Multichannel Resonance Raman Experiments on Carboxypeptidase A Catalyzed Ester Hydrolysis under Cryoenzymological Conditions

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Two principal reaction pathways have been considered in many laboratories for the action of carboxypeptidase A (CPA) on ester or peptide substrates.[1,2] In one route, residue Glu-270, an essential active site group, acts as a general base catalyst;[3,4] in the other pathway, Glu-270 acts as a nucleophile forming a transient enzyme-bound mixed anhydride intermediate. In 1976 one of us (E.T.K.), together with Makinen and Yamamura,[5] showed that, under conditions of enzyme in excess, biphasic kinetics could be observed at subzero temperatures in the CPA-catalyzed hydrolysis of *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllacetate (**1**). The kinetic observations were interpreted as being consistent with the transitory formation of a mixed anhydride intermediate, involving the carbonyl group of the substrate, **1**, and the γ-carboxylate of Glu-270, with the slow step in the hydrolysis reaction at low temperature being the breakdown of the anhydride. Subsequent to the report of these kinetic observations, Makinen and co-workers published an extensive series of articles[6-8] on fundamental properties of the anhydride species including the coordination state of the active site metal ion. The validity of the interpretation of their results rests upon the premise that the anhydride intermediate accumulates at low temperatures, allowing them to observe its physical properties directly. To the best of our knowledge, there has been no direct test of the extent to which an anhydride intermediate, if formed, accumulates at subzero conditions in carboxypeptidase A catalyzed ester hydrolysis. The present communication contains results we have obtained at low temperatures using multichannel resonance Raman (RR) spectroscopy to ascertain the extent to which acyl-enzyme intermediates accumulate in the CPA-catalyzed hydrolysis of ester substrates.

A problem with the use of **1** in mechanistic studies has been that the difference in the observed rate constants measured for the fast and slow phases of its CPA-catalyzed hydrolysis is small. In view of the success Breaux and Bender had in using (*trans*-*p*-(dimethylamino)cinnamoyl)imidazole to generate a relatively stable acyl-enzyme in the case of elastase,[9] we anticipated that the use of *O*-(*trans*-*p*-(dimethylamino)cinnamoyl)-L-β-phenyllacetate (**2**), as an ester substrate for CPA might lead to a stabilized intermediate in the action of CPA.[10a]

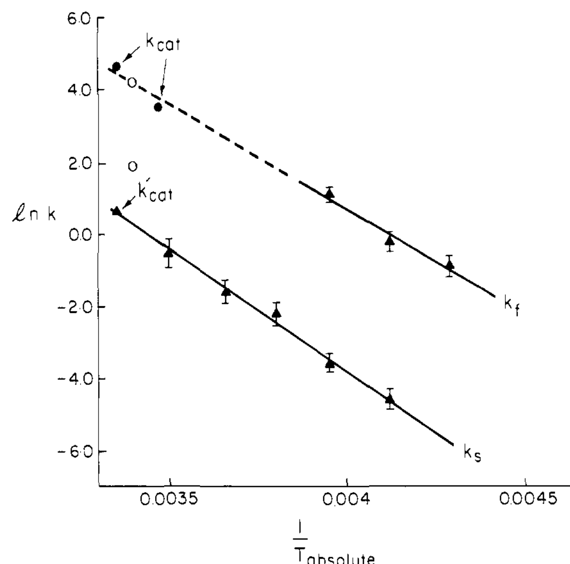

Figure 1. Temperature dependence of rate constants for CPA-catalyzed hydrolysis of **2**. Reaction solutions: (●) an aqueous buffer consisting of 20 μM sodium cacodylate, pH 7.5 containing 1.0 M NaCl; (▲) the aqueous cacodylate buffer (20%), ethylene glycol (40%), and methanol (20%); (○) the aqueous cacodylate buffer (55%), ethylene glycol (30%), and methanol (15%). The k_{cat} values estimated at 25 (●, ▲) and 12 °C (●) were obtained from measurements with substrate in excess. All other measurements were made under conditions of enzyme in excess.

