inconsistent with the experimental results. In any event, this study indicates that temperature-jump relaxation studies on heterogeneous dispersions of phospholipids are possible and is indicative of the feasibility of applying this technique to the study of the dynamics of membrane-medicated processes.

A Search for an Intermediate in Carboxypeptidase A Catalyzed Ester Hydrolyses

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Abstract: The kinetics of the carboxypeptidase A catalyzed hydrolyses of *O*-(*trans*-*p*-nitrocinnamoyl)-L-β-phenyllactate (I) and *O*-(*trans*-*p*-nitrocinnamoyl)-L-mandelate (II) have been studied under substrate in excess and enzyme in excess conditions in an attempt to obtain evidence concerning the possible formation of covalent enzyme–substrate complexes as intermediates in these reactions. Stopped-flow kinetic measurements with I at high enzyme and substrate concentrations failed to reveal the accumulation of any detectable concentration of an intermediate differing significantly in its ultraviolet–visible absorption spectrum from a spectrum of a mixture of the substrates or of the products. Furthermore, a comparison of the k_{cat} values for I and II indicates that the breakdown of an acyl enzyme, *trans*-*p*-nitrocinnamoyl-carboxypeptidase A, cannot be rate limiting in the hydrolysis of II. Also the k_{cat} value is increased and the K_M value is decreased for the reaction of I relative to the corresponding kinetic parameters for the unsubstituted ester, *O*-(*trans*-cinnamoyl)-L-β-phenyllactate. The lack of evidence for the transient formation of covalent enzyme–substrate complexes in the hydrolyses of the *p*-nitrocinnamoyl esters is not surprising when two of the reasonable mechanisms for carboxypeptidase action which involve the catalytic participation of glutamate 270 in the enzyme are considered. The hypothetical intermediate in one mechanism would be a tetrahedral adduct formed by the attack of water on the carbonyl group of the ester assisted by the participation of glutamate 270 as a general base catalyst and the zinc ion at the active site as an electrophilic catalyst. In the other proposed mechanism the hypothetical intermediate might be an acid anhydride formed from the attack of glutamate 270 acting as a nucleophile on the carbonyl group of the ester. In either of the mechanisms considered the intermediates formed would be expected to be very labile indeed.

Bovine pancreatic carboxypeptidase A (CPA) is a zinc-containing metalloenzyme with a molecular weight of approximately 34,000 which catalyzes the hydrolyses of polypeptides at the C-terminal peptide bond, especially if the terminal residue contains an aromatic group. CPA also catalyzes the hydrolysis of the analogous types of ester substrates, *O*-acyl derivatives of α-hydroxy acids.[4-11] This paper is concerned with the esterase action of CPA.

In many cases the "turnover" kinetics observed for the CPA-catalyzed hydrolysis of ester substrates has been complicated by competitive product inhibition,[4,7-9] substrate inhibition,[8] and substrate activation.[6] Much of the previous work with ester substrates has been concerned with describing these effects. In the present work an attempt has been made to probe the mechanism of action of CPA by trying to identify the individual steps of the catalytic reaction.

The existence of acyl-enzyme intermediates has been suggested for CPA-catalyzed reactions, although there is, as yet, no experimental evidence for such species.[12] It might be mentioned that a covalent intermediate has been detected for another zinc metalloenzyme, *viz. E. coli* alkaline phosphatase.[13] These factors led us to conduct ester hydrolysis experiments with CPA under conditions that would favor the detection of any intermediate(s). Accordingly, we employed high enzyme and substrate concentrations in our study. Further, we utilized substrates which had low K_M values and which contained suitable chromophores. Finally, we made use of rapid reaction techniques.

Previous work in this laboratory had made use of cinnamoyl esters which have an absorption maximum in the vicinity of 280 mμ.[10] These esters were unsuitable for studies employing high enzyme concentrations since CPA has a very strong absorption band in this

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region (λ_{max} 278 mμ). For this reason we have synthesized *p*-nitrocinnamoyl esters which have absorption maxima at longer wavelengths (λ_{max} 308 mμ) and used them in our search for an intermediate in CPA-catalyzed ester hydrolyses.

