in its reaction with (+)-I is in progress.

In conclusion, our isolation of cinnamoyl-CPA from the reaction of the enzyme with (+)-I constitutes the first instance in which an acyl-enzyme has been observed directly during the esterase action of CPA. Our findings indicate that different mechanistic pathways are followed in the CPA-catalyzed hydrolysis of

TABLE II

Rates of Hydroxaminolysis of Cinnamoyl-CPA and Phenyl Cinnamate

	Gel-filtered protein solution after incubation with				Phenyl Cinnamate[a]	
	(+)-I	(+)-I	(+)-I	(-)-I		
pH	8.63	8.63	7.50	7.50	8.63	7.50
NaCl(M)	0.43	0.43	0.46	0.46	0.47	0.44
Tris(M)	0.0085	0.0085	-	0.0090	0.0093	-
MES(M)	-	-	0.018	-	-	0.017
NH_2OH(M)	0.091	0.091	0.091	0.09	0.088	0.087
$k \times 10^3 (sec^{-1})$[b]	1.74	1.98	0.649	no reaction	0.458	0.188

[a] Reaction solutions contained 3.88 (v/v) percent CH_3CN.

[b] Pseudo-first-order rate constant.

the (+)- and (-)-enantiomers of I. We believe that this is the first example in which an experimental demonstration has been made that different enantiomers of a substrate can react with an enzyme by different pathways.

References

(1) E. T. Kaiser, T.-W. Chan and J. Suh, in "Protein-Metal Interactions," M. Friedman, ed., Plenum Publishing Corp., New York (1974), p. 59.

(2) T.-W. Chan, Ph. D. Thesis, University of Chicago, 1974.

(3) E. T. Kaiser and B. L. Kaiser, Accounts Chem. Res., 5, 219 (1972).

(4) F. A. Quiocho and W. N. Lipscomb, Adv. Protein Chem., 25, 1 (1971).

(5) R. T. Simpson, J. F. Riordan and B. L. Vallee, Biochemistry, 2, 616 (1963).