Resonance Raman Carbonyl Frequencies and Ultraviolet Absorption Maxima as Indicators of the Active Site Environment in Native and Unfolded Chromophoric Acyl-α-chymotrypsin†

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ABSTRACT: The imidazole of chromophoric *p*-(dimethylamino)benzoic acid, DABIm, reacts with the serine protease α-chymotrypsin in the pH range of 4–7 to form a stable acyl intermediate that gives very good resonance-enhanced Raman spectra. The resonance Raman and absorption spectra of the acyl enzyme intermediate have been compared with the spectra of simple model compounds such as the corresponding chromophoric methyl ester, aldehyde, and imidazole. The resonant Raman and ultraviolet absorption spectra of these simple chromophoric model compounds change considerably with the solvent. However, each of the model compounds exhibits a linear correlation between the maximum wavelength of absorption and the frequency of the carbonyl vibration. The observed values of the acyl intermediate do not fall on the line for the methyl ester but rather on the line for the aldehyde. This shows that the chromophoric serine ester of the acyl enzyme behaves differently than an ordinary ester, which cannot be explained as a solvent effect. Thermal unfolding of the acyl enzyme brings the spectroscopic parameters close to those of the model ester. We conclude that it is the specific conformation of the native enzyme and not solvent effects that change the spectroscopic properties of the acyl chromophore. It is reasonable that these changes arise from the same forces that cause the catalytic events. The carbonyl frequencies of a series of para-substituted benzoyl methyl esters show a remarkably linear correlation with the rate of deacylation of the corresponding acyl enzymes.

The mechanism of serine protease activity is now understood to occur in several steps, which include a tetrahedral intermediate, an acyl enzyme plus first product, and a second tetrahedral intermediate that dissociates to give the free enzyme plus the final product. Throughout this series of steps, the carbonyl bond plays a primary role as it changes bond order from two in the acyl enzyme to one in the tetrahedral intermediate and back again to two in the product. What is not so well understood is the role played by the structure of the native enzyme in its catalytic activity; i.e., what is the nature of the forces that are brought to bear on the substrate and its subsequent intermediates in the active site of the native protein?

Catalytic intermediates have been detected in the past by means of absorption spectroscopy; generally, the acyl intermediates exhibit absorption maxima that are red-shifted compared to those of the corresponding model serine or methyl esters [see, for example, Bernhard & Lau (1971) and Bernhard & Malhotra (1974)]. Upon denaturation, the acyl enzyme absorption spectrum approaches that of the corresponding methyl ester. Thus, it has been concluded that there is something about the environment in the active site that shifts the absorption band and that this environmental effect also plays a role in the catalytic mechanism. One difficulty of this approach is that the absorption spectra of model compounds change substantially upon going from one solvent to another. Consequently, it is not clear which solvent for the model compound produces an absorption spectrum that is the most appropriate for comparison with the native acyl enzyme spectrum. This is particularly true if the possibility of an unusual dielectric behavior at the active site is considered. In this investigation, we have obtained both the absorption maximum and the carbonyl frequency of the chromophoric acyl enzyme and compared these two values with those of likely model compounds in a variety of solvents. This approach is made more meaningful because, as we show below, the wavelengths of the absorption maxima and the carbonyl frequencies for a given chromophoric model compound obtained in solvents of various dielectric constant show a linear correlation.

There are at least two possible explanations for the origin of the red shift in the absorption spectrum of the acyl enzyme compared to those in model esters. The structure of [(3-indolylacryloyl)Ser^{195}]acyl-α-chymotrypsin determined by Henderson (1970) shows that the ester group of the acyl enzyme, unlike other esters of known structure, is not entirely in the *s*-trans configuration. Bernhard & Malhotra (1974) have speculated that the electronic consequence of such an out-of-plane bend would be to eliminate the π-bond electron withdrawing of the alkyl oxygen from the carbonyl, thereby converting the ester to the electronic equivalent of an aldehyde or ketone. In support of this conjecture, the absorption spectra of the aldehyde and ketone show an absorption maximum close to that of the acyl enzyme. Another possibility is that the ionic–dipolar forces at the active site change the electronic quantum energy levels of the chromophore and hence its absorption band. [For details of this approach, see Warshel & Levitt (1976) and Warshel & Weiss (1980).] We propose that by making measurements of two spectroscopic parameters, the carbonyl frequency and the wavelength of maximum absorption, it may be possible to obtain some new insight into the nature of the environment at the active site of α-chymotrypsin.