Experimental Section

Syntheses. ***O*-(*trans*-*p*-Nitrocinnamoyl)-L-β-phenyllactic Acid (I).** A solution of *p*-nitrocinnamoyl chloride (1.1 g, 5.2 mmol) in tetrahydrofuran (5 ml) was brought to 5° and mixed with a similarly chilled solution of L-β-phenyllactic acid (0.86 g, 5.2 mmol) in tetrahydrofuran (THF). The mixture was magnetically stirred at 5°, and a solution of pyridine in tetrahydrofuran (1.2 ml in 2 ml of THF) added at such a rate that the temperature did not rise sharply. The solution was stirred at 5° for 1 hr and then for a further hour at room temperature. The solvent was removed under reduced pressure and the residue taken up in chloroform (100 ml). This solution was washed once with dilute HCl and then twice with water, dried over anhydrous Na_2SO_4, and evaporated to dryness. The residual yellow solid was recrystallized twice from benzene, mp 139–141° (uncorrected).

Anal. Calcd for $C_{18}H_{15}NO_6$: C, 63.34; H, 4.43. Found: C, 63.49; H, 4.53.

***O*-(*trans*-*p*-Nitrocinnamoyl)-L-mandelic Acid (II).** (a) A solution of *p*-nitrocinnamoyl chloride (1.16 g, 6.0 mmol) in dry benzene (5 ml) was added dropwise to a refluxing stirred solution of L-mandelic acid (0.912 g, 6.0 mmol) in dry benzene (10 ml). Refluxing was continued for 4–5 hr after which the solution was cooled, washed with three portions of water, filtered, and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure leaving a yellow oily residue which was recrystallized twice from a mixture of benzene and hexane.

(b) The procedure for this preparation was identical with that for *O*-(*trans*-*p*-nitrocinnamoyl)-L-β-phenyllactic acid. The following quantities were used: *p*-nitrocinnamoyl chloride, 1.16 g, 6.0 mmol; L-mandelic acid, 0.912 g, 6.0 mmol; pyridine, 1.2 ml. The product obtained was an uncrystallizable yellow oil which was finally successfully seeded with a crystal from preparation a above, mp 149–151°, equiv wt (as determined by slow titration of the product with NaOH) 326 (calcd 327).

Anal. Calcd for $C_{17}H_{13}NO_6$: C, 62.39; H, 4.00. Found: C, 62.45; H, 3.93.

Substrate Solutions. The substrates that were to be used in this work were found to be only sparingly soluble in water in their acid forms. Hence they were all converted to their sodium carboxylate forms before use.

Preparation of Enzyme Stock Solutions. For the attempts to detect enzyme–substrate intermediates in CPA-catalyzed ester hydrolyses enzyme solutions of high concentrations were required. Saturated solutions of the enzyme were prepared therefore directly from material supplied by Worthington Biochemical Corporation (code COA).

Enzyme Concentration. The concentrations of enzyme solutions were determined by the method of Simpson, *et al.*,[14] based on the measurement of the protein light absorption at 278 mμ.

Enzyme-Activity Measurements. There is no direct method at present of measuring the active-site concentration of CPA solutions. Relative activity measurements for enzyme stock solutions were determined using a standard ester substrate as previously described.[10] It was observed that during rate assay measurements using these conditions the pH of the reactant solutions dropped by 0.2–0.3 pH unit. This was undoubtedly caused by the low buffering capacity of the Tris–HCl system at the low concentration employed (0.005 *M* Tris) at pH 7.5. A series of parallel runs using a buffer that was 0.05 *M* in Tris did not suffer this defect. We recommend that buffers not less than 0.05 *M* in Tris be used in future work at pH 7.5.

Enzyme Concentration and Activity. The concentrations of the stock solutions from the two enzyme batches employed determined spectrophotometrically, and the activities determined kinetically, are given in Table I.

