Raman Spectral Evidence for an Anhydride Intermediate in the Catalysis of Ester Hydrolysis by Carboxypeptidase A[†,‡]

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Abstract: Single crystals of carboxypeptidase A, grown at pH 7.5, have been soaked with solutions of a chromophoric substrate, the ester of *p*-dimethylaminobenzoic acid and L-β-phenyllactic acid, at pH = 8. The enzyme in the crystal catalyzes the conversion of the ester to the acid and alcohol products. During this process, pre-resonance-enhanced Raman spectra of the interior of the crystal were taken using a laser Raman microscope. In addition to the Raman bands characteristic of the substrate and the acid product, weak but reproducible bands are observed in the frequency region of 1700–1800 cm^{-1}. Bands in this region are unique to organic acid anhydrides and indicate the accumulation of an anhydride intermediate during catalysis. A comparison of these spectra with those of model compounds designed to mimic a pure anhydride intermediate and a Zn(II)-complexed anhydride intermediate suggests the active-site Zn(II) is sometimes bound to the carbonyls of the anhydride intermediate. In an attempt to observe the intermediate in the reverse reaction, the hydrolysis products of the ester substrate were combined with the enzyme crystal, and Raman spectra were obtained from the interior of the crystal. Since the Raman spectrum taken during the forward reaction is dominated by the presence of the excess substrate while the Raman spectrum taken during the reverse reaction contains no substrate bands, the two spectra are quite different. However, there are Raman bands in the spectrum taken during the reverse direction that correlate well with the bands produced from the forward reaction and with the bands of the model compounds that are assigned to the anhydride intermediate. This indicates that the reverse reaction proceeds as far as the formation of the intermediate, but no measurable substrate is formed. Since carbonyl vibrational frequencies are very sensitive to the molecular environment, the carbonyl frequencies of the ester substrate, protonated acid product, and mixed anhydride model compounds were measured in various solvents. Plots were made of the carbonyl frequencies versus Gutmann's electron acceptor numbers of the solvents. It is shown that only the mixed anhydride can possess carbonyl frequencies in the region 1720–1800 cm^{-1}. This appears to rule out the possibility that the bands observed from the enzyme/ligand spectra are attributable to ester substrate or acid product. These data are consistent with a mechanism that involves a mixed anhydride intermediate that becomes activated toward hydrolysis through binding of one or both carbonyls to the active-site Zn(II).

Introduction

Carboxypeptidase A (CPA) is a Zn(II)-containing enzyme that hydrolyzes the carboxyl-terminal peptide bond of proteins and peptides for substrates with a hydrophobic side chain on the C-terminal residue. The enzyme will also hydrolyze similarly configured esters. The Zn(II) ion, complexed to three amino acid residues and situated at the active site, is essential for activity in the native enzyme although other transition metal ions may be substituted to give varying degrees of activity.[1,2] Elucidation of the active-site structure of CPA suggested to early workers two general mechanisms to account for the enzyme's action.[3] In either of these schemes the Zn(II) serves to activate the substrate by polarizing the carbonyl of the scissile ester or peptide bond. In one proposed mechanism, Glu-270 serves as a general base delivering a hydroxide ion to the carbonyl carbon to cleave the amide or ester bond. In the alternate scheme, Glu-270 itself attacks the carbonyl to form an anhydride intermediate which is subsequently hydrolyzed.

There have been several previous efforts directed toward the detection of an anhydride intermediate during the hydrolysis of chromophoric ester substrates by CPA.[4-10] From data obtained using cryoenzymologic techniques at low temperature, Makinen and co-workers have concluded that a covalent enzyme intermediate accumulates during the hydrolysis of the L-β-phenyllactic acid ester derivative of *p*-chlorocinnamic acid.[4-6] Although these conclusions appear to be quite convincing, Christianson and Lipscomb have suggested that the apparent intermediate may have been an enzyme–substrate or an enzyme–product complex.[11] A parallel study by Carey and co-workers employing resonance Raman spectroscopy failed to detect a covalent intermediate.[10] However, Kuo and Makinen[6] have pointed out that the failure of Carey et al. to detect an intermediate is possibly tied to inadequate mixing of the viscous solvents employed. Further evidence for an anhydride intermediate in CPA catalysis has also been offered from nucleophile trapping studies of Sander and Witzel[7] and kinetic studies of Suh et al.[12] Our aim in this study is to look for further evidence for the existence of the anhydride intermediate using a chromophoric substrate with a carbonyl band that is strongly active in the Raman effect and that has a low k_{cat} so that the rate of hydrolysis of the acyl enzyme is slow.

It must be acknowledged at the outset that the isotope experiments of Nau and Riordan[8] and Breslow and Wernick[9] appear to set rather severe limitations on the anhydride intermediate mechanism. The former authors note that their experiments require that the "hypothetical anhydride intermediate be cleaved asymmetrically...." In reviewing the work of the latter authors, Fersht[13] concludes that the anhydride intermediate is ruled out by these isotope exchange experiments "unless the added amino acid [phenylalanine] is an activator of the exchange reaction... or unless the $H_2{}^{18}O$ released [at the active site] does not exchange with the medium but remains attached to the enzyme." Fersht

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[‡] Abbreviations: carboxypeptidase A (CPA); L-β-phenyllactyl-*p*-dimethylaminobenzoate (BPLDAB); L-β-phenyllactic acid (PLA); *p*-dimethylaminobenzoic acid (DA); electron acceptor number (AN).[23]

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goes on to conclude that "The mechanistic situation is thus unresolved."[13]

To seek further evidence for the occurrence of an anhydride intermediate in CPA catalysis and to better characterize the putative intermediate, we have conducted a preresonance Raman investigation of the interaction of a chromophoric substrate with single crystals of CPAα. A laser Raman microscope was used to obtain Raman spectra from the interior of a single CPAα crystal during catalysis. The chromophoric ester substrate L-β-phenyllactyl-*p*-dimethylaminobenzoate (BPLDAB) was used because it binds strongly to the enzyme active site, turns over very slowly, and gives a strong Raman signal in the carbonyl region of the spectrum. A comparison of the enzyme–ligand spectra with the Raman spectra of a model compound designed to mimic an anhydride intermediate, the mixed anhydride of *p*-dimethylaminobenzoic acid and acetic acid, and its complex with Zn(II) was taken to determine evidence for an anhydride intermediate. (It might be noted that we have found that ester and anhydride derivatives of cinnamic and furylacrylic acid give very weak carbonyl bands in the preresonance Raman effect because the carbonyl bands are coupled to the intense Raman active C=C ethylene stretching mode. Since the esters of these acids turn over much more quickly, it is difficult to isolate an intermediate.)

In an attempt to induce the formation of an anhydride using the reverse reaction, the hydrolysis products of BPLDAB were combined with the CPAα crystals and Raman spectra were obtained. Again, the spectra were compared with those of the model mixed anhydride and its Zn(II) complex in order to determine evidence for the formation of an anhydride intermediate. At pH = 8 the formation of the free ester in the back-reaction is thermodynamically unfavorable, but the formation of the anhydride intermediate at the active site could conceivably take place if there is a distinct potential energy minimum for this intermediate that is not too much greater than that of the products. As will be discussed below, the formation of this intermediate appears to be very slow. It should be noted that Breslow and Wernick found that the closely related chromophoric acid *p*-chlorocinnamic acid did not exchange ^{18}O, on the time scale of their experiment, when reacted with CPA in the presence of added L-phenylalanine or L-β-phenyllactic acid (PLA).[9] This is somewhat surprising since both the amide and ester of this acid are good substrates. However, it may be that the rate of the back-reaction is very slow relative to the rate of hydrolysis or that the water released upon intermediate formation is retained at the active site of the enzyme and taken up again upon hydrolysis, thus preventing isotope exchange.