Phelps et al. (1981) and MacClement et al. (1981) have obtained the resonance Raman and absorption spectra of a series of substituted [(furylacryloyl)acyl]-α-chymotrypsins. However, these particular chromophores do not exhibit a strong resonance enhancement of their carbonyl vibrations. Rather, it is the —C=C— bond stretching vibration that is resonance enhanced, and this band is due to a part of the molecule that may not be acted upon by the enzyme. Thus,

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Table I: Analytical Data on Compounds Synthesized for This Work

compd	mass spectroscopic mol wt		calcd (%)			found (%)			mp (°C)
	calcd	found	C	N	O	C	N	O	
DABIm	215.28	215	66.95	19.52	7.43	67.2	19.27	7.78	116
DAB-O-Me	179.24	179	67.01	7.82	17.85	66.7	7.95	18.22	99

we have reexamined this problem by using a chromophore that shows a strongly resonance-enhanced Raman band corresponding to the carbonyl group.

As possible candidates for chromophoric substrates, we have examined a series of para-substituted benzoylimidazoles that were previously studied by Caplow & Jencks (1962). The carbonyl group of para-substituted benzoyl compounds always appears to be strongly active in the resonance Raman spectra. These compounds fall in the class of compounds described by Nishimura & Tsuboi (1980) in which the π-electrons of the carbonyl group are involved in the transition to the excited state. We chose [*p*-(dimethylamino)benzoyl]-α-chymotrypsin[1] for (pre)resonance Raman investigation because its absorption maximum at 330 nm avoids interference with tryptophan fluorescence, and due to its small Hammett σ value (−0.6), the acyl enzyme was expected to be stable at pH 7 for a sufficient period of time to be thoroughly examined by Raman and other techniques. As discussed below, the spectroscopic parameters of DAB-α-chymotrypsin indicate that the environment in the active site of the enzyme is unique and cannot be explained in terms of any possible solvent effect.

We examined the spectroscopic properties of the other para-substituted substrates to see if their carbonyl frequencies correlated with their rate of deacylation. A correlation between the rate of deacylation and carbonyl frequency has been observed, a result that will be discussed in terms of bond-order changes in the carbonyl group.

Materials and Methods

p-(Dimethylamino)benzoic acid (DAB acid) was purchased from Aldrich; [*p*-(dimethylamino)benzoyl]imidazole (DABIm) and *p*-(dimethylamino)benzoic acid methyl ester (DAB-O-Me) were prepared from the acid chloride of DAB acid, which was prepared by the procedure of Schonbaum et al. (1961). The physical characteristics of these two compounds are summarized in Table I.

Substituted Benzaldehydes. *p*-Methoxy-, *p*-chloro-, *m*-fluoro-, *p*-methyl-, *p*-bromo-, *p*-nitro-, and *p*-(trifluoromethyl)benzaldehydes were purchased from Aldrich, benzaldehyde was purchased from Fisher Scientific, and *p*-(dimethylamino)benzaldehyde was purchased from Matheson Coleman and Bell.

Substituted Methyl Benzoates. Methyl benzoate was purchased from Fisher Scientific; *p*-(trifluoromethyl)benzoate was synthesized from the corresponding acid chloride (Aldrich) by adding an excess of methanol with pyridine and subjecting the mixture to rotary evaporation for 1 h; *p*-(methoxymethyl)benzoate was also synthesized from the corresponding acid chloride (Sigma) by similar method.

Preparation of DAB-α-chymotrypsin. For absorption measurements, α-chymotrypsin (Sigma, 3 times crystallized) was brought to a concentration of about 40 mM in appropriate buffer. An equivalent of DABIm in dimethylformamide was then added. Buffers used were pH 7.0 0.1 M phosphate and pH 4.0 0.1 M formate. The corresponding concentrations of α-chymotrypsin and DABIm for Raman measurements were 2.8 mM.