Inhibition Study with Sodium *p*-Nitrocinnamate. Standard assay runs were made with *O*-(*trans*-cinnamoyl)-L-β-phenyllactate ($[S]_0 = 1 \times 10^{-4}$ *M*) using various concentrations of sodium *p*-nitrocinnamate as inhibitor. When $[I]_0 = 7 \times 10^{-3}$ *M* the pseudo-first-order rate constant observed for the hydrolysis reaction was only reduced by about 10%. It was concluded that *p*-nitrocinnamic acid is a very weak inhibitor of the esterase activity of CPA.

Table I

Batch no.	COA 8AB	COA 8DD	Assay conditions
$10^4[E]_0$, *M*/l.	4.54	2.37	
$v_0/[E]_0$, sec^{-1}	12.4 ± 0.1	11.2 ± 0.1	0.005 *M* Tris, 0.5 *M* NaCl
$v_0/[E]_0$, sec^{-1}	12.7		0.05 *M* Tris, 0.5 *M* NaCl
$v_0/[E]_0$, sec^{-1}	14.1 ± 0.1	12.2 ± 0.1	0.05 *M* Tris, 1.00 *M* NaCl

Spectra. Spectra of the products obtained after complete hydrolysis of *O*-(*trans*-*p*-nitrocinnamoyl)-L-β-phenyllactic acid and *O*-(*trans*-*p*-nitrocinnamoyl)-L-mandelic acid by CPA were obtained and agreed within experimental error with the spectra measured for the appropriate mixtures of *O*-*trans*-*p*-nitrocinnamate and L-β-phenyllactate or *O*-*trans*-*p*-nitrocinnamate and L-mandelate, respectively, in the same buffer. Since in previous studies with racemic esters closely related structurally to I and II we found that only the L isomers were hydrolyzed,[10] the spectral observations we made on the products of the reactions of I and II with CPA indicate that I and II are optically pure within the accuracy of our measurements.

Kinetic Measurements. "Turnover" Kinetics. The CPA-catalyzed hydrolyses of *O*-(*trans*-*p*-nitrocinnamoyl)-L-β-phenyllactate and *O*-(*trans*-*p*-nitrocinnamoyl)-L-mandelate were followed on a Cary 15 spectrophotometer. The 0–0.1 optical density scale of the instrument was used and the wavelength for each individual run was chosen so that the optical density change was of the order 0.08–0.095. Runs at higher substrate concentration were followed at the higher wavelengths in order that the reference cell blanks would not be needed and that runs would not be made at high slit widths with consequent loss of sensitivity.

Rapid Reaction Kinetics. The instrument used for this work was a Durrum Gibson stopped-flow apparatus. Particulars of this instrument and the procedures used in this laboratory have already been described in some detail.[15]

Results

"Turnover" Kinetics. The kinetics of the CPA-catalyzed hydrolyses of the nitrocinnamoyl esters I and II were observed to be closely related to the kinetics described in earlier papers for the CPA-catalyzed hydrolyses of *O*-acetyl-L-mandelate and *O*-(*trans*-cinnamoyl)-L-β-phenyllactate.[4,7,10] Our kinetic results could be treated in terms of a simple Michaelis–Menten scheme, complicated only by competitive product inhibition (see eq 1). In the case of ester I the inhibitory product was L-β-phenyllactate and in the case of II it was L-mandelate. The steady-state rate expression for the scheme of eq 1 is given by eq 2 where $K_i = k_{-i}/k_i$, and the quantity $([S]_0 - [S])$ represents the concentration of the inhibitory product P, at any time *t*. A computer program was used to calculate the parameters K_i, K_M, and k_{cat}. The program is adequately described elsewhere.[10,16,17]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P_1 + P_2 \qquad (1)$$

$$E + P_1 \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} EP_1$$

$$v = \frac{-d[S]}{dt} = \frac{k_{cat}[E]_0[S]}{K_M + [S] + K_M([S]_0 - ([S])/K_i} \qquad (2)$$

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(16) F. W. Carson, Ph.D. Thesis, University of Chicago, Chicago, Ill., 1965.