Raman spectroscopy is useful for distinguishing an acid anhydride from the corresponding protonated acid or ester derivative because, as we will discuss below, the carbonyl bands of the acid anhydride are well separated from the carbonyls of the latter materials. In this respect it differs from electronic absorption spectroscopy which is much less sensitive to these chemical changes. Esters in solution exhibit a single carbonyl stretching band with a frequency below 1720 cm^{-1}, while protonated carboxylic acids give a single carbonyl band below 1730 cm^{-1}. Acid anhydrides, on the other hand, have two carbonyl bonds attached through an intermediate oxygen atom. As will be discussed below, they are well known to give multiple bands with frequencies from 1700 to 1840 cm^{-1}. Neither the substrate nor the enzyme gives any Raman bands above 1700 cm^{-1}. The occurrence of a number of bands above 1725 cm^{-1} in the reactions of CPA with chromophoric ligands would therefore be consistent with the formation of an anhydride intermediate.

Raman spectroscopic studies of the mechanism of CPA are hampered somewhat by the enzyme's low solubility in water at low ionic strength. However, the crystalline α form of the enzyme is at approximately 20 mM[14] and retains roughly one-third of the activity of the solubilized enzyme.[15] The crystalline enzyme therefore affords a high concentration of active enzyme. Recent work by Bicknell and co-workers suggests that the presence of C-terminal products of ester or amide hydrolysis greatly facilitates the binding of anions to the enzyme active site.[16] It therefore seemed reasonable to attempt to induce intermediate formation by combining the C-terminal product PLA with the chromophoric acid *p*-dimethylaminobenzoic acid (DA) and CPA.

A comparison of the Raman spectra of BPLDAB/CPAα with the spectra of the model compounds indicates the occurrence of an anhydride within the CPAα crystal, suggesting that the hydrolysis of BPLDAB by crystalline CPAα proceeds through an anhydride intermediate. The carbonyl region of the Raman spectrum is perturbed in a way that suggests that interaction of the active-site Zn(II) with one or both of the anhydride carbonyls. Bands characteristic of both types of anhydride intermediate are also observed from the reverse reaction. Such an interaction of the active-site Zn(II) with the anhydride intermediate would activate the intermediate toward hydrolysis and suggests a novel mechanism may account for the hydrolysis of chromophoric ester substrates.

Experimental Section

Sources of Chemicals. *p*-Dimethylaminobenzoic acid (DA), L-β-phenyllactic acid (PLA), and oxalyl chloride were obtained from Sigma Chemical Co. of St. Louis, MO. DA was further purified by recrystallization from ethanol. Acetyl chloride was obtained from Mallinckrodt of West Germany. BPLDAB was synthesized by Professor Lloyd Dolby of the chemistry department of the University of Oregon. All organic solvents were of standard reagent grade.

Carboxypeptidase A (CPA) type I, the α form of the enzyme, was obtained from Sigma. The enzyme was recrystallized according to the procedure of Lipscomb.[17] Studies with solubilized CPA were done with the less expensive type II enzyme, also from Sigma. The type I enzyme (α form) differs from the type II enzyme (γ form) by the presence of a pentapeptide at the amino terminus. Both forms of the enzyme possess the same kinetic parameters in solution.[15]

Synthesis of BPLDAB. L-β-Phenyllactyl-*p*-dimethylaminobenzoate (BPLDAB) was synthesized from *p*-dimethylaminobenzoyl chloride (DAB-Cl) and the benzyl ester of PLA. The acid chloride of *p*-dimethylaminobenzoic acid (DA) was synthesized by slowly adding 0.012 mol of DA dissolved in tetrahydrofuran to 0.036 mol of oxalyl chloride in a flask maintained at 50 °C. The reaction mixture was allowed to cool to room temperature and stirred for 12 h. A Drierite trap was placed on the flask to allow gases to escape while keeping the solution dry. At completion, solvent and excess oxalyl chloride were removed. An IR spectrum gave a band at 1725 cm^{-1} confirming synthesis.

The carboxyl function of PLA was protected to prevent its participation in the esterification step to yield the substrate. This was done by combining 0.0295 mol of PLA with equimolar amounts of diazobicycloundecane and benzyl chloride in acetonitrile and stirring at room temperature. Thin layer chromatography (TLC) revealed that after 2 days of stirring the reaction was incomplete. The reaction flask was then warmed on a steam bath for 1 h. TLC confirmed completion of the reaction. The desired product was then isolated with dichloromethane, washed with sodium carbonate, and dried on a vacuum pump.

The synthesis of the substrate was accomplished by combining 0.0085 mol of protected PLA with 0.0109 mol of DAB-Cl in 10 mL of acetonitrile with 3 mL of pyridine. TLC revealed that synthesis was incomplete after standing for 2 h. At this point, 100 mg of dimethylaminopyridine were added, and after 2 h the solution was washed with water and $KHCO_3$. The desired product was then extracted with CH_2Cl_2 and recrystallized from CH_3OH to yield 2.4 g. The protective benzyl group was removed by hydrogenation in 50 mL of ethanol and 300 mg of 10% palladium catalyst. After 3 h the solvent was removed and the product was recrystallized twice to yield the ester substrate. Elemental analysis: %C, 68.91 (calc, 69.98); %N, 4.23 (calc, 4.47); %H, 5.90 (calc, 6.12).

Synthesis of the Model Mixed Anhydride. *p*-Dimethylaminobenzoic acetic anhydride (DAA) was prepared by combining equimolar amounts of DA, acetyl chloride, and triethylamine in a minimum of dry tetrahydrofuran. After stirring for approximately 30 min, the reaction was essentially complete. The triethylammonium chloride was then removed by filtration, and an equal volume of diethyl ether was added to the remaining organic phase which was then washed successively with H_2O, 0.2 M $NaHCO_3$, 10^{-4} M HCl, and brine. The remaining organic phase

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Table I. Raman Bands of the Model Compounds for the Study of the Interaction of the *p*-Dimethylaminobenzoyl Chromophore with Crystalline CPA

DABPLA[a]	DAA[b]	DAA/Zn(II)[c]	DA[d]	DA/Zn(II)[e]	DAanion[f]	DAanion/Zn(II)[g]
	1795 m	1787 w				
	1765 w	1758 m				
	1718 m		1714 s			
		1707 m				
1685 m						
1605 s	1606 s	1605 s	1613 s	1609 s	1610 s	1608 s
1560 w	1558 w	1558 w	1564 w			
1536 w	1534 w	1534 w	1534 w	1537 w	1533 w	1534 w
			1497 w			
1451 w	1446 w	1446 w				
				1408 s		1406 s
					1389 s	
	1376 w	1373 w				
1355 m						
		1321 w				
		1296 w				
1286 m	1281 m	1280 w				
			1260 m			
		1247 w				
				1204 m	1204 m	1202 m
1193 m	1195 w	1193 m	1188 s			
	1154 m	1162 m		1154 m		
1137 w				1139 m		

[a] From 0.15 M Tris (pH = 8). [b] From $CDCl_3$. [c] From $CDCl_3/ZnCl_2$. [d] From tetrahydrofuran (THF). [e] From THF/$ZnCl_2$. [f] DA Na^+ salt from EtOH. [g] DA Na^+ salt from EtOH/$ZnCl_2$; letters with band assignments refer to band intensities: s = strong, m = medium, w = weak.

was then dried with $MgSO_4$ and the solvent removed. The product was purified by recrystallization twice from tetrahydrofuran. Elemental analysis: %C, 64.10 (calc, 63.94); %H, 6.35 (calc, 6.27); %N, 6.59 (calc, 6.68).