To assure that the substrate DABIm reacted at the active site, two control experiments were performed. The enzyme was inhibited by incubation overnight with a 10-fold excess of TPCK in pH 7.0 0.05 M phosphate buffer. Excess TPCK was removed via Sephadex G-25 gel filtration. The inhibited α-chymotrypsin was treated with DABIm in the same way as uninhibited α-chymotrypsin. No DAB-α-chymotrypsin was formed as evidenced by the lack of change in the absorption spectrum from that of the substrate DABIm to that of the acyl enzyme. TPCK is known to inhibit proteolysis by binding at histidine-57 [see, for example, Lehninger (1970)]. In the second experiment, a 50-fold excess of the standard substrate BTpNA was added to a 5 mM solution of α-chymotrypsin in pH 7.0 0.05 M phosphate buffer and also to the DAB-α-chymotrypsin. The initial rate of formation of pNA in the native enzyme was 0.165 OD unit/min as monitored at 375 nm while with the acyl enzyme a rate of 0.002 OD unit/min was observed. The first experiment showed that the DABIm will not bind if the active site is blocked while the second experiment showed that the acyl enzyme is not catalytically active because of acylation at the active site. These experiments clearly establish the binding of the DABIm to the active site serine.

Thermal Denaturation of α-Chymotrypsin. The absorption maximum of DAB-α-chymotrypsin in pH 4.0 0.1 M formate buffer was followed as the temperature was changed from 20 to 60 °C with a Hewlett-Packard HP 8450A spectrophotometer equipped with a Peltier heating jacket. If the thermal unfolding of DAB-α-chymotrypsin is carried out at concentrations of about 2 mM (which is required for Raman measurements), substantial irreversible aggregation leading to turbidity resulted. This behavior is in contrast to the native enzyme, which can be thermally unfolded reversibly (Privalov, 1974). The turbidity in these samples precluded obtaining the Raman spectrum of the thermally unfolded acyl enzyme with them. However, Raman samples of the unfolded acyl enzyme could be prepared by first diluting the acyl enzyme 10-fold and slowly heating it to 60 °C. The resulting sample was then slowly cooled to room temperature, centrifuged to remove precipitate, and concentrated in an Amicon concentrator to about 2.8 mM. All attempts to obtain Raman spectra of the unfolded samples at high temperatures failed due to the presence of fluorescence.

Raman Spectroscopy. Raman spectra were recorded on a Spex Model 1400 double monochromator with a thermoelectrically cooled RCA C31034 photomultiplier tube operated in the photon-counting mode. A 90° scattering geometry was used. The exciting source was a Spectra Physics Model 165 or Coherent Model CR 5 argon ion laser. A Varian 620/I minicomputer was used to acquire data on-line and for data analysis. Raman samples were contained in melting point capillaries. Spectra were repetitively scanned to improve the signal to noise ratio. The spectral resolution was 3 cm^{-1}.

[1] Abbreviations: DABIm, [*p*-(dimethylamino)benzoyl]imidazole; DAB-O-Me, *p*-(dimethylamino)benzoic acid methyl ester; DABA, *p*-(dimethylamino)benzaldehyde; DAB-α-chymotrypsin, [*p*-(dimethylamino)benzoyl]-α-chymotrypsin; BTpNA, (*N*-benzoyl-L-phenylalanyl)-*p*-nitroaniline; pNA, *p*-nitroaniline; TPCK, N^{α}-tosyl-L-phenylalanine chloromethyl ketone.