Table II. Collected Kinetic Data for the CPA-Catalyzed Hydrolysis of Some Ester Substrates[a]

Substrate	K_M, M	k_{cat}, sec^{-1}	k_{cat}/K_M, M^{-1} sec^{-1}	K_i, M
O-(*trans*-Cinnamoyl)-L-β-phenyllactate[b]	$(1.87 \pm 0.07) \times 10^{-4}$	67.2 ± 1.7	3.67×10^5	$(5.8 \pm 0.3) \times 10^{-5}$
O-(*trans*-*p*-Nitrocinnamoyl)-L-β-phenyllactate	$(0.99 \pm 0.02) \times 10^{-4}$	230 ± 1.0	2.32×10^6	$(9.0 \pm 0.3) \times 10^{-5}$
O-(*trans*-*p*-Nitrocinnamoyl)-L-mandelate	$(0.88 \pm 0.03) \times 10^{-3}$	5.12 ± 0.12	5.82×10^3	$(2.5 \pm 0.2) \times 10^{-3}$
O-Acetyl-L-mandelate[c]	$(6.00 \pm 0.75) \times 10^{-2}$	0.488 ± 0.038	8.13	$(1.8 \pm 0.4) \times 10^{-3}$

[a] Medium: aqueous buffer, 1.00 *M* NaCl, 0.05 *M* Tris; pH 7.50; temperature, 25.0° [b] Medium: 0.500 *M* NaCl, 0.005 *M* Tris; pH 7.48; temperature, 25.0°; ref 10. [c] Medium: 0.500 *M* NaCl, 2% acetonitrile or dimethylformamide; pH 7.5; temperature, 25.0°; ref 16.

The kinetic parameters for the CPA-catalyzed hydrolysis of *O*-(*trans*-*p*-nitrocinnamoyl)-L-β-phenyllactate (I) were obtained from the analysis of rate data using ten substrate concentrations over a range of 6.65×10^{-5} to 9.69×10^{-4} *M* and an enzyme concentration range of 1.097×10^{-8} to 2.215×10^{-8} *M* at pH 7.49 ± 0.03 and 25.0°. The results are summarized in Table II.

In the case of the hydrolysis of *O*-(*trans*-*p*-nitrocinnamoyl)-L-mandelate (II) the substrate concentration range employed was 3.8×10^{-5} to 1.95×10^{-3} *M* and the enzyme concentration varied from 0.95×10^{-7} to 7.64×10^{-7} *M*. Kinetic measurements were carried out at pH 7.50 ± 0.02 and 25.0°, and the kinetic parameters found are also recorded in Table II.

The Findings of the Search for a Transient Intermediate. A search for a transient intermediate in the reaction of CPA with *O*-(*trans*-*p*-nitrocinnamoyl)-L-β-phenyllactate (I) spectrophotometrically using a stopped-flow apparatus showed negative results. The maximum decrease in absorbance on mixing was at 300 mμ. It changed to zero at 330 mμ corresponding to the isosbestic point in the substrate–product difference spectrum. Above 330 mμ the displacement corresponded to an increase in the absorbance. All of these observations correlated to the difference spectrum of the substrate and product. There was no variation in the half-life of the signal with wavelength. It was concluded that this method was not capable of providing evidence for any transient intermediate in the hydrolysis of I, even at the highest enzyme concentrations available, $[E_0] = 1 \times 10^{-4}$ *M*.

Kinetic Results for the Reactions of Substrates with an Excess of CPA. The kinetics of the reactions of *O*-(*trans*-*p*-nitrocinnamoyl)-L-β-phenyllactate (I) and *O*-(*trans*-*p*-nitrocinnamoyl)-L-mandelate (II) with CPA in excess were measured with the stopped-flow apparatus. The reactions were followed at 300 mμ, the wavelength at which the largest signal was observed. The reactions followed pseudo-first-order kinetics in all cases.

In these experiments the substrate concentrations were kept as low as possible but high enough so that there was a sufficient absorbance change to follow the reaction. An excess of enzyme over substrate was always present. In this way complications due to product inhibition were avoided.