CPA-Catalyzed Hydrolysis of BPLDAB. Kinetic parameters for the hydrolysis of BPLDAB by CPA were determined in solution by monitoring the increase in absorbance at 274 nm corresponding to the production of DA. Reactions were carried out in 0.025 M Tris, 0.5 M NaCl (pH = 7.5), using 0.1 μM CPA and BPLDAB concentrations ranging from 10 μM to 100 μM. Absorbance readings were made on a Beckman DU-40 spectrophotometer. Eadie–Hofstee plots of the data gave K_M = 65 μM and k_{CAT} = 0.6 s^{-1}.

To establish that BPLDAB is a substrate for crystalline CPAα, the following experiment was performed. A 1 mM solution of BPLDAB in 0.025 M Tris, pH = 8.0, was made to pass through an open-ended capillary containing CPAα crystals using a Sage Instruments syringe pump. The effluent was collected and its absorbance at 318 nm, the λ_{max} of the substrate, was determined. A comparison of the resulting value to that of the freshly prepared substrate solution and to that of a 1 mM solution of DA revealed that 21% of the substrate had been hydrolyzed. A concurrent measurement of the absorbance at 318 nm of a portion of the original substrate solution that did not pass over the enzyme crystals revealed no hydrolysis.

Raman Spectra of the Model Compounds. Raman spectra of BPLDAB and of the model compounds for the reaction of BPLDAB with CPAα were obtained using either the 458-nm, 488-nm, or 515-nm line from a Spectra Physics Model 165 Ar^+ laser with the laser output power at approximately 100 mW. Samples were placed in melting point capillaries and the scattered light was collected at 90° from the incident light. The scattered light was then dispersed through a Spex 1301 double grating monochromator onto a S-20 type photomultiplier tube. Interfacing of a Hewlett-Packard 9121 computer with the monochromator permitted computer-controlled acquisition of spectra. Several scans were usually necessary to obtain acceptable signal-to-noise ratios.

Zn(II) complexes of the model compounds were prepared by stirring a solution of the compound over dry $ZnCl_2$. Raman spectra of these solutions were then compared to Raman spectra of aliquots of the solutions obtained before the addition of $ZnCl_2$.

Reactions of DA and DA anion with Zn(II) were essentially 100% complete as revealed by comparison of these spectra to the spectra of the chromophores in the absence of Zn(II). This was not the case with DAA. In this case, the extent of reaction was determined from ^1H NMR by the upfield shift of the δ = 2.3 ppm resonance of the acetylic protons upon addition of the anhydride to Zn(II). Such a displacement (of approximately 0.2 ppm) is consistent with the deshielding of these protons resulting from a polarization of the carbonyls by Zn(II). The ratio of the area of the Zn(II)-perturbed resonance to that of the δ = 2.3 ppm resonance revealed the fraction of DAA that had reacted with Zn(II). An equivalent component of the DAA Raman spectrum was then subtracted from the Raman spectrum of the mixture of DAA and DAA/Zn(II) to yield the Raman spectrum of DAA/Zn(II). It was assumed that the position and intensity of the 1605-cm^{-1} band, due to a ring mode of the phenyl group, was independent of the bound state of the carbonyls. To determine relative intensities of the Raman bands, some of the spectra were fit to a minimum number of Lorentzians using a program developed in our laboratory.

Raman Spectra of the Interaction of Ligands with Crystalline CPA. The Raman spectra of the interaction of the chromophore, PLA, and inhibitors with crystalline CPAα were obtained with a custom-built laser Raman microscope which has been described elsewhere.[18] Ligand solutions were made up in either 0.2 M Tris (pH = 8.0) or 0.3 M Tris (pH = 8.0). Spectra of the interaction of BPLDAB with CPAα were obtained from anywhere between 5 to 90 min after the addition of a solution of BPLDAB in buffer to the enzyme crystal. Complexes of the enzyme with DA and PLA were prepared by soaking a few crystals in the ligand solutions for approximately 48 h. Longer soaking of the crystals resulted in a their solubilization. All crystal spectra were obtained from single, oriented (with respect to the incident light polarization) crystals using the 457.9-nm or 514.5-nm line from a Spectra Physics 171 Ar^+ laser at an output power of approximately 50 mW. Scans were acquired every 0.6 s and spectra consisted of approximately 150 scans.

Results

Raman Spectra of the Model Compounds. During the hydrolysis of BPLDAB by CPA there are at least eight different molecular species containing the *p*-dimethylaminobenzoyl chromophore that may be present within the enzyme crystal. These are: free BLPDAB that is located within the crystal lattice but not at the enzyme active site; BPLDAB at the active site and, possibly, bound to the Zn(II) ion; an anhydride intermediate; an anhydride intermediate that is bound to the active-site Zn(II) via one or both carbonyls; the protonated acid product; the latter complexed with Zn(II); the DA anion; and the Zn(II)-complexed DA anion. It was therefore necessary to determine the Raman spectra of the substrate and acid product (DA and DA anion) and of an appropriate model compound corresponding to the anhydride intermediate so that the detection of an anhydride intermediate in the hydrolysis of BPLDAB by CPA could be unambiguously identified. Because of the possibility of interaction of the active-site Zn(II) with each of these chromophoric species, it was necessary to determine the effects of complexation with Zn(II) on their Raman spectra.

Table I lists the Raman bands from 1100 to 1900 cm^{-1} of these compounds. These bands were used to identify the chromophoric

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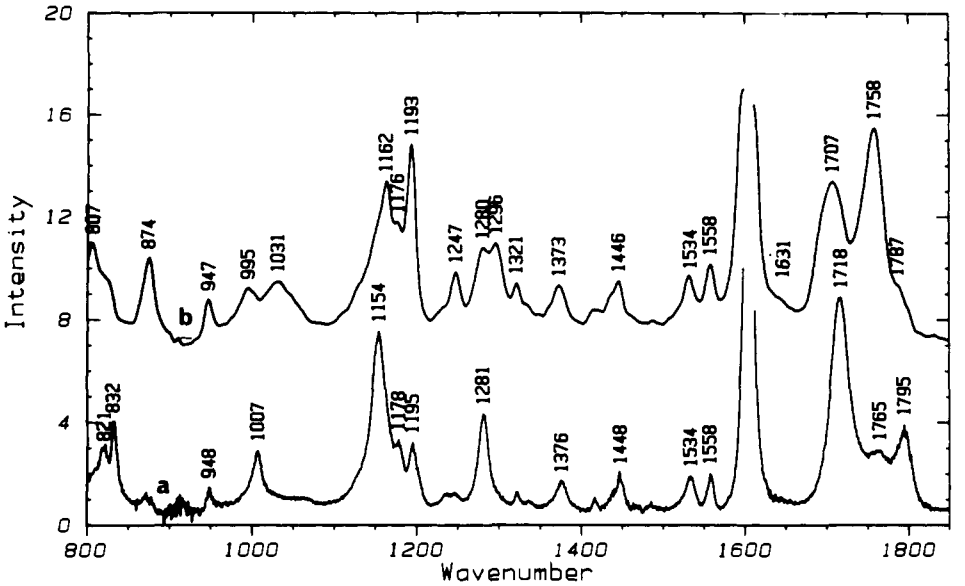