Table II: Summary of Absorption Spectroscopic Data

derivative	pH	λ_{max} (nm)
DABIm	7.0	355
DABIm	4.0	365
DAB acid	7.0	290
DAB acid	3.0	316
DAB-chymotrypsin	7.0	328
DAB-chymotrypsin	4.0	331
thermally denatured DAB-chymotrypsin	4.0	318
DAB-O-Me	7.0	314
DAB acid and chymotrypsin	7.0	290

Results and Discussion

Throughout the pH range of 4–7, the chromophoric substrate DABIm reacted with α-chymotrypsin to form a relatively stable acyl enzyme with an absorption maximum of 328 nm compared to 355 nm for the substrate at pH 7.0. When this acyl enzyme was passed through a Sephadex G-25 gel filtration column, the absorption spectrum did not change from its 328-nm absorption maximum. This plainly indicates that the chromophore is covalently bound to the enzyme. The half-life for deacylation of the acyl enzyme to DAB acid at pH 7 is about 3 days. Deacylation was monitored by the change in absorption maximum from 328 to 290 nm for the DAB acid. [Table II gives the wavelength of the absorption maxima for each of the related compounds containing the *p*-(dimethylamino)benzoyl chromophore.] It was also verified that DAB acid does not bind to α-chymotrypsin to give an absorption band at 328 nm. Other tests demonstrating that this substrate is bound at the active site are described under Materials and Methods.

Figure 1 shows Raman spectra for compounds containing the DAB chromophore. Figure 1A is a spectrum of DAB-α-chymotrypsin. Figure 1B is a spectrum of the unreacted enzyme recorded under the same conditions. Comparison of these two spectra shows that in Figure 1A there is considerable preresonance enhancement of several bands of the DAB chromophore. The spectrum in Figure 1B was subtracted from that in Figure 1A to eliminate the bands due to the enzyme, and the resulting difference spectrum is shown in Figure 1C. Although the disadvantage of using only the preresonance Raman effect is that it is necessary to subtract the classical Raman spectra of the protein, the advantage is that there are no problems either with fluorescence or with photodecomposition of the chromophoric acyl enzyme. (At 333-, 351-, and 363-nm exciting laser lines, the Raman spectrum was masked by fluorescence.)

There is now much evidence that the carbonyl band of the DAB chromophore is strongly active in the resonance-enhanced Raman spectrum. For example, Jagodzinski et al. (1982) have shown that a band at 1696 cm^{-1}, which occurs in the resonance-enhanced Raman spectrum of the chromophoric aldehyde, DABA, in ether, shows a pronounced shift of 40 cm^{-1} to lower frequency when ^{18}O is substituted for ^{16}O at the carbonyl position. Furthermore, in the DAB chromophore, this band is strong in the infrared spectrum. This infrared evidence, in addition to the fact that this is the only vibration of the chromophore that is strongly dependent on the dielectric constant of the solvent, permits the unambiguous assignment of this band as the carbonyl band. Thus, we assign with confidence the 1697-cm^{-1} band in Figure 1C to the carbonyl vibration of the chromophoric acyl enzyme. As will be discussed below, the carbonyl band in model compounds such as the methyl ester of DAB acid, DAB-O-Me, also changes with solvent, giving further evidence of the correctness of this assignment. Jagodzinski et al. (1982) also showed that upon action of certain Lewis acids, such as Zn(II) in dry organic solvents, the aldehyde went into a quinonoid form resulting in a complete change in the Raman spectrum. It was, therefore, of interest in this investigation to see if the acyl enzyme showed evidence of going into this quinonoid form. This possibility can be ruled out on the basis that the Raman spectrum of the acyl enzyme does not show the characteristic Raman bands of the quinonoid structure.