At 25.0 °C, CPA$_\alpha$ catalyzed the hydrolysis of **2** monitored at 325 nm with $k_{cat}/K_m = 4.2 \times 10^4\ M^{-1}\ min^{-1}$ in 20 mM sodium cacodylate buffer, pH 7.5 containing 1 M NaCl. With a mixed aqueous-organic solvent (40% buffer, 40% ethylene glycol, 20% methanol), k_{cat}/K_m was $10^3\ M^{-1}\ min^{-1}$, substantially less.[10b] Under conditions of enzyme in excess (typically, [E]/[S] = 3.4), the reaction kinetics of the CPA-catalyzed hydrolysis of **2** in the mixed solvent at temperatures ≤-20 °C were biphasic. Using these conditions, the two phases were readily separated and could be analyzed as consecutive first-order reactions.

The values of the observed first-order rate constant measured for the fast phase, k_f, at temperatures ≤-20 °C are shown in Figure 1. As can be seen from Figure 1, the values for k_{cat} estimated under conditions of $[S]_0 >> [E]_0$ at 12.0 and 25.0 °C in purely aqueous buffer[10b] fall nicely on the extrapolated line for k_f. The value estimated at 25.0 °C in the cryosolvent, k_{cat}', falls on the line for the rate constants for the slow step, k_s, measured at subzero temperatures. These results are consistent with the hypothesis that it is the nature of the solvent that determines which is the rate-determining step in the CPA-catalyzed hydrolysis of esters like **2** and not the temperature, as was previously thought.[5]

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(10) (a) The procedure for the synthesis of **2** involved the following steps: *p*-(Dimethylamino)cinnamic acid (1 mmol, 191 mg) was dissolved in 20 mL of dry, amine free DMF and cooled to 0 °C under N_2. PCl_5 (1 mmol, 209 mg) was added in DMF slowly, and the solution was stirred at 0 °C for 1 h before being allowed to warm to room temperature. The red solution was concentrated under reduced pressure and cooled to 0 °C under N_2. L-β-Phenyllactic acid (1 mmol, 166 mg) was added, the mixture washed down with DMF, and the solution stirred overnight. After removing the solvent under reduced pressure, the red residue was dissolved in EtOAc and extracted with 4 × 25 mL of 50% $NaHCO_3$. The combined aqueous extracts were vigorously stirred with 75 mL of $CHCl_3$ and brought to pH 5 (indicating paper) with acetic acid. The aqueous phase was separated and washed with $CHCl_3$. The combined organic extracts were washed once with saturated NaCl and dried over Na_2SO_4. At this stage TLC (silica; EtOAc, 1% AcOH) showed the presence of *p*-(dimethylamino)cinnamic acid (R_f 0.77) and product (R_f 0.53). The product was purified by silica gel chromatography, eluting with $CHCl_3$ and $CHCl_3$/EtOAc. NMR ($CDCl_3$) δ 3.01 (s, 6 H), 3.25 (m, 2 H), 5.4 (t, 1 H), 6.22 (d, 1 H), 6.65 (d, 2 H), 7.29 (m, 5 H), 7.40 (d, 2 H), 7.63 (d, 1 H); mp 178-180 °C; yield 16 mg (4.5%). (b) The highest substrate concentration used in these experiments was 3×10^{-4} M. Because the K_m values for the reaction of **2** with CPA are high we could only estimate them. Approximate values for k_{cat} and K_m at 25.0 °C were $10^2\ min^{-1}$ and 2.5×10^{-3} M in aqueous solvent and 2 min^{-1} and 2×10^{-3} M in mixed solvent, respectively.

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Table I. Resonance Raman Positions for the *p*-Chlorocinnamoyl Chromophore[a]

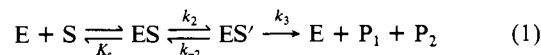
system	temp, °C	$\nu_{C=O}$, cm^{-1}	$\nu_{C=C}$, cm^{-1}	benzene ring mode, cm^{-1}
CPA$_\alpha$ (4 × 10^{-4} M) + ester 1 (3.8 × 10^{-4} M)	−40	undetected	1636, s	1595.5, s
CPA$_\alpha$ (4 × 10^{-4} M) + 1 (3 × 10^{-4} M) + inhibitor (racemic benzylsuccinate, 1.5 × 10^{-3} M)	−40	undetected	1636.5, s	1597.5, s
CPA$_\alpha$ (4 × 10^{-4} M) + 1 (3.8 × 10^{-4} M)	−20	undetected	1642, s	1596, s
p-chlorocinnamic acid (2.3 × 10^{-3} M) in CH_3CN		1719, m	1640, s	1595, s
ester 1 (2.4 × 10^{-3} M) in CH_3CN		1720, m	1636, s	1595, s
p-chlorocinnamic propionic anhydride (3.25 × 10^{-3} M) in CH_3CN		1730, m, 1800, vw	1630, s	1595, s

[a] s = strong, m = medium, vw = very weak.