Figure 1. Raman spectra of the mixed anhydride between *p*-dimethylaminobenzoic acid and acetic acid (DAA), 0.1 M (bottom), and (top) the DAA/$ZnCl_2$ complex in $CDCl_3$ solution. Spectra were obtained with the 514.5-nm line from an Ar^+ laser.

species present in the *p*-dimethylaminobenzoyl (DAB) chromophore/CPAα spectra. For the back-reactions of DA and coproducts with CPAα at pH = 8, the DA anion would be expected to be the most abundant chromophoric compound. The 1387-cm^{-1} band, assigned to the carboxylate symmetric stretch, provides a marker band for this particular chromophore. It is possible that protonated DA could also be present given the hydrophobic nature of many enzyme active sites. The 1260-cm^{-1} band (see Table I) serves as a marker band for the protonated acid. Both the protonated DA and the DA anion interact strongly with Zn(II) as may be seen from the dramatic changes in the Raman frequencies upon complexation (Table I). The occurrence of strong bands in their respective spectra at 1413 cm^{-1} and 1406 cm^{-1} indicates that the chromophores have assumed a quininoid-like structure as occurs in the reaction of *p*-dimethylaminobenzaldehyde with Zn(II).[19]

The Raman spectra of DAA (bottom) and of Zn(II)-complexed DAA (top) from $CDCl_3$ are shown in Figure 1. A very distinguishing feature of the Raman spectra of acid anhydrides is the multiplicity of bands in the high frequency end of the carbonyl stretching region that results from the coupling of the symmetric and asymmetric stretching vibrations of the two carbonyls. For example, acetic anhydride in the vapor phase has two carbonyl Raman bands that are found at 1779 and 1837 cm^{-1}.[20] In the liquid phase, two weaker bands at 1778 and 1800 cm^{-1} are found in addition to the main components at about 1756 and 1828 cm^{-1}.[20] These additional bands arise from combinations of lower frequency carbonyl bending modes. The frequencies of these bands are extraordinarily sensitive to environment. For example, crystalline acetic anhydride shows a multiplicity of seven or more bands in the carbonyl region at 1735, 1748, 1766, 1777, 1790, 1808, 1821, 1828, and 1836 cm^{-1}.[20] The origin of this multiplicity of bands is partially due to the multiplicity of the environments found in the crystal. We would therefore expect that, as substrate diffuses into the crystal and an anhydride intermediate is formed, a multiplicity of bands in the 1730–1830-cm^{-1} range will occur. The intensity of these bands will change with time as the environment in the crystal changes with time due to diffusion of the substrate into the active site, and the formation of products and their diffusion out of the active sites. Any Raman bands in this frequency region must be due to anhydride since, as can be seen from Table I, there are no other bands from any other chromophore that occur in this region. The occurrence of bands in this region of CPA/DAB chromophore spectra would then appear to be uniquely characteristic of an anhydride intermediate.

In the spectrum of DAA, three bands are clearly resolvable in $CDCl_3$. These occur at 1718, 1765, and 1795 cm^{-1}. Complexing of DAA with Zn(II) produces several changes in the Raman spectrum. A shift to lower frequency of the carbonyl bands is consistent with the polarization of the carbonyls by Zn(II). ^{13}C NMR results also suggest that the interaction of Zn(II) with the anhydride results in a polarization of the carbonyls rather than a complexation to form covalent bonds.[21] This type of interaction is characteristic of the interaction of Zn(II) with acetylacetone.[22] Other changes include the appearance of new bands at 1631, 1296, 1247, 1031, and 874 cm^{-1} and changes in the positions and relative intensities of the bands at 1154 and 1195 cm^{-1} from DAA.

Because Raman carbonyl frequencies are very solvent dependent, it was necessary to definitively rule out the possibility of confusing the spectra of the ester substrate carbonyl band or the un-ionized acid carbonyl band with the carbonyl bands of an anhydride intermediate. In order to do this we adopted the procedure of measuring the environmentally induced displacement of the carbonyl frequencies of each of these model compounds in a systematic way. Raman spectra of DAA and DAA/Zn(II) were determined in different solvents. Plots were made of the carbonyl frequency, ν(C=O), versus Gutmann's electron acceptor number (AN)[23] for each of the resolvable carbonyl bands for both DAA and the DAA/Zn(II) complex—four bands in the case of DAA and three bands in the case of DAA/Zn(II). The seven straight lines demonstrating the linear dependence of the C + O frequencies on the AN are shown in Figure 2. The Zn(II)-complexed DAA possesses a weak, fourth band at approximately 1785 cm^{-1}, but we were unable to determine an accurate dependence of ν(C=O) versus AN for this weak band because of its proximity to the strong band at 1758 cm^{-1}. The carbonyl regions of the spectra were resolved into the individual components by fitting the spectra to a mixture of Lorentzians/Gaussian functions. It has been shown that the ν(C=O) of tetramethylurea has a linear dependence upon AN.[24] This is also the case with the model anhydrides and is a way of correlating the frequency with the electron-accepting nature of the environment. This aided in the

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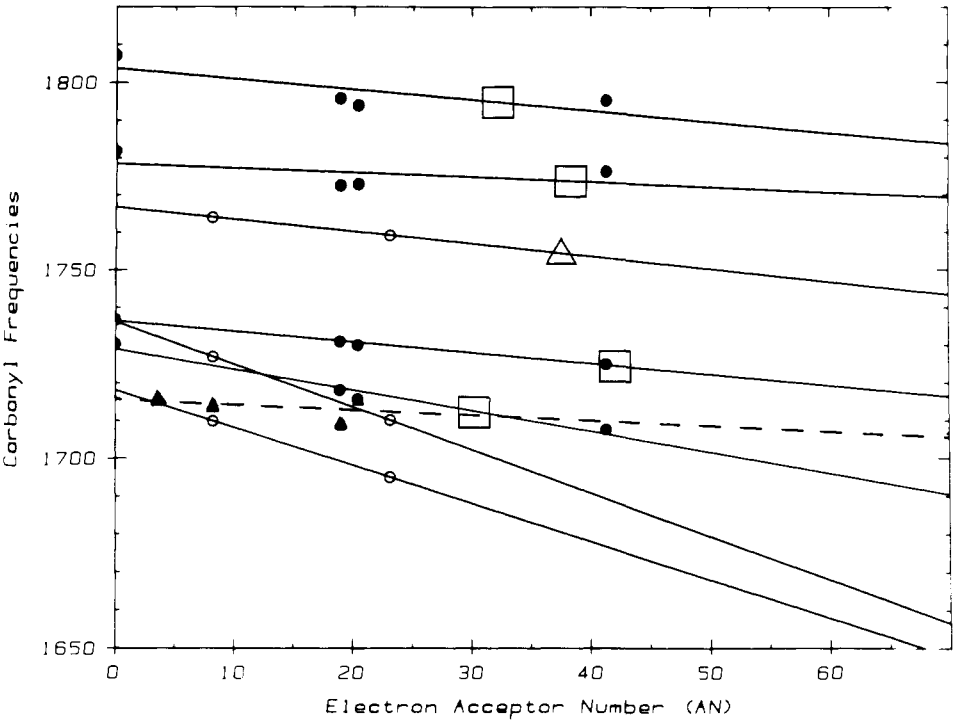

