Chromophoric Cinnamic Acid Substrates as Resonance Raman Probes of the Active Site Environment in Native and Unfolded α-Chymotrypsin†

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Received August 28, 1985

ABSTRACT: Chromophoric [4-(dimethylamino)cinnamoyl]imidazole reacts with the serine protease α-chymotrypsin to form an acyl enzyme. At pHs below 4.0, the acyl enzyme turns over very slowly to yield the free acid. During this slow deacylation it is possible to obtain a very good resonance Raman spectrum of the acyl intermediate by using the 350.7-nm line of the krypton laser. The resonance Raman carbonyl frequency of the covalently bonded substrate and its wavelength at maximum intensity in the absorption spectrum of the acyl enzyme have been taken and used to monitor the active site environment. A comparison has been made of the absorption and Raman spectra of the acyl enzyme and those of the corresponding chromophoric methyl ester, aldehyde, and imidazole model compounds. A linear correlation is found between the wavelength of maximum absorption and the Raman frequency of the carbonyl group over a wide range of solvent conditions for each of the model compounds. By combining the Raman carbonyl frequency with the absorption maximum, we can determine that the bond order changes in the carbonyl bond of the bound substrate are not due to changes in the solvent, since the carbonyl frequency and the absorption maximum of the acyl enzyme do not fall on any of the linear correlations for the model compounds. The unusual spectroscopic properties of the bound substrate appear to be due to some specific enzyme-induced change in the substrate when it is bound at the active site. Thermal unfolding of the acyl enzymes changes both the carbonyl frequency of the acyl enzyme and its absorption maximum to completely different values. This change is strikingly similar to that obtained in going from the model aldehyde to the corresponding ester. This leads us to the conclusion that the native acyl enzyme is more "aldehyde-like". We conclude that the environment in the active site of the native protein induces a conformational change in the bound substrate that is responsible for a change in the spectroscopic properties and for the catalytic activity of the enzyme. It is suggested that the observed spectroscopic properties are consistent with a change in the all-planar sp^2 structure of the acyl serine ester.

In the previous paper of this series (Argade et al., 1983), it was shown that an aqueous solution of the chromophoric acyl enzyme [4-(dimethylamino)benzoyl]-α-chymotrypsin shows a characteristic ultraviolet absorption maximum and carbonyl frequency that is unlike that of the corresponding model compound 4-(dimethylamino)benzoic acid ethyl ester in any solvent. Since both the model compound and the acyl enzyme are similar chromophoric esters, it was concluded that specific interactions between the enzyme and the bound substrate were responsible for both the unusual spectroscopic properties and the catalysis. In this paper we will explore these same properties using another chromophoric substrate and model compounds, the 4-(dimethylamino)cinnamic acid derivatives, in order to formulate a reason for these unusual spectroscopic properties of the acyl enzyme and to determine what they can tell us about catalysis.

The substrate used previously is rather special since it requires several days to turn over—even at pH 7. Consequently, it seems of interest to repeat this earlier work with an acyl group that turns over more rapidly and in which the carbonyl group frequency is easily obtained from rigorous resonance Raman spectroscopy. For this purpose we have chosen the DACA[1] derivatives discussed above. These chromophoric materials have strong absorption bands in the region of 360–420 nm and give good resonance Raman spectra with the 350.7-nm line of an krypton laser. The use of this line well to the blue of the absorption maximum allows the observation of the resonance Raman spectrum in the presence of the rather strong fluorescence which is Stokes-shifted well to the red of the absorption maximum. As in our previous paper (Argade et al., 1983), we have made a detailed study of the spectroscopic properties of the chromophoric aldehyde, the acid, and the methyl ester in a variety of solvents and compared these results with those of the chromophoric acyl enzyme.

Previous work by a number of workers (Caplow & Jencks, 1962; Bernhard et al., 1965; Bernhard & Lau, 1971) has shown that simple benzoyl derivatives turn over considerably slower than the corresponding cinnamoyl or furylacryloyl derivatives. Presumably, this is because in benzoyl derivatives the position normally occupied by the α-carbon in the peptide substrates is occupied by a branched carbon, while this is not

† This work was supported in part by grants from the National Science Foundation (DMB-8417199) and the National Institutes of Health (5 R01 GM 15547-18).

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[1] Abbreviations: DAC, 4-(dimethylamino)cinnamoyl; DACA, 4-(dimethylamino)cinnamic acid; DAC-O-Me, methyl 4-(dimethylamino)-cinnamate; DAC-O-NASA, N^{α}-acetyl-L-serine amide 4-(dimethylamino)cinnamate; DAC-Im, [4-(dimethylamino)cinnamoyl]imidazolate; DACHO, 4-(dimethylamino)cinnamaldehyde; DAB, 4-(dimethylamino)benzoyl.