According to the reaction scheme suggested in eq 1, in the presence of a sufficient excess of enzyme, a pseudo-first-order reaction should be observed. The pseudo-first-order rate constants, k_{obsd}, measured for the formation of *p*-nitrocinnamate are related to the quantities of eq 1 and 2 by eq 3.

$$k_{obsd} = \frac{k_{cat}[E_0]}{K_M + [E_0]} \quad (3)$$

The rate constants obtained for the hydrolysis of *O*-(*trans*-*p*-nitrocinnamoyl)-L-β-phenyllactate in the presence of excess CPA have a relatively high experimental error (*ca.* 10%) due to the extremely short half-lives of this reaction under the conditions used and also the relatively small signal obtainable (sometimes less than 2% transmittance). Therefore, although it was possible to work with concentrations of enzyme close to K_M with this substrate, we could only calculate the ratio k_{cat}/K_M with any degree of confidence. The results obtained for the hydrolysis of *O*-(*trans*-*p*-nitrocinnamoyl)-L-mandelate (II) on the other hand had relatively large half-lives and were hence far more accurate. However, it was not possible to approach close to the value of K_M for II with the enzyme concentration, and once again we could only obtain a satisfactory value for the ratio k_{cat}/K_M. From a comparison of the results in Tables II and III it can be seen that the k_{cat}/K_M *ratios* for both I and II obtained from the [S] ≫ [E] and [E] ≫ [S] conditions agree very satisfactorily. In summary, the experiments we performed at high enzyme concentrations showed that there was no detectable accumulation of spectrally absorbing transient intermediates in the *p*-nitrocinnamoyl ester hydrolyses.

Table III. Results from Stopped-Flow Kinetic Measurements with Enzyme in Excess

	O-(*trans*-*p*-Nitrocinnamoyl)-L-β-phenyllactate	*O*-(*trans*-*p*-Nitrocinnamoyl)-L-mandelate
k_{cat}/K_M	$(2.6 \pm 0.3) \times 10^6$	$(4.95 \pm 0.4) \times 10^3$

Conclusions

We have found that the introduction of a *p*-nitro substituent in the cinnamoyl residue of *O*-(*trans*-cinnamoyl)-L-β-phenyllactate causes a substantial increase in the k_{cat}/K_M ratio for the hydrolysis of the ester. This increase is due to both an increase in the value of k_{cat} and a decrease in the value of K_M for the nitro-substituted compound (I) relative to the corresponding parameters for the unsubstituted ester (see Table II).

The k_{cat} and K_M values measured in the CPA-catalyzed hydrolysis of the nitrocinnamoyl esters may be complex quantities. Intermediate enzyme–substrate

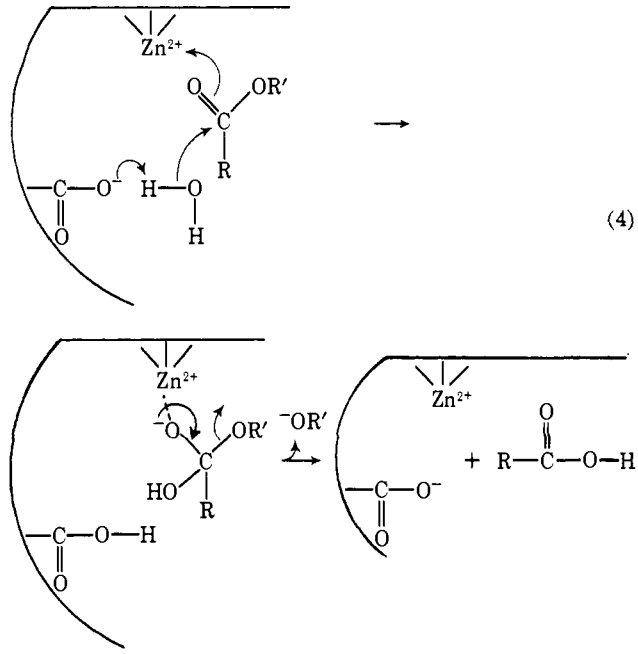
(17) P. L. Hall, Ph.D. Thesis, University of Chicago, Chicago, Ill., 1967.