The reaction of substrate **2** and to a somewhat lesser extent that of **1** with CPA appear to be excellent choices for examination by RR spectroscopy. It was our hope that we could detect spectroscopically the chromophores corresponding to mixed enzyme anhydrides for these substrates. We wish now to report that our RR data show that the spectra of the putative enzyme–substrate complexes, taken at low temperature under conditions where biphasic kinetics are seen for these esters, are indistinguishable from those of the spectra of the substrates added to an enzyme–benzylsuccinate inhibitor complex. In Figure 2 this is shown for a mixture of substrate **2** with CPA at −25 °C. An exactly analogous result was obtained for CPA + **1** at −40 °C (Table I). Moreover, identical results are obtained for the α and β forms of the enzyme. Upon allowing the temperature of the sample to rise, the spectrum changes and becomes that of the COO^- form of the respective acid. Thus, in Figure 2, at 6 °C, the 1600.5- and 1623.5-cm^{-1} peaks seen at low temperature for the mixture of **2** and CPA have moved to 1606 and 1628 cm^{-1}, respectively. These features correspond to a phenyl ring mode and the ethylenic stretching frequency of the cinnamoyl group. The strong electron-donating power of the *p*-$N(CH_3)_2$ group results in the vibrational coupling of these modes while in the case of ester **1** the corresponding modes are not coupled appreciably. Consequently, chemical changes about the ester bonds bring about synchronous shifts in both these features for the *p*-$N(CH_3)_2$ derivatives, whereas for the *p*-Cl compounds only $\nu_{C=C}$ shifts markedly (Table I).

The immediate conclusion from the RR data is that no evidence has been found for the accumulation of an intermediate with spectral properties differing from those of the substrate. In order to find out how sensitive the spectral signature is to chemical changes, the RR spectra were recorded in CH_3CN for the ester substrates **1** and **2** and the corresponding cinnamic acids and cinnamic propionic acid anhydrides.[11] In this solvent $\nu_{C=C}$ in the RR spectrum of each species is characteristic (e.g., Table I) and may be interpreted in terms of the π-electron polarization model.[12] For any of the *p*-Cl or *p*-$N(CH_3)_2$ model compounds in CH_3CN, the cinnamoyl carbonyl band is clearly identified near 1720 cm^{-1}. The absence of this feature in the enzymatic solutions (e.g., Figure 2) is significant and can be attributed to multiple environments (caused, e.g., by varying contacts with H_2O molecules) broadening the band profile so that it is lost in the background. The failure to observe $\nu_{C=O}$ in the low-temperature RR spectra of the enzyme–substrate complexes indicates that the carbonyl moiety is not in an hydrophobic environment.[13] Given the overall similarity to the spectrum in the presence of inhibitor, it is probably in a poorly defined, heterogeneous, hydrogen-bonding milieu. One result of the hydrogen-bonding interactions at the carbonyl group would be to increase electron polarization and to reduce $\nu_{C=C}$ for a putative anhydride intermediate a few reciprocal centimeters below the value observed for the anhydride in CH_3CN. Major twisting about the C=C—C=O single bond would result in the loss of the $\nu_{C=O}$ in the RR spectrum but would also change the position of $\nu_{C=C}$.

From the results on models it is apparent that although the differences are modest, it is possible to distinguish the RR spectrum of an anhydride from that of an acid or ester when the molecules are in the same solvent and are not subject to differing stereochemical constraints. The question then arises as to whether dielectric or stereochemical effects in the enzyme active site could alter the RR spectral signature of an anhydride species so that it is identical with that of the corresponding ester. It is impossible to refute this hypothesis with certainty, and it can only be stated, on the basis of experience with related chromophoric enzyme–substrate complexes, that, for two systems with such differing electronic properties as the *p*-Cl and *p*-$N(CH_3)_2$ derivatives, such a coincidence is unlikely. Unquestionably, we can find no evidence for a major population of anhydride or any other complex whose cinnamoyl moiety has a vibrational signature and distribution of π-electrons differing significantly from that of the substrate,



$$E + S \underset{K_s}{\rightleftharpoons} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES' \xrightarrow{k_3} E + P_1 + P_2 \quad (1)$$

(where E is the free enzyme, S is **2**, ES is the Michaelis complex, ES′ is an intermediate, P_1 is *trans-p*-(dimethylamino)cinnamate, and P_2 is L-β-phenyllactate).