0006-2960/86/0425-1912$01.50/0

the case in the longer, more linear cinnamoyl or furylacryloyl derivatives. As was pointed out in the previous paper (Argade et al., 1983), the furylacryloyl derivatives are difficult to use for such a detailed study of the carbonyl frequencies because their carbonyl vibrations are insufficiently resonance enhanced. [See, for example, Figure 2 in MacClement et al. (1981).] It appears that the DACA derivatives may offer the best choice of a true substrate that can be studied by the resonance Raman technique.

Materials and Methods

Isobutyl chloroformate from Sigma, 4-(dimethylamino)-cinnamaldehyde (DACHO) from Aldrich, 4-(dimethylamino)cinnamic acid (DACA) from Sigma or Pfaltz and Bauer, and triethylamine from BDH Chemicals were o btained and used without further preparation. Imidazole obtained from Aldrich was 2× recrystallized from benzene before use. All other solvents and reagents were reagent grade. Double glass distilled water was used to prepare the buffers.

Syntheses. (*A*) [*4-(Dimethylamino)cinnamoyl*]*imidazole* (*DAC-Im*). To 1 equiv of DACA (2 gm) in 200 mL of anhydrous tetrahydrofuran at 0 °C was added 1 equiv of triethylamine (1.45 mL). Then 1 equiv of chilled isobutyl chloroformate (1.37 mL) was added dropwise. After about 15 min the mixture was rapidly filtered, and 2 equiv of imidazole (1.43 gm) in tetrahydrofuran was added. The solution was stirred overnight at 4 °C and filtered, and the solvent was removed. The solid was taken up in chloroform, and most of the residual DACA was removed by fractional precipitation. The DAC-Im was purified on a silica gel column in chloroform. The yellow solid was crystallized from ethyl acetate and diethyl ether with a melting point of 148 °C. The yield was 45% on the basis of DACA.

(*B*) *4-(Dimethylamino)cinnamic Acid Methyl Ester* (*DAC-O-Me*). To 100 mg of DAC-Im (prepared above) in 50 mL of methanol was added 1 equiv of triethylamine (0.06 mL). After 30 min at room temperature, the mixture was filtered and the solvent removed. The product was dissolved in chloroform and purified on a silica gel column in chloroform. Purified DAC-O-Me was crystallized from methanol and water to give pale yellow crystals with a melting range of 124–126 °C. The yield was stoichiometric with respect to DAC-Im.

Preparation of Acyl Enzyme. A stock solution of 5×10^2 M DAC-Im was prepared in dimethylformamide. Aqueous solutions of α-chymotrypsin (3 × crystallized from Sigma) were anywhere from 6 to 100 mg/mL in 0.5 M phosphate, pH 7.0. Sufficient DAC-Im was added to an α-chymotrypsin solution to give a 2-fold excess of DAC-Im over α-chymotrypsin. After 10–15 s, the mixture was simultaneously quenched to pH 4.0 and the acyl enzyme isolated centrifuging through a column of Sephadex G-25 fine (Pharmacia) that had been equilibrated with the pH 4.0 acetate quench buffer (0.5 mL of solution per 5 mL of packed gel). Acyl enzyme prepared in this manner is stable for days at room temperature. The centrifuge columns used above were made from a 5-cm^2 syringe into which a fritted disk was fitted. The column was poured from water and centrifuged at full speed for 30 s in a clinical benchtop centrifuge using a swinging bucket rotor. At least two washes of the desired buffer, centrifuging each time, were required to equilibrate the column. Dilution was negligible, and recovery of protein was greater than 95%. Separation from small molecules was on the order of 10^5-fold.

Thermal Denaturation of Acyl Enzyme. A solution of DAC-α-chymotrypsin acyl enzyme (2.5 mg/mL) was heated by increments. Absorption spectra were recorded after equilibration at each temperature on a Hewlett-Packard HP 8450A spectrophotometer equipped with a Peltier heating jacket. After denaturation was complete, the enzyme was cooled slowly by increments, and absorption spectra were again recorded.

In the preparation of denatured acyl enzyme for Raman measurements, a solution of 5 mg/mL DAC-α-chymotrypsin was slowly heated to 60 °C in a water bath and then cooled slowly. Due to precipitation of the denatured acyl enzyme, this solution was centrifuged to remove precipitate and then concentrated to about 25 mg/mL by ultrafiltration on a PM 10 membrane (Amicon).