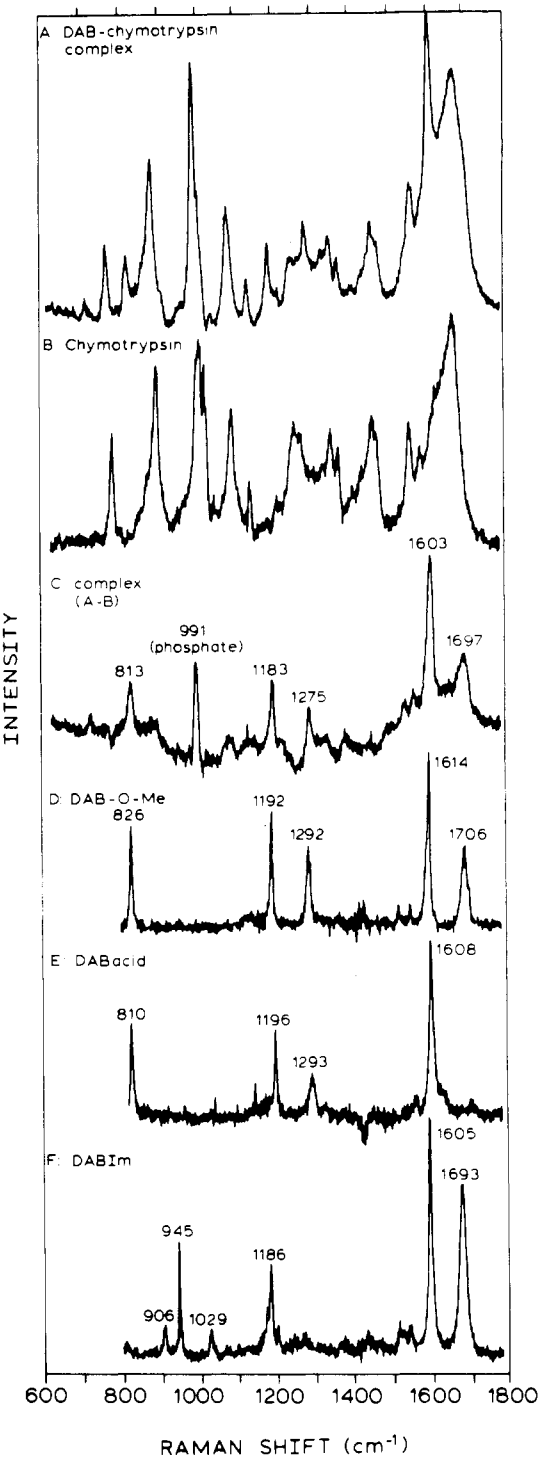

FIGURE 1: Raman spectra of (A) DAB-α-chymotrypsin acyl enzyme (2.8 mM in pH 7.0 0.25 M phosphate buffer) and (B) α-chymotrypsin (2.8 mM in the same buffer) and (C) difference spectrum of DAB acyl enzyme obtained by subtracting (B) from (A) to eliminate the bands due to α-chymotrypsin. (D) DAB-O-Me; (E) DAB acid; (F) DABIm. Spectra D–F were obtained at 0.1 M concentration in acetonitrile; the solvent bands have been subtracted. All the spectra were recorded with a 4579-Å exciting laser line.

Table III: Variation of λ_{max} and $\nu_{C=O}$ with Solvent for DABIm, DABA, and DAB-O-Me[a]

solvent	dielectric constant ϵ	DABIm λ_{max} (nm)	DABIm $\nu_{C=O}$ (cm^{-1})	DABA λ_{max} (nm)	DABA $\nu_{C=O}$ (cm^{-1})	DAB-O-Me λ_{max} (nm)	DAB-O-Me $\nu_{C=O}$ (cm^{-1})
diethyl ether	4.3	332	1702	325	1697	300	1719
carbon tetrachloride	2	335	1701	329	1696	306	1716
acetonitrile	37.5	342	1694	335	1668/1682	308	1707
methylene chloride	9.1	344	1691	345	1667	310	1703
formamide	111	352	1680	349	1657	315	1686
water	80	355		350	1647	314	

[a] The error in $\nu_{C=O}$ is ± 2 cm^{-1} and that in λ_{max} is ± 1 nm. Due to low solubility of DABIm and DAB-O-Me in water, the corresponding $\nu_{C=O}$ could not be determined by Raman. The carbonyl band of DABA is split in acetonitrile.