Figure 2. Plots of the frequencies of the carbonyl vibrations in DAA and Zn(II)-complexed DAA taken in different solvents versus Gutmann's solvent electron acceptor number (AN):[23] (●) DAA ν(C=O)s from hexane (AN = 0), acetonitrile (AN = 18.9), dichloromethane (AN = 20.4), and methanol (AN = 41.3); (○) Zn(II)-complexed DAA (C=O)s from C_6D_6 (AN = 8.2) and $CDCl_3$ (AN = 23.1). Solvents for the Zn(II)-complexed DAA plots were limited by the availability of solvents that did not interact strongly with Zn(II). The dashed line is fit to data for the protonated acid, DA. It is important to note that there is no environment in which the carbonyl frequency of the protonated DA could lie in the range 1754–1800 cm^{-1} which is the common frequency range for acid anhydrides. The open triangles and squares are the frequencies observed in the spectra of single crystals of CPAα soaked with substrate (see text).

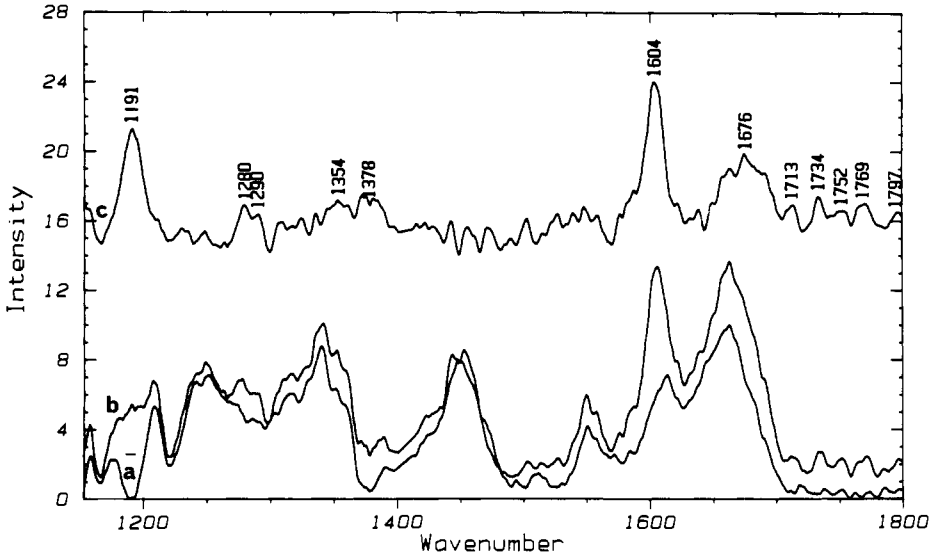

Figure 3. Raman spectrum of the interaction of the substrate, BPLDAB with CPA: (bottom line a) a single CPAα crystal at pH = 8; (middle line b) a single CPAα crystal soaked in an approximately 20 mM solution of BPLDAB at pH = 8 taken 33 min after the initiation of the reaction; (top line c): the difference spectrum BPLDAB/CPA minus CPA. The spectra were taken with a laser Raman microscope using the 457.9-nm line from an Ar^+ laser. The data are unsmoothed, and the spectrum is obtained by drawing a line between the experimental points taken from the diode array.

identification of bands from the CPA/DAB chromophore spectra since carbonyl bands from either type of anhydride intermediate must be in a similar but not necessarily identical environment. Also included in Figure 2 is a ν(C=O) versus AN plot for protonated *p*-dimethylamino acid (DA) shown as a dotted line. As can be seen, the acid ν(C=O) has a very weak dependence upon the environment as given by AN. It is obvious from Figure 2 that the frequencies of most of the anhydride carbonyl bands in any environment are much higher than the possible frequency of the carbonyl band of the protonated acid, DA. For the carbonyl band frequency of protonated DA to fall in the range of the higher frequency anhydride carbonyl vibrations would require that the environment have an infinitely large negative acceptor number. In this way one can rule out the protonated acid product, DA, as the origin of the Raman bands in the 1720–1800-cm^{-1} region that occur when substrate or products are infused into the CPA crystals. Figure 2 also shows four large square boxes and three large triangles. As will be discussed below, these are the bands that occur in the CPA crystals during catalysis.

Reaction of BPLDAB with Crystalline CPA. Figure 3 (middle line b) shows a Raman spectrum taken from a CPAα single crystal in 0.2 M Tris buffer (pH = 8) 33 min after the addition of an approximately 20 mM buffered solution of BPLDAB compared to that of a single CPAα crystal in the same buffer (bottom line

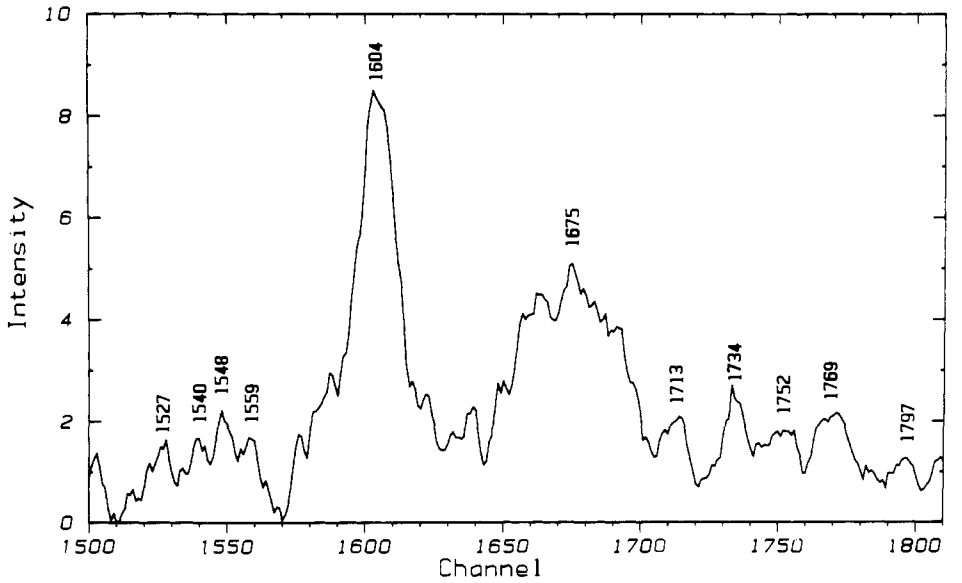

Figure 4. An expansion of the difference spectrum (top line c) in Figure 3 in the region 1500–1800 cm^{-1}.

a). Also shown is the difference spectrum (top line c). Figure 4 shows the difference spectrum enlarged in the 1500–1800-cm^{-1} region. It should be emphasized that none of these spectra are smoothed, but each spectrum is obtained by drawing a line connecting the measured intensities at integral wavenumbers. Although the spectra appear somewhat noisy, they are reproducible.

The broad band centered at approximately 1675 cm^{-1} in the difference spectrum is assigned to the substrate carbonyl in a variety of environments. Such behavior for a substrate carbonyl is not unknown. Tonge and Carey have reported the appearance of several carbonyl bands of the acyl enzyme of α-chymotrypsin in going from the inactive to the active form of the enzyme, indicating different populations at the active site.[25] It is reasonable to suppose that the carbonyl groups of the ester substrate in the CPAα crystal are in more than one environment.