Spectral Measurements. Absorption spectra of all model compounds and acyl enzyme in the various solvents were obtained on a Cary 14 spectrophotometer. Raman spectra were obtained with the 350.7-nm line of a krypton laser. A Spec 1400 double grating monochromator equipped with a cooled RCA 31034A photomultiplier tube was used to monitor the intensity of the scattered light by using a backscattering geometry. The scattered photons were counted and stored as a function of their frequency by using a Varian 620i computer. Frequency assignments for the carbonyl stretching vibrations were verified in most of the solvents for the model compounds from their observed intensity in the infrared by using a Sargent Welch 3-200 infrared spectrophotometer. Although the two double bond vibrations, —C═C— and —C═O, of α,β-unsaturated esters, aldehydes, and acids are coupled, the —CH═CH— vibration is weak or nonexistent in the infrared so that the carbonyl frequency could be identified by this technique. For each of the model compounds, the carbonyl frequency was verified by infrared, as well as Raman, measurements in at least one solvent. This aids in the definitive establishment of the assignment of the carbonyl band frequency.

Results

Chromophoric acyl imidazoles react with α-chymotrypsin to form the acyl enzyme in which the Raman spectrum of the bound chromophore can be studied to obtain structural information. The acyl enzyme contains an ester linkage between the acyl group and the serine-195 hydroxyl at the active site. Consequently, the properties of the acyl enzyme can be compared with those of a suitable model compound. The model compounds studied in this work are the corresponding methyl ester of 4-(dimethylamino)cinnamic acid, DAC-O-Me, and the aldehyde, 4-(dimethylamino)cinnamoylaldehyde, DACHO. In addition, the corresponding imidazole amide of the DACA chromophoric groups was also studied.

Figure 1 shows resonance Raman spectra of 4-(dimethylamino)cinnamic acid methyl ester (DAC-O-Me) taken with the 350.7-nm line of krypton. The lower spectrum was taken in the relatively lower dielectric solvent dioxane, and the upper spectrum was taken in the relatively higher dielectric constant solvent formamide. In this figure, a change of 18 cm^{-1} is observed in the frequency of the carbonyl group of the chromophore. Clearly, the resonance Raman frequency of the carbonyl group of this chromophore varies greatly from solvent to solvent. As discussed in our earlier paper (Argade et al., 1983), the frequency of the carbonyl tends to decrease with an increase in the dielectric constant of the solvent due to the spontaneous increase in the dipolar polarization of the carbonyl bond, which decreases the bond order of the carbonyl group. H-bonding solvents also tend to reduce the bond order of the carbonyl group, probably due to a stabilization of an increased negative charge on the oxygen through H bonding with the solvent. It should be noted that for the model compounds in

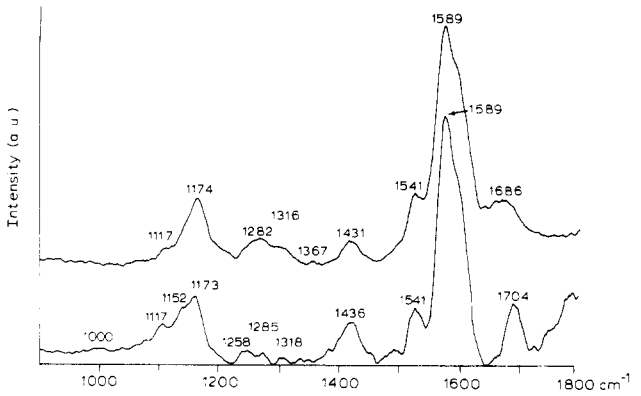

FIGURE 1: Resonance Raman spectra of DAC-O-Me. The upper spectrum was taken in formamide; the lower spectrum was taken in dioxane. The spectrum was taken in backscattering geometry by using the 350.7-nm line of a krypton laser.

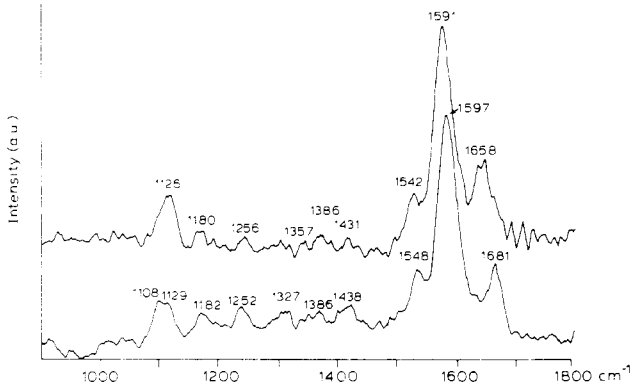

FIGURE 2: Resonance Raman spectra of DACHO. The upper spectrum was taken in chloroform; the lower spectrum was taken in cyclohexane.

different solvent environments, an increase of the bond order of the carbonyl group is always accompanied by a blue shift in the absorption spectrum. Furthermore, as the bond order of the carbonyl increases, it is reasonable to suppose that the bond order of the bond between the carbonyl carbon and the ester oxygen, i.e., the C–O bond of the ester linkage, decreases, thus increasing the basicity of the ester oxygen. As we shall see, the acyl enzyme appears to be anomalous when compared with the model compounds, thus giving a clue as to its mechanism of activity.