To further characterize the DAB-α-chymotrypsin acyl enzyme, it is desirable to compare its spectrum with that of model compounds containing the DAB chromophore. Figure 1D–F shows Raman spectra of 0.1M DAB-O-Me, DAB acid, and DABIm in acetonitrile. Table III summarizes the observed frequencies of the carbonyl group and the wavelength of maximum absorption for each of these small molecules dissolved in various solvents. As may be seen in Table III, the absorption maxima and the carbonyl frequencies are solvent dependent. Although the dielectric constant of the solvent correlates to some extent with these changes, other factors such as specific solute–solvent interaction may also be involved. It has been suggested that the decrease in the carbonyl frequency with increasing polarity is due to the polarization of the C=O bond with a corresponding lowering of the bond order (Jagodzinski et al., 1982). Because of this solvent dependence, we decided to look for a linear correlation between the observed carbonyl frequency and the absorption maximum for a given compound in a variety of solvents. Such a linear correlation between absorption maxima and carbonyl vibrational frequencies has previously been reported from this laboratory for the aldehyde, DABA (Peticolas, 1982). Similar linear correlations are well-known for —C=C— stretching vibrations and absorption maxima in both retinal-containing proteins (Heyde et al., 1971; Aton et al., 1977) and certain chromophoric acyl enzymes such as the (furylacryloyl)acyl enzyme of α-chymotrypsin (Phelps et al., 1981; MacClement et al., 1981). However, we point out that in all of the previous work on the chromophoric acyl enzymes in which the resonance Raman spectrum contains a strong band due to the —C=C— group, the protein-chromophore value for the —C=C— stretching frequency and the absorption maximum falls on the same line as do the values for all of the small model compounds.

The origin of the linear correlation between absorption maximum and the carbonyl frequency is qualitatively understood in terms of an environmentally induced increase in π-electron delocalization, which gives rise to a red shift because of the lower energy levels of the delocalized electron system. This electron delocalization also reduces the bond order of double bonds such as the —C=C— or the carbonyl bond. The decreased bond order of the double bond reduces its vibrational frequency so that it is correlated with the shift in the value of the absorption maximum. The origin of this correlation is not yet quantitatively understood, although we will give evidence that it is related to linear free-energy changes such as those that are well-known to occur in aromatic molecules upon substituent-induced electronic displacement. In our case, we are observing the carbonyl frequency that is due to a group intimately involved in the catalytic process. It is of interest to see whether or not the values of the spectroscopic parameters for the DABA acyl enzyme fall on the same curve as obtained for the small model ester, DAB-O-Me, since it has been shown in the (furylacryloyl)acyl enzyme that the —C=C— stretching vibration and the absorption maximum fall on the same curve as does the corresponding furylacryloyl methyl ester (Phelps et al., 1981; MacClement et al., 1981).

We propose an interpretation of these observed linear correlations. If the spectroscopic parameters for an acyl enzyme fall on the linear correlation plot for the model compound, then the changes induced in the spectroscopic parameters by the environment at the active site of the enzyme are simply solvent effects. Thus, the changes in the —C=C— stretching frequency and the absorption maximum produced in the furylacryloyl chromophore at the active site of the enzyme (MacClement et al., 1981; Phelps et al., 1981) appear to be due to a simple solvent effect, since the values for the acyl enzyme fall on the line of the corresponding methyl ester. However, the —C=C— bond is not intimately involved in the catalytic process. For this reason, we have examined DAB-α-chymotrypsin since in this acyl enzyme, the catalytically important carbonyl vibration of the chromophore is strongly resonance enhanced. In view of the suggestion of Bernhard & Malhotra (1974) that the acyl enzyme should behave like the corresponding aldehyde or ketone, the absorption maxima and carbonyl frequencies of the DAB aldehyde and the imidazole as well as the methyl ester were determined. If there is a different linear correlation for each of these compounds, we can determine on which linear correlation plot the values of the spectroscopic parameters of the acyl enzyme fall.