Above 1700 cm^{-1} there are a number of weak, yet distinct, Raman bands. These bands are reproducible and are independent of the wavelength of the incident laser light and the position of the spectrum on the photodiode array. These bands do not appear in the CPA crystals treated with the substrate if the crystals are pre-equilibrated with the competitive inhibitor DL-benzylsuccinic acid before or with the addition of the substrate. It would be difficult to assign these bands to anything other than an anhydride intermediate since no other organic compounds have vibrational bands in this region of the spectrum. The marker band at 1260 cm^{-1} for protonated DA is not observed in the spectrum, indicating that protonated DA is not present in the crystal. In any event these bands cannot be due to this species because their frequencies are too high. The observed bands above 1700 cm^{-1} fall into five frequencies centered about 1713, 1734, 1752, 1769, and 1796 cm^{-1}. From the data on the model compounds shown in Figure 2, one would expect two other frequencies in this spectrum below 1700 cm^{-1}. It is likely that the higher frequency part of the broad band centered at 1675 cm^{-1} in Figure 4 does indeed contain these bands overlapping with the environmentally broadened carbonyl bands of the substrate.

To further characterize these bands in Figure 4, which are assigned to an anhydride intermediate, an attempt was made to assign the bands to either of the DAA or the Zn complexed DAA compounds using the ν(C=O) versus AN plots. The position of these five bands is given in Figure 2 as either a large empty square or triangle. The 1797- and 1770-cm^{-1} bands fall nicely upon the two highest plots for the uncomplexed DAA. It is not possible to assign these bands to any other plot at meaningful AN values. The 1713- and 1734-cm^{-1} bands also fall upon one of the DAA carbonyl plots. These bands may be correlated to the non-Zn-(II)-complexed model anhydride. The band at 1750–5 cm^{-1} will not fall on any of the lines in Figure 2 for the uncomplexed DAA, but only on the line for the Zn(II)-complexed DAA. This is consistent with a model in which at least one of the carbonyls of an anhydride is bound to the Zn(II) in some of the sites. It seems reasonable then to assign the bands at 1713, 1734, 1770, and 1797 cm^{-1} to the anhydride intermediate, and the band at 1750–5 cm^{-1} to a Zn(II)-complexed anhydride intermediate in which one or both of the carbonyls are polarized by the active-site Zn(II).

Interaction of Crystalline CPA with DA and PLA. Bicknell and co-workers have reported that PLA binds to Co(II) CPAα in a 1:1 complex in a competitive mode and that the presence of this C-terminal product of ester hydrolysis facilitates the binding of anions to the active-site metal ion.[16] It therefore seemed reasonable to attempt to form the anhydride intermediates by combining DA and PLA with the enzyme crystals. Although the formation of ester substrate from this reaction would be energetically unfavorable, it is possible that the DA bound at the active site could exist in an equilibrium between the acid form and the acid anhydride intermediates. Such a mixture would have the advantage of producing less complicated spectra since the bands from the ester substrate would not be present and the bound DA anion lacks a carbonyl. Assuming that the dissociation constants of PLA to crystalline Zn(II) CPAα is 0.5 mM as it is with solubilized Co(II) CPA, then the saturation of PLA at the active site of the crystalline enzyme would be 86% with the concentrations of ligands employed. If the addition of DA anion to the mixture results in synergistic binding of both ligands as occurs with other anions,[16] then the enzyme active sites may be regarded as essentially saturated. This is a reasonable assumption since the binding site of the C-terminal product is removed from the metal ion, and the crystalline enzyme is essentially as active as the solubilized enzyme, suggesting that there are no major conformational differences.

Figure 5 (middle b line) shows a Raman spectrum of the interaction of DA and PLA with CPA; the bottom line a shows the Raman spectrum of the CPA crystal while the top spectrum c shows the difference. Figure 6 shows an expansion of the difference spectrum. In Figure 6 each data point is the difference in the number of counts in the enzyme/substrate spectrum minus the number of counts in the enzyme spectra as obtained from the diode array. The line is a calculated sum of 20 Lorentzian functions that were fit to this spectrum using a least-squares procedure.[18] If the competitive inhibitor glycyl-L-tyrosine (GT) is added to the crystal of CPA prior to adding the DA/PLA mixture, there is a total suppression of these Raman bands in the 1650–1800-cm^{-1} range. Note that in Figure 6 there is no smoothing of the data. Although the use of the computer fit program effectively smooths the spectrum by drawing a series of

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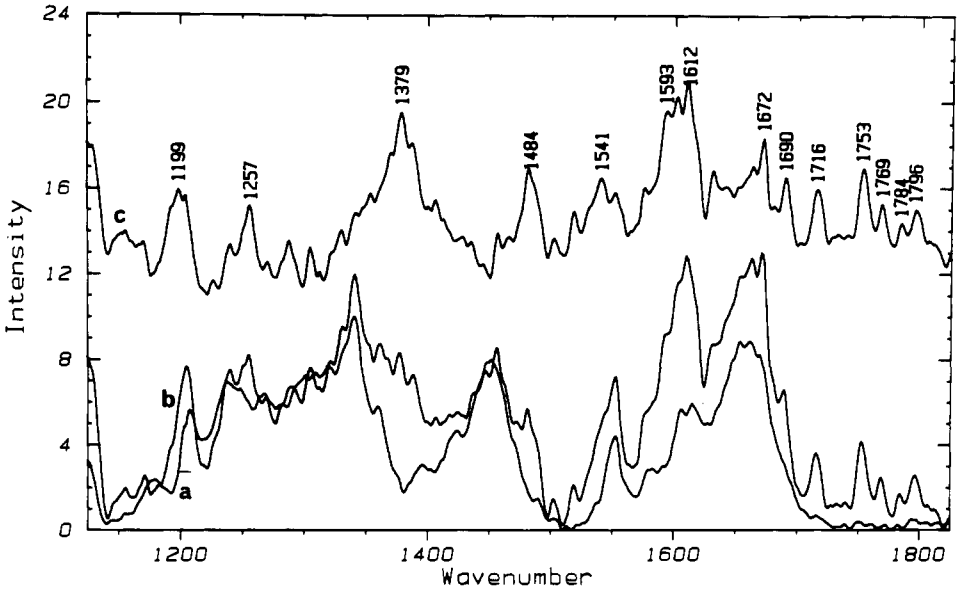

Figure 5. Preresonance Raman spectrum of the interaction of *p*-dimethylaminobenzoic acid (DA) and β-phenyllactic acid (PLA) with crystalline CPA taken from CPA crystals that have soaked for 48 h in a 20 mM buffered solution of DA and PLA (line b) versus an enzyme crystal in mother liquor (bottom line a). If the solution of DA and PLA is made 20 mM in the competitive inhibitor glycyl-L-tyrosine (GT), then the line becomes identical with the line in the region from 1700 to 1800 cm^{-1}.