Previous work has shown that the absorption spectra of chromophoric acyl enzymes appear to more closely resemble those of the corresponding aldehyde or ketone rather than those of the model esters (Bernhard & Lau, 1971; Bernhard & Malhotra, 1974; Argade et al., 1983). Consequently, as mentioned above 4-(dimethylamino)cinnamaldehyde (DACHO) had been included in this study. The resonance Raman spectra of DACHO in chloroform and cyclohexane are shown in Figure 2. Excellent quality resonance Raman spectra are obtained, and the frequency of the carbonyl band is very sensitive to solvent environment.

Figure 3 shows the resonance Raman spectrum of the native DAC-α-chymotrypsin in pH 4.0 acetate buffer prepared from the reaction of DAC-Im and α-chymotrypsin as discussed under Materials and Methods. An excellent spectrum is exhibited, and the carbonyl frequency is easily obtained as 1677 cm^{-1}. The band in Figure 3 at 1600 cm^{-1} is assigned to the C=C double bond stretch of the DACA chromophore while the band at 1625 cm^{-1} is most likely due to the 1622-cm^{-1} band of tryptophan, which is preresonance-enhanced, combined with the band that appears as a shoulder in Figure 1 for the resonance spectrum of the chromophoric ester DAC-O-Me.

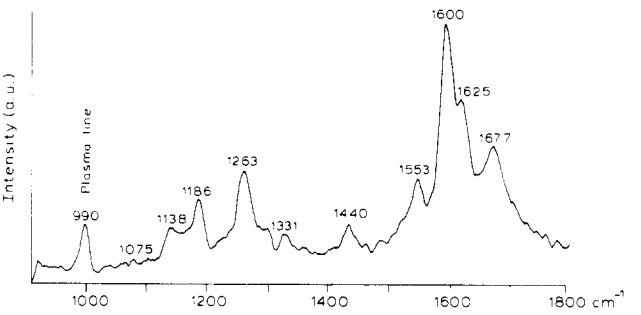

FIGURE 3: Resonance Raman spectrum of native DAC-α-chymotrypsin taken in 0.05 M acetate buffer, pH 4.0, at room temperature.

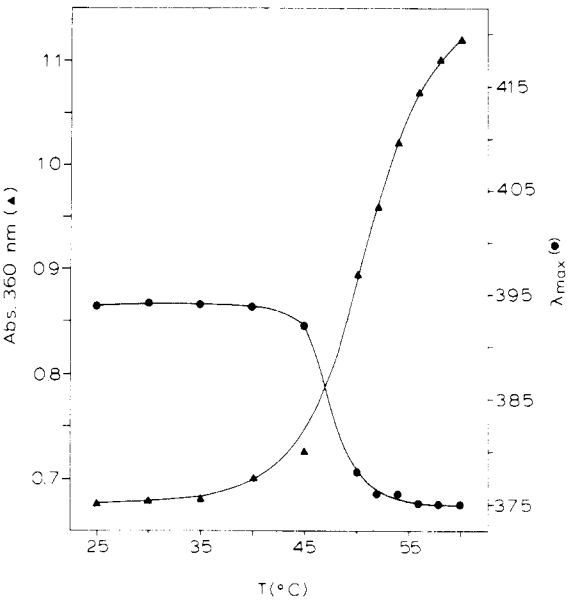

FIGURE 4: Thermal denaturation profile of DAC-α-chymotrypsin taken in 0.05 M acetate buffer, pH 4.0. The absorption λ_{max} was obtained from a difference spectrum between acylated and nonacylated α-chymotrypsin at the same temperature. Each enzyme's concentration was about 5 mg/mL. Symbols: filled circles, λ_{max} vs. temperature; filled triangles, absorption at 360 nm as a function of the temperature.