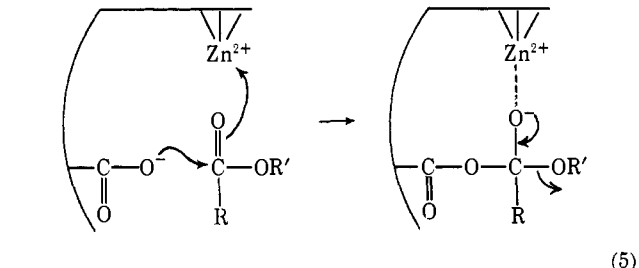
complexes may lie along the reaction pathway leading from the Michaelis complex ES (eq 1) to the regeneration of the free enzyme and the formation of products P_1 and P_2. Specifically, the products P_1 and P_2 could be formed in a stepwise fashion by a mechanism involving the intermediate formation of an acyl-enzyme species.[11,12,18] If we consider this possibility it is clear that the decomposition of a *p*-nitrocinnamoyl-CPA species cannot be rate limiting in the hydrolysis of II since the k_{cat} value in the case of II is lower than that seen for I. Furthermore, in the hydrolysis of I studied by the stopped-flow method at high enzyme and substrate concentrations no spectrophotometric evidence could be obtained for the formation of an intermediate. If *p*-nitrocinnamoyl-CPA would accumulate to a significant extent in the hydrolysis of I it seems likely that we would have detected it in the stopped-flow experiments (unless the ultraviolet–visible spectrum of the intermediate is identical with that of the mixture of the starting materials or of the products).

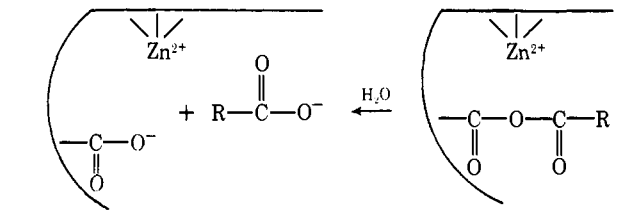
We believe therefore that the breakdown of a *p*-nitrocinnamoyl-CPA species is not rate controlling in the reaction of I or II. Also, by comparing the k_{cat} values for *O*-(*trans*-cinnamoyl)-L-β-phenyllactate and I given in Table II, it can be concluded that if a covalent complex of L-β-phenyllactate with CPA forms as an intermediate, the breakdown of the complex cannot be rate limiting at least in the case of the former ester. From a comparison of the data for *O*-acetyl-L-mandelate to those for the hydrolysis of II a similar conclusion can be reached in the case of the acetyl ester concerning a possible L-mandelate-CPA intermediate.

On the basis of their X-ray crystallographic studies on CPA, Lipscomb and his coworkers have suggested[12] that a carboxylate group of glutamate 270 in the enzyme might be catalytically important, acting either as a nucleophile or as a general base. The results of solvent isotope effect measurements on the CPA-catalyzed hydrolysis of *O*-(*trans*-cinnamoyl)-L-β-phenyllactate which were reported recently[11] favor somewhat the hypothesis that the carboxylate group is functioning as a general base catalyst although the findings were certainly not conclusive in this regard. If the carboxylate of glutamate 270 were indeed acting as a general base catalyst as shown in eq 4 below, it would not be surprising that we found no evidence in the present study for an intermediate species other than the Michaelis complex since the tetrahedral species formed from the ester as an intermediate in the reaction pathway of eq 4 should be very labile.

On the other hand if the carboxylate residue of glutamate 270 acts as a nucleophile and the reaction pathway of eq 5 holds, the intermediate species might be an anhydride, and one might expect that such a species would hydrolyze extremely rapidly making it very difficult to detect.







Further studies using direct and indirect approaches for the detection of intermediates in the CPA-catalyzed hydrolyses of esters and peptides are in progress in our laboratory.

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