In summary, the RR data do not indicate that an enzyme-bound mixed-anhydride intermediate accumulates in the CPA-catalyzed hydrolysis of the esters **1** or **2** at low temperature. The kinetic scheme of eq 1 which was proposed by Kaiser and Kaiser[2] for ester substrates is consistent with our results on **1** and **2** as well as with the recent findings reported by Galdes et al.[14] on the reactions of depsipeptide and peptide substrates with CPA at low temperature. According to eq 1, the fast phase of the reaction observed between **2** and CPA with enzyme in excess at low temperature would correspond to the establishment of the equilibrium between enzyme, substrate, and ES′. In the slow phase of the reaction after this equilibrium is established, breakdown of ES′ is rate controlling. It should be pointed out that our kinetic and RR observations are not incompatible with the transient formation of an anhydride intermediate undergoing rate-controlling breakdown in mixed aqueous–organic solvent at low temperature, providing that the anhydride intermediate is in an unfavorable equilibrium with the starting substrate and enzyme. Model building at the active site of CPA shows that if a mixed anhydride were formed between the γ-carboxylate of Glu-270 and the carbonyl group of *O*-(*p*-*trans*-chlorocinnamoyl)-L-β-phenyllacetate, the leaving alcohol L-β-phenyllactate would be trapped in a hydrophobic binding pocket of the enzyme, making it quite reasonable that reversal of the anhydride-forming step could occur readily.[15] This situation

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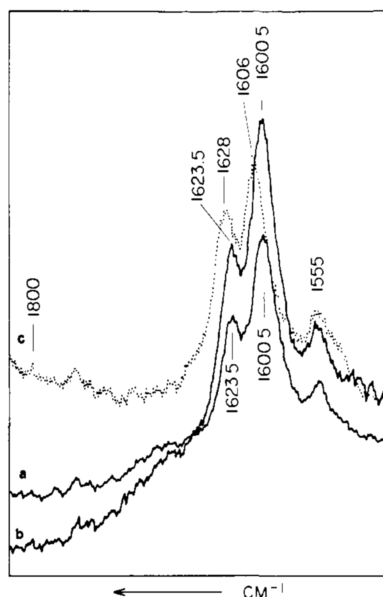

Figure 2. Resonance Raman spectra: (a) Spectrum of a mixture of ester substrate **2** (3.3×10^{-4} M) with CPA_α (4.5×10^{-4} M) at −25 °C (solid line, top). The solvent that had an apparent pH of 7.5 consisted of 50% ethylene glycol, 50% aqueous 0.1 M sodium cacodylate, and 0.5 M NaCl. Recording of the spectrum was started approximately 5 s after mixing, at a point where the reaction of **2** with CPA was in the "slow" phase of the biphasic kinetics seen. The peaks observed for *p*-(dimethylamino)-cinnamic propionic anhydride in CH_3CN occur at 1622 and 1598 cm^{-1} and are both expected to be 1–2 cm^{-1} lower under the solvent conditions of this experiment. The spectra were obtained using a diode array-based multiplex instrument described in ref 17. Approximately 200-mW, 350.7-nm laser excitation with 20-s total acquisition time was employed. Minor features in the 1650–1800-cm^{-1} region are also present in the spectra of the solvent–enzyme mixture alone. (b) Spectrum of ester **2** (3.3×10^{-4} M) added to a mixture of the competitive inhibitor benzylsuccinate (1.5×10^{-3} M racemate) with CPA_α (4.5×10^{-4} M) at −25 °C (solid line, bottom). The conditions employed were similar to those described above. (c) Spectrum of the solution resulting from allowing a mixture of ester **2** (3.3×10^{-4} M) with CPA_α (4.5×10^{-4} M), to be incubated at −25 °C and then maintained at 6 °C for 5 min (dotted line). The solvent conditions corresponded to those employed in parts a and b above. Under these conditions turnover had occurred.

is reminiscent of the hydrolysis of 3,5-dinitroaspirin where an equilibrium with an anhydride intermediate favoring the starting material is thought to occur but anhydride breakdown is rate limiting.[16] In any event, what does emerge clearly from the present study is that any interpretation of physical data, based on the premise that the mixed anhydride accumulates in mixtures of esters such as **1** or **2** with CPA at low temperatures in aqueous–organic solvents, is fraught with danger.