Upon heating the acyl enzyme to 60 °C, the wavelength of the absorption maximum shifts to the blue as the protein unfolds. Unfortunately, it proved to be impossible to monitor the carbonyl frequency as the protein unfolded because of a strong fluorescence induced by the heating process. Two plots of absorption spectra of the acyl enzyme vs. the temperature are given in Figure 4. First, a plot of λ_{max} vs. temperature is presented, which shows the shift in the wavelength of maximum absorption from 391 to 375 nm upon denaturation (solid circles). Second, the absorption at 360 nm measured as a function of the temperature shows a continuous increase as the protein unfolds (solid triangles). The transition from native acyl enzyme (λ_{max} = 391 nm) to denatured acyl enzyme (λ_{max} = 375 nm) is unusual for two reasons. The thermal denaturation transition with DAC-α-chymotrypsin is not nearly as sharp as with DAB-α-chymotrypsin (Argade et al., 1983) or with the calorimetry measurement of Privalov (1974). Also, a unique isosbestic point was not obtained during the transition between native and denatured DAC-α-chymotrypsin but was obtained with DAB-α-chymotrypsin. The transition temperatures of 47 °C at pH 4.0 and 43 °C at pH 3.0 are not in agreement with the calorimetric measurements of the thermal unfolding of this protein by Privalov (1974). Privalov found transition temperatures of 56 °C at pH 4.0 and 52 °C at pH 3.0. This discrepancy may indicate that the protein

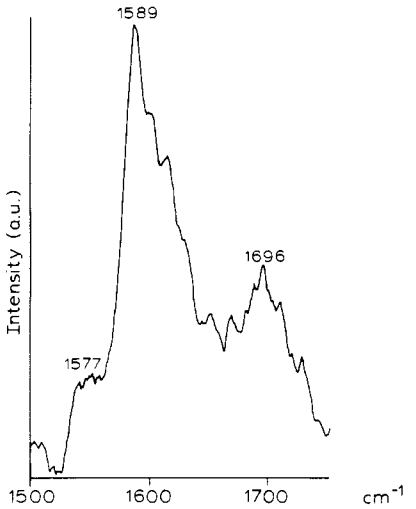

FIGURE 5: Raman spectrum of thermally denatured DAC-α-chymotrypsin in 0.05 M acetate buffer, pH 4.0, taken after cooling and concentrating to about 20 mg/mL.

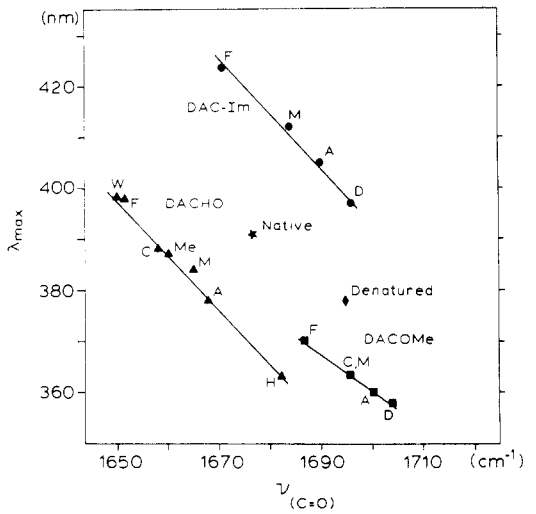

FIGURE 6: Linear correlation diagram of the wavelength of maximum absorption vs. the carbonyl frequency in cm^{-1} for DACHO, DAC-O-Me, and DAC-Im in various solvents. Solvents used are W = water, F = formamide, C = chloroform, Me = methylene chloride, A = acetonitrile, H = cyclohexane, and D = dioxane. For DACHO the slope is $s = -1.079$ and the linear correlation coefficient is $r = 0.995$; for DAC-O-Me, $s = -0.712$ and $r = 0.998$; for DAC-Im, $s = -1.066$ and $r = 0.996$.

active site environment may become disordered before the protein as a whole unfolds—particularly if it is acylated. Upon cooling, the λ_{max} does not return to the value for the native acyl enzyme but remains blue-shifted. It appears that complete renaturation of refolding of the denatured acyl enzyme upon cooling does not occur. Thus, we were able to obtain the Raman spectrum and the absorption spectrum of the cooled, and at least partially denatured, acyl enzyme.

Figure 5 shows a portion of the resonance Raman spectrum of the cooled thermally denatured acyl enzyme. Although the thermally denatured acyl enzyme gave a rather noisy Raman spectrum, the carbonyl frequency is still observable at 1696 cm^{-1}. The measured absorption maximum of the cooled, thermally denatured enzyme was found to be 375 nm—a shift to the blue of 16 nm from the 391-nm absorption maximum of the native acyl enzyme.

The effect of solvent on the absorption maximum and the carbonyl frequency of DAC-O-Me, DACHO, and DAC-Im and the values for native and partially denatured DAC-α-chymotrypsin are shown in Figure 6. The solvent dependencies are consistent with the explanation discussed in Argade et al. (1983).