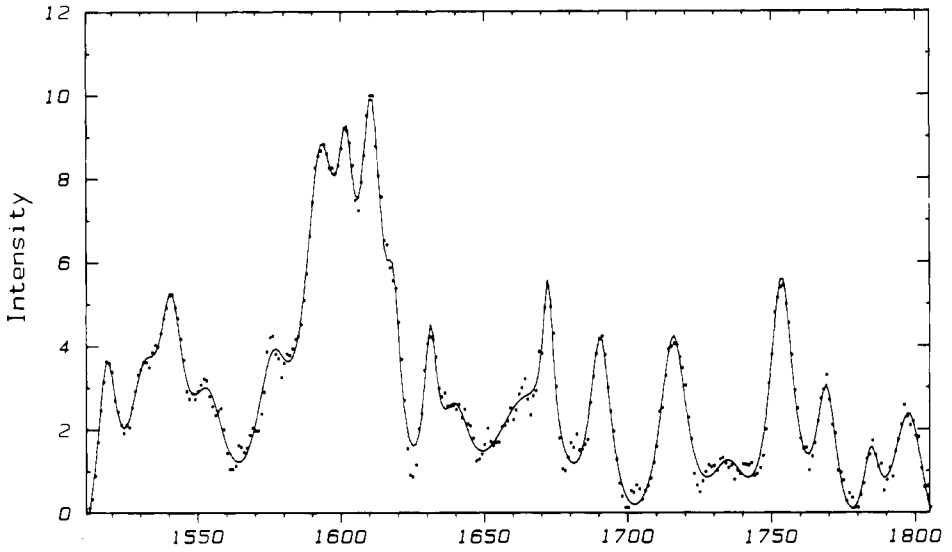

Figure 6. The difference spectrum of Figure 5 plotted as the raw data (points) taken from the diode array and the calculated sum of 20 Lorentzian functions (solid line).

Lorentzian functions, each of the points is a data point obtained by subtraction of the counts from the CPA crystal alone from the counts for the enzyme crystal submerged in the solution containing DA + PLA. Figure 6 is an alternative way of presenting the data but is equivalent to a line drawn from point to point at 1-cm^{-1} intervals since in each case there is no actual smoothing of the data; each experimental data point is specifically exhibited. In one case the points are connected by straight lines, and in the other the best possible Lorentzian is drawn through the points which are shown. In this way one can see the scatter in the experimental data points. Although there is a scatter between the points and the calculated lines, the agreement appears to be acceptable.

There are several interesting aspects of this difference spectrum. The strong band at 1379 cm^{-1}, indicative of DA anion, indicates that this species is a contributor to the spectrum. The band at 1256 cm^{-1} is assignable to protonated DA. From this we conclude that at least some of the intensity of the 1716-cm^{-1} band may be due to this species. The occurrence of the protonated acid at pH = 8 is possibly significant. In order for DA anion to react with the enzyme active site to form an anhydride intermediate, it must become at least transiently protonated in order to give a good leaving group (hydroxide ion) upon reaction with the active-site glutamate residue. The intensity of the 1256- and 1716-cm^{-1} bands indicates that there may be a significant contribution of this species to the spectrum.

The 1605-cm^{-1} region of the spectrum is resolvable into at least four components. Two of these are most likely due to DA and DA anion. The lack of a band at approximately 1410 cm^{-1} seems to indicate that there is no Zn(II)-complexed DA or DA anion. The absence of a band at approximately 1355 cm^{-1}, characteristic of BPLDAB, seems to indicate that none of the bands at 1605 cm^{-1} may be assigned to the ester substrate. It is probable that two of the phenyl modes are due to anhydride intermediates.

Between 1650 and 1800 cm^{-1} are nine distinct bands. These occur at 1655 (shoulder), 1670, 1690, 1715, 1735, 1754, 1770, 1785, and 1790 cm^{-1}. The omission of PLA from the reaction mixture illuminates or greatly diminishes the intensities of these bands, indicating that the binding of both products may facilitate the formation of the acid anhydride. If only PLA alone is added to the CPA crystals, there is no noticeable change in the Raman

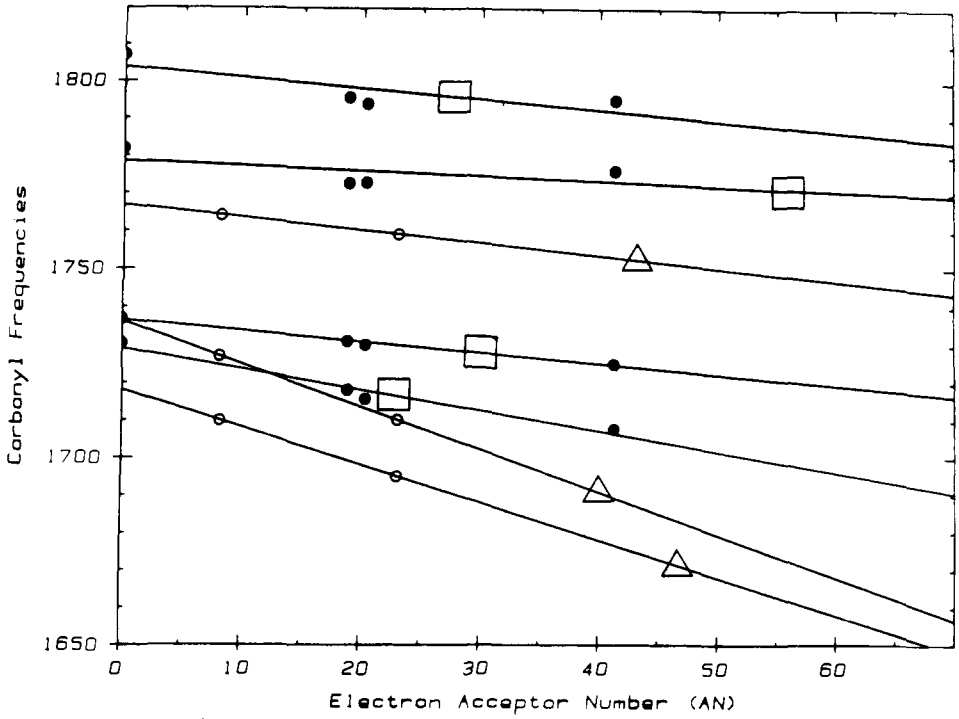

Figure 7. Correlation of the $\nu > 1650$ cm^{-1} bands from the interaction of DA and PLA with CPA to the plots of ν(C=O) for DAA, Zn(II)-complexed DAA, and DA. Large open squares denote bands from the back-reaction that are attributed to an anhydride intermediate. Open triangles denote bands that are attributed to a Zn(II)-complexed anhydride intermediate.

spectrum because CPA is a very weak Raman scatterer and does not appear to react with the enzyme. Raman spectra obtained with the long axis of the enzyme crystal oriented perpendicular to the laser light polarization consistently gave stronger spectra in the >1600-cm^{-1} region than from crystals oriented parallel to the incident light polarization. Such a difference would be expected if the chromophore were confined to a particular orientation within the crystal lattice as would be the case for a covalent enzyme intermediate.

The broad shoulder at 1660 cm^{-1} is assigned to a change in the amide I conformation of the protein induced by ligand binding. There are no anhydride bands in this region in the model compounds. The correlation of the remaining bands lying above 1650 cm^{-1} to the model anhydride compounds is shown in Figure 7. Again the lines for the carbonyl frequencies were acceptor numbers are obtained from the model compounds as shown in Figure 2. The 1770- and 1795-cm^{-1} bands fall nicely on the two highest (C=O) versus AN plots for DAA and correlate well with the 1774- and 1796-cm^{-1} bands from the BPLDAB/CPAα complex. Although the 1770-cm^{-1} band lies at a large AN, the flat line means that this could be moved to the left without much error and this would bring it into the 30–50 AN region of the other points. The 1754-, 1735-, 1715-, 1690-, and 1670-cm^{-1} bands fit nicely on the plots of carbonyl frequency versus AN. The 1754-cm^{-1} band, which correlates well with the 1759-cm^{-1} band from the BPLDAB/CPAα complex, falls upon the plot for Zn(II)-complexed DAA. Furthermore, the 1672- and 1690-cm^{-1} bands fall upon the lower (C=O) plots at nearly the same AN value for Zn(II) complex anhydrides.