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(15) The observation that L-β-phenyllactate acts as a competitive inhibitor in the CPA-catalyzed hydrolysis of several esters has been cited in kinetic arguments in ref 14 that the complexes related to ES′ of eq 1 cannot be acyl-enzymes. This argument is based upon the assumption that L-β-phenyllactate must be released to solution if it is formed by acylation of CPA with a corresponding ester. However, if trapping of L-phenyllactate in the hydrophobic binding pocket of the enzyme occurs due to steric hindrance resulting from acylation of CPA by the acyl group of the acyl-L-β-phenyllactate, as is suggested by a molecular model of the enzyme, then release of L-β-phenyllactate to solution would take place only as the acyl-enzyme decomposes. In this case the observation of competitive inhibition by L-β-phenyllactate would not be inconsistent with the intermediacy of an anhydride species.

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Selective DEPT Pulse Sequence. A Rapid One-Dimensional Experiment for the Simultaneous Determination of Carbon–Proton Chemical-Shift Correlations and CH_n Multiplicities

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Multipulse techniques are finding increasing use for facilitating the structure elucidation of organic molecules by NMR spectroscopy. Much structural information can be gleaned by the generation of $^{13}C,^{1}H$ chemical-shift correlation maps, and a variety of two-dimensional NMR procedures are available for this purpose.[1] A number of one-dimensional editing pulse sequences have been developed recently for the determination of CH_n multiplicities with the DEPT pulse sequence being probably the procedure of choice.[2] Ernst and co-workers have developed a two-dimensional version of DEPT that generates simultaneously the required chemical-shift correlation *and* edits the two-dimensional contour map into CH, CH_2, and CH_3 subregions.[3] In this paper, we describe a selective version of DEPT that can be utilized as a one-dimensional NMR procedure for the generation of $^{13}C,^{1}H$ chemical-shift correlations (to an accuracy of 0.05 ppm in the ^1H spectrum) and, if desired, to acquire information concerning CH_n multiplicities.

The selective DEPT pulse sequence is as follows:

$$\pi(H_a)(\pi/2)[H,\pm x](\pi/2) \times [C,y]\text{–}1/(2J)\text{–}\pi[C,\pm x]\theta[H,y]\text{–}1/(2J)\text{–}(\pi/2)[C,\pm y] \text{ acquire } ^{13}C \text{ with receiver phase cycling, decouple proton} \quad (A)$$

$\pi(H_a)$ is a selective pulse applied to one-half of the $^{13}C,^{1}H$ doublet in the ^1H spectrum. The chemical-shift correlation is generated because of the selective nature of the soft $\pi(H_a)$ pulse. The remaining pulses are hard pulses. The soft $\pi(H_a)$ pulse causes a selective population inversion; double-quantum coherence is now generated for CH_n spin systems following the hard-pulse combination $(\pi/2)[H,\pm x](\pi/2)[C,y]$. This coherence is allowed to evolve for a time period $(2J)^{-1}$ after which it is converted to observable single-quantum coherence by the $\theta[H,y]$ pulse. These processes have been discussed in detail elsewhere.[3,4] Because of the selective nature of the experiment, no $\pi[H]$ pulses are required. The pulse combination $\pi(H_a)(\pi/2)[H,\pm x]$ is equivalent to the initial pulse segment $(\pi/2)[H,\pm y]\text{–}1/2J\text{–}$ of the basic DEPT pulse sequence

$$(\pi/2)[H,\pm y]\text{–}1/(2J)\text{–}\pi[H](\pi/2) \times [C,y]\text{–}1/(2J)\text{–}\pi[C]\theta[H,x]\text{–}1/(2J)(\pi/2)[C,\pm y] \text{ acquire } ^{13}C \text{ with receiver phase cycling, decouple proton} \quad (B)$$

The difference in phase of the θ-pulses between sequences A and B arises as a consequence of $\pi(H_a)$ being applied $J/2$ Hz off resonance.

Pulse seqeunce A can be implemented in a variety of ways dependent upon the structural information sought. Because of the functional dependence of the intensity[4] of the observed ^{13}C signal with θ ($\sin\theta$ for CH, $\sin\theta\cos\theta$ for CH_2, and $\sin\theta\cos^2\theta$ for CH_3 carbon signals) if θ is set equal to $\pi/2$, $^{13}C,^{1}H$ chem-

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