Table I: Absorption Maxima and Carbonyl Frequency of DAC-α-chymotrypsin and Selected Model Compounds in Various Solvents

compd	solvent	λ_{max} (nm)	$\nu_{C=O}$ (cm^{-1})
DAC-O-Me	10% dioxane	363	
	30% dioxane	369	
	50% dioxane	370	1687
	85% dioxane	362	1697
	100% dioxane	358	1705
native DAC-α-chymotrypsin	water	391	1677
denatured DAC-α-chymotrypsin	water	375	1696
DAC-α-chymotrypsin	20% dioxane	391	1677
	40% dioxane	391	1677
	50% dioxane	385	
	60% dioxane	376	
	70% dioxane	362	
	80% dioxane	358	
	100% dioxane	358	

To obtain further information about native and denatured DAC-α-chymotrypsin, both absorption and Raman spectra of DAC-α-chymotrypsin were obtained in varying concentrations (v/v) of dioxane/water up to 100% dioxane (Table I. At concentrations of dioxane greater than 40%, DAC-α-chymotrypsin denatures, accompanied by a shift in the absorption maximum from 391 to 358 nm. In up to 40% dioxane, the Raman spectrum of DAC-α-chymotrypsin is superimposable on the Raman spectrum taken in aqueous buffer. Unfortunately, due to the poor solubility of denatured acyl enzyme in high concentrations of dioxane, the Raman spectrum was not obtainable. To help interpret these results, the absorption Raman spectra of DAC-O-Me were obtained as a function of dioxane concentration (Table I). The values for the carbonyl frequency and the adsorption maxima yield coordinates that fall directly on the DAC-O-Me line in Figure 6.

Discussion

Chromophoric acyl enzymes have been used for several years to study the mechanism of serine protease catalysis. Generally, these acyl enzymes exhibit a red-shifted absorption spectrum compared to the corresponding model methyl ester (Argade et al., 1984; Bernhard et al., 1965, 1971, 1974). It has been proposed that this red shift in the absorption maximum from that found in esters to that of the corresponding aldehyde is caused by bending the normal s-trans configuration about the acyl enzyme ester bond. Thus, this out-of-plane bend reduced the π-bond electron withdrawing of the alkyl ester oxygen from the carbonyl, thereby converting the acyl enzyme into the electronic equivalent of an aldehyde or ketone (Bernhard & Malhotra, 1974).

Another way of formulating this idea is to realize that an ester (or corresponding amide) may be considered as a resonance structure between the two structures, C—C(=O)—O—C and C—C(C—O^-)=O^+—C. It is this resonance that gives partial double bond character or sp^2 hybridization to the ester alkyl oxygen (or amide nitrogen). This is the simple explanation of the well-known experimental observation that all of the five atoms in simple esters and amides are coplanar with either an s-trans or an s-cis conformation—even in the gas phase (Henderson, 1970). The bending of the torsional angle about the C–O ester bond or the C–N amide bond would change the hybridization of the ester alkyl oxygen or amide nitrogen from the trigonal planar sp^2 to something approaching

sp^3 hybridization. This would have the effect of increasing the carbonyl frequency and making the ester spectroscopically more like an aldehyde or ketone, which do not have this ability to form these two resonating structures. The change of the ester alkyl oxygen from the sp^2 planar hybridization to the sp^3 out-of-plane hybridization must also be accompanied by a large increase in the basicity of this ester oxygen, and this may play a role in the catalysis. Komiyama and Bender (1979) have already suggested that hydrolysis of amides by serine proteases may involve an enormous increase in the basicity of the amide nitrogen. However, they do not appear to have specifically invoked the change in the torsional angle defined by the C–C–N–C bonds from 180° to 109° in their mechanism. In this paper we suggest that, on the basis of the spectroscopic evidence, it is likely that an out-of-plane torsional twist is induced about the C–O ester bond in the acyl enzyme.

By correlating the effect of solvent polarity on the carbonyl frequency and the absorption maximum of a family of related compounds, we can distinguish between bond order changes due to solvent polarity effects and bond order changes due to changes in chemical structure of these chromophoric materials. As can be seen in Figure 6, varying the solvent polarity with the various model compounds gives a good linear correlation between the carbonyl frequency and the absorption maximum. These lines can be described as polarity vectors. These vectors go from the lower right-hand part of the diagram corresponding to solvents of generally lower dielectric constant to the upper left-hand part corresponding to solvents of higher dielectric constant. In our previous paper (Argade et al., 1983), the polarity vectors for 4-(dimethylamino)benzaldehyde, DAB ester, and DAB-iinidazole were nearly parallel but were displaced relative to each other. In that case, it was easy to tell to which chemical structure the acyl enzyme belonged. In the present experiments with the DACA derivatives, the polarity vectors for the aldehyde, DACHO, and the ester intersect. However, if lines are drawn connecting the pair of different model compounds (i.e., DACHO and DAC-O-Me) for each of the solvents, then a series of nearly parallel lines results. Such lines can be obtained by drawing connecting lines between the two M's, the two A's, or the two F's on the DACHO and DAC-O-Me polarity vectors. These lines give the result of changing the structure of the model compound but keeping the solvent constant. This set of lines can be used to describe a bond order change vector that results from changes in the chemical structure. Together, these two vectors predict the combined effect of changes in the solvent polarity at the active site and the change in chemical structure that results from the special nature of the acyl enzyme.