We assign the 1770- and 1795-cm^{-1} and some of the intensity of the 1716-cm^{-1} band to a pure (uncomplexed) anhydride intermediate. Likewise, the 1672-, 1690-, 1754-, and 1785-cm^{-1} bands are assigned to a Zn(II)-complexed anhydride intermediate. It is impossible that any of these bands, with the exception of the 1716-cm^{-1} band, can be attributed to protonated DA because of the weak dependence of the (C=O) on electrophilic environment for this compound (see Figure 2). The average AN environments for the bands assigned to either type of anhydride intermediate formed from the BPLDAB/CPAα complex or the DA/PLA/CPAα complex are in good agreement: AN = 36 and 35 for the uncomplexed intermediate and AN = 37 and 43 for the Zn(II)-complexed intermediate from the forward and reverse reactions, respectively.

Conclusions

Raman spectra of the reaction of the chromophoric substrate BPLDAB with crystalline CPA gives bands above 1700 cm^{-1} that can be most easily attributed to an acid anhydride. There appears to be no satisfactory alternative assignment. Raman bands at approximately the same frequencies result from the addition of the hydrolysis products of BPLDAB with the crystalline enzyme, demonstrating that the acid anhydride may be formed from the reverse reaction. Furthermore, the intensities of these bands from either direction are greatly diminished upon the inclusion of competitive inhibitor before or in the reaction mixture. This fact strongly suggests that the place of origin of these bands is the enzyme active site and implies that the ligands have reacted with the enzyme to form a covalent, anhydride intermediate. These results appear to confirm the work of Makinen and co-workers who have found evidence for anhydride intermediate formation using an analogous chromophore, *p*-chlorocinnamic acid.[4-6]

A correlation of these bands to model compounds designed to mimic an anhydride intermediate and a Zn(II)-complexed anhydride intermediate reveals that several of the bands observed in the reaction mixtures correlate very well with an anhydride intermediate in which the carbonyls are substantially polarized by the active-site Zn(II). Similar lowering of the substrate carbonyl frequencies at enzyme active sites has been detected by others[25-31] and interpreted in terms of polarization of the carbonyl group to lower the bond order of the C=O bond. We cannot say from our measurements if one or both of the carbonyl groups are polarized by the Zn(II). The next step of the proposed mechanism involves the attack of the glutamate residue to form an anhydride

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intermediate. Because of the polarization by the Zn(II), this intermediate is activated toward hydrolysis through polarization of the carbonyl bonds by the active-site Zn(II). Such an interaction would result in a decrease in electron density about the carbonyl carbons, thereby making them more susceptible to nucleophilic attack. While each carbonyl is activated toward attack, it is possible that the enzyme has evolved to hydrolyze preferentially at only one of the sites. Work by Nau and Riordan suggests this may actually be the case.[8] The remainder of the mechanism involves the hydrolysis of the activated intermediate to liberate the acid product.

Registry No. CPA, 11075-17-5; BPLDAB, 140176-20-1; DA, 619-84-1; DAA, 140176-21-2; DAB-Cl, 4755-50-4.

Synthesis and Structure Elucidation of a New [2]-Catenane

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Abstract: A supramolecular self-assembly process has lead to the synthesis of new type of [2]-catenane. A 34% yield of catenane was obtained from a one-pot double macrocyclization reaction. Its three-dimensional structure was determined by using two-dimensional ^{1}H NMR spectroscopy. This structure agrees well with the predictions of molecular mechanics calculations, although there is some ambiguity in the orientation of one of the amide bonds. The molecule is locked into a well-defined conformation by several intermacrocycle H-bonds and π-π interactions and it is these interactions that template the assembly of the interlocked ring system. The dynamic properties of the catenane were investigated by variable-temperature ^{1}H NMR. The "inside" and "outside" parts of the molecule do not exchange with one another because of bulky cyclohexyl groups, which act as "spokes" preventing free rotation of the macrocycles. However, the system does possess some conformational mobility, which can be followed on the ^{1}H NMR time scale. A chiral NMR shift reagent was used to show that the [2]-catenane adopts a chiral ground state. It switches between two enantiomeric conformations at a rate of 1 s^{-1} at room temperature.

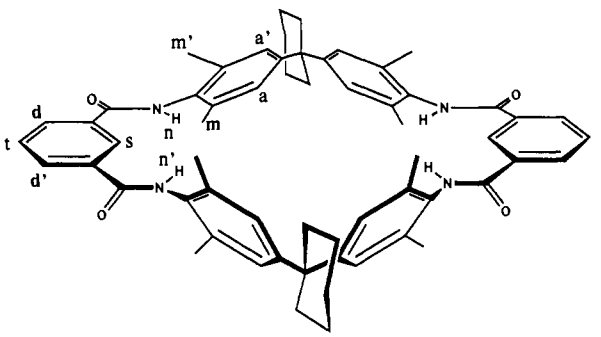
Introduction

Supramolecular chemistry has revolutionized approaches to the synthesis of topologically complex molecules.[1] Early catenane syntheses relied purely on chance to thread the macrocyclic link.[2] The first efficient catenane synthesis was developed by Schill and Lutteringhaus who used covalent bonds to direct formation of the interlocked ring system.[3] More recently, Sauvage's introduction of the metal ion template has afforded an impressive array of catenanes and molecular knots.[4] In nature, weaker intermolecular forces such as H-bonding and π-π interactions are used to template the extremely efficient syntheses that characterize biological self-assembly and self-replication.[5] Stoddart has demonstrated the power of this approach with his supramolecular catenane and rotaxane syntheses.[1,6] In these remarkable systems, π-π interactions provide the major driving force for self-assembly: they direct and template threading of the macrocyclic links.

In this paper, I report the synthesis and NMR structure determination of a new class of [2]-catenane. The molecule is locked into a well-defined conformation by a combination of H-bonds and π-π interactions and it is these interactions that template the formation of the interlocked ring system. A supramolecular self-assembly process results in a 34% yield of [2]-catenane in a one-pot double-macrocyclization reaction.

Results and Discussion

Synthesis. I recently reported the synthesis of **1**, a receptor for *p*-benzoquinone.[7] This synthesis employed the macrocyclization reaction shown in Scheme I. In an attempt to improve the yield of **1**, I developed the two-step synthesis shown in Scheme II. This reaction yielded three major products, fractions A, B, and C (see Experimental Section). Fraction B was identical with the cyclic dimer, **1**, synthesized via Scheme I. FAB mass spectra of fractions A and C showed molecular ions that corresponded to tetrameric species. Fraction C had a ^{1}H NMR spectrum that was almost identical with that of **1** in $CDCl_3/CD_3OD$. Thus all four components of the tetramer were equivalent and had retained their symmetry. In contrast, fraction A had a very complex ^{1}H NMR spectrum (see below). There were several sets of nonequivalent protons and some signals showed large changes in chemical shift



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