Figure 2 shows a plot of the wavelength at maximum absorption in nanometers as a function of the frequency of the carbonyl vibration in reciprocal centimeters. Each of the small chromophoric molecules, the ester, the aldehyde, and the imidazole, shows a reasonably linear correlation but with considerably different slopes. For comparison with these model compounds, the values for the native acyl enzyme and the heat-denatured acyl enzyme have been placed on this plot in Figure 2. The native acyl enzyme shows a coordinate in this plot far removed from the model ester compound and, in fact, on the line for DABA. Thus, although the acyl enzyme and DAB-O-Me are both esters, they differ considerably in the relative value of their spectroscopic parameters, i.e., in the values of their carbonyl frequencies and their absorption maxima. Indeed, these results are consistent with the conjecture of Bernhard & Malhotra (1974) that the chromophore in the acyl enzyme is in an electronic configuration more like that of the aldehyde. It is apparent that for its carbonyl frequency, the acyl enzyme is far too red to resemble the simple methyl ester. On the other hand, the coordinates for the thermally denatured enzyme approach more closely those for the simple ester. Indeed, it must be realized that the actual

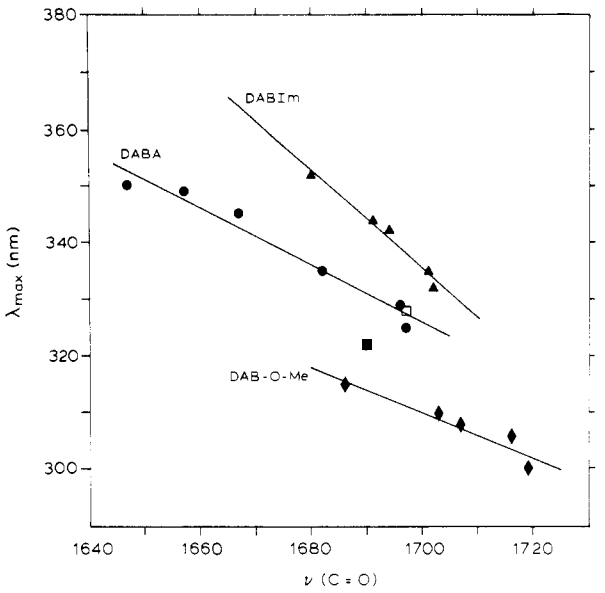

FIGURE 2: Linear correlation plot of wavelength of maximum absorption in nanometers vs. the C=O frequency in reciprocal centimeters for DABIm, DABA, and DAB-O-Me in various solvents. For DABIm, slope $s = -0.87 \pm 0.08$ and linear correlation coefficient $r = 0.99$; for DABA, $s = -0.51 \pm 0.05$ and $r = 0.98$; and for DAB-O-Me, $s = -0.40 \pm 0.08$ and $r = 0.94$. Native DAB-α-chymotrypsin is given by (□), while denatured DAB-α-chymotrypsin is given by (■).

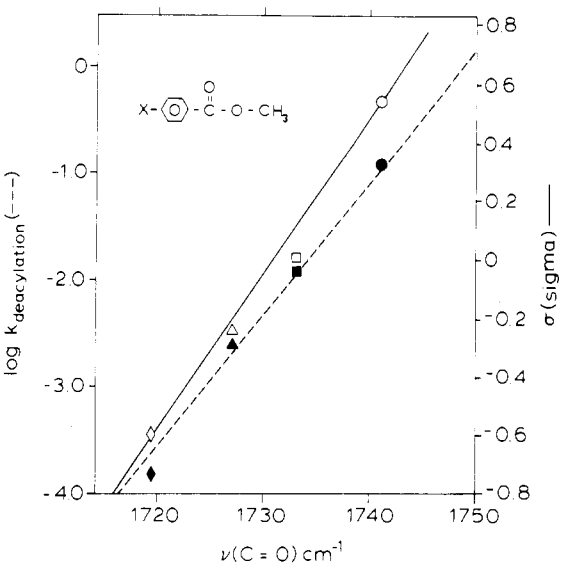

FIGURE 3: Linear correlations between Raman carbonyl frequency of para-substituted methyl benzoates in diethyl ether and (a) the Hammett σ value of the para substituent (—) and (b) the log of the rate of deacylation of the corresponding para-substituted benzoyl-α-chymotrypsins (--). For the σ values, the slope $s = 19.1 \pm 1.8$ and the linear correlation coefficient $r = 0.99$. For the deacylation rate