The active site of α-chymotrypsin is thought to be relatively nonpolar and should cause spectral shifts in the acyl chromophore analogous to those of a nonpolar solvent like acetonitrile (Berhard & Lau, 1971). Since nonpolar solvents cause blue shifts in the absorption spectra of model compounds (Figure 6), forces or factors other than the nonpolar nature of the active site must be responsible for the red shift in the acyl enzyme that is observed. If the red shift in the absorption spectrum is due to breaking the electron withdrawal from the carbonyl by the alkyl oxygen of the active site serine, then native acyl enzyme should behave spectrally like an aldehyde. Furthermore, denatured acyl enzyme should behave spectroscopically more like an ordinary serine ester.

Although the effect of "solvent environment" at the active site in perturbing the spectrum of the acyl chromophore is unknown, the solvent polarity vector predicts the way the spectrum changes with solvent polarity. If the acyl chromophore is sequestered from bulk solvent in native acyl enzyme, its spectrum should be insensitive to changes in bulk solvent polarity as long as the enzyme's structure remains native. Similarly, the effect of solvent environment on the acyl chromophore in denatured acyl enzyme is unknown although presumably the acyl group is now substantially exposed to bulk solvent. The extent to which the acyl group is exposed to bulk solvent should be reflected in the absorption maximum. If the denatured acyl enzyme chromophore is solvent-exposed, it should be sensitive to the bulk solvent composition.

As can be seen in Figure 6, neither the native nor the denatured acyl enzyme fall on any of the lines corresponding to examined model compounds. Thus, native acyl enzyme is not exactly an aldehyde in a nonpolar solvent environment, and denatured acyl enzyme is not exactly an ordinary serine ester in a mainly polar environment. However, a line drawn from the point for the active enzyme to the point for the denatured enzyme in Figure 6 almost exactly parallels the lines drawn from the aldehyde to the ester for the model compounds in the same solvent; i.e., it parallels the bond order change vector. Clearly, the change from the native DAB-α-chymotrypsin to the denatured acyl enzyme is very similar to the change from being "aldehyde-like" to being "ester-like". Indeed, if we assume that part of the red shift (about 10–12 nm) is environmentally induced because the chromophore is in the active site and the points in Figure 6 for the native and the denatured enzyme are each dropped vertically by this amount, then the point for the native enzyme falls on the aldehyde line and the point for the denatured enzyme falls on the line for the ester. It is reasonable to assign these changes to a nonplanar to planar structural change that occurs upon denaturation of the acyl enzyme since aldehydes and ketones cannot have the possibility of resonance in the second form given above. However, we cannot rule out the fact that the denaturation may disturb other specific substrate–enzyme interactions that occur at the active site and that give rise to these unusual spectroscopic properties of the bound substrate. Clearly, however, we can rule out these effects being due to a simple change in the environment which would be similar to changes induced by a change in solvent. In the native acyl enzyme, the breaking of the normal s-trans structure of the serine ester by the torsional twist in the C–O ester bond would tend to make the ester oxygen more basic. As discussed above, our proposal of a greatly increased basicity induced by a torsional twist in the C–O ester bond appears to be consistent with the proposal for serine proteolysis of amides suggested by Komiyama and Bender (1979).

Regardless of the polar environment in the active site of native DAC-α-chymotrypsin, the acyl chromophore is effetively sequestered from the bulk solvent. Both the absorption spectrum and the Raman spectrum of DAC-α-chymotrypsin were insensitive to changes in the concentration of dioxane up to 50% dioxane. A large change in the bulk solvent polarity occurs over this range of dioxane concentration as evidenced by the effect of varying dioxane on the absorption of DAC-O-Me (Table I). At high concentrations of dioxane, the DAC-α-chymotrypsin denatures, accompanied by a shift in the absorption spectrum of the same value as DAC-O-Me (Table I). Unfortunately, the dentured acyl enzyme is insufficiently soluble in high concentrations of dioxane to obtain a Raman spectrum. The expectation is that the correlated value would shift along the DAC-O-Me solvent polarity vector.

The thermal denaturation is not as sharp when the absorption maximum of DAC-α-chymotrypsin is monitored and compared with the calorimetric data of Privalov (1974). In

addition, the transition between native and denatured DAC-α-chymotrypsin occurs at a lower temperature than measured on the protein as a whole (Privalov, 1974). This transition occurs at an even lower temperature than with the DAB-α-chymotrypsin, which is 52 °C at pH 4.0 (Argade et al., 1983). The region around the active site appears to denature earlier with the acyl enzyme than with the protein as a whole and even depends on the nature or size of the acyl group. These observations, coupled with the lack of a single isosbestic point, are consistent with there being more than two spectrally distinct species along the denaturation pathway. Whether these species are local or more generalized intermediates might be of interest to investigate. We were not able to obtain Raman spectra of partially denatured acyl enzyme at high temperatures.

Added in Proof

An alternative explanation for the experimental results given here is that of Warshel and Russell (1984), who propose that the reduced bond order of the acyl carbonyl in the native acyl enzyme compared to that in the denatured enzyme could be due to a positive charge in the protein located close to the carbonyl oxygen. [See Figure 42 of Warshel and Russell (1984).] The negative charge on the carbonyl oxygen could also perturb the sp^2 hybridization and give rise to a nonplanar conformation of the ester linkage proposed above.

Registry No. DACHO, 6203-18-5; DACA, 1552-96-1; DAC-Im, 59708-13-3; DAC-O-Me, 7560-48-7; triethylamine, 121-44-8; imidazole, 288-32-4; α-chymotrypsin, 9004-07-3.

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Identification of Coenzyme Aldimine Proton in ^1H NMR Spectra of Pyridoxal 5′-Phosphate Dependent Enzymes: Aspartate Aminotransferase Isoenzymes†

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Received September 19, 1985

ABSTRACT: The pyridoxal form of the α subform of cytosolic aspartate aminotransferase (EC 2.6.1.1) is fully active and binds pyridoxal 5′-phosphate via an aldimine formation with Lys-258 whereas the γ subform is virtually inactive and lacks the aldimine linkage. Comparison of ^1H NMR spectra between the α and γ subforms suggested that peak 1 of the α subform at 8.89 ppm contains a resonance assignable to the internal aldimine 4′-H. Reaction with a reagent that cleaves or modifies the internal aldimine bond [(aminooxy)acetate, L-cysteinesulfinate, NH_2OH, $NaBH_4$, or $NaCNBH_3$] caused the disappearance of a resonance line at 8.89 ppm that possessed a broad line width and corresponded in intensity to a single proton. These reagents were also used successfully for the identification of the aldimine 4′-H resonance in the mitochondrial isoenzyme. In contrast to the cytosolic isoenzyme whose resonance for the 4′-H did not show any detectable change in chemical shift with pH, the corresponding resonance in the mitochondrial isoenzyme exhibited pH-dependent chemical shift change (8.84 ppm at pH 5 and 8.67 ppm at pH 8) with a pK value of 6.3, reflecting the interisozymic difference in the microenvironment provided for the internal aldimine. Validity of the signal assignment was further shown by the two findings: (a) the resonance assigned to the 4′-H emerged upon conversion of the pyridoxamine into the pyridoxal form, and (b) the resonance appeared upon reconstitution of the apoenzyme with [4′-^{1}H]pyridoxal phosphate but not with [4′-^{2}H]pyridoxal phosphate.

The importance of imine bond formation is apparent in many enzymic reactions catalyzing the transformation of carbonyl compounds and amines. Occurrence of such bonds seems to be universal in pyridoxal-P[1]-dependent enzymes, in which the formyl group at position 4 of the coenzyme forms an aldimine bond with the ε-amino group of a specific lysyl residue (Snell & Di Mari, 1970). Since this binding mode of pyridoxal-P was shown for glycogen phosphorylase (Fischer et al., 1958)

† This paper is dedicated to Professor Esmond E. Snell on the occasion of his 70th birthday. This work was supported by a Grant-in-Aid for scientific research from the Ministry of Education, Science and Culture. Preliminary reports on this work have been presented (Morino et al., 1982; Morino, 1984).

[1] Abbreviations: pyridoxal-P, pyridoxal 5′-phosphate; pyridoxamine-P, pyridoxamine 5′-phosphate; NMR, nuclear magnetic resonance; cAspAT, cytosolic aspartate aminotransferase; mAspAT, mitochondrial aspartate aminotransferase; EDTA, ethylenediaminetetraacetate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/86/0425-1917$01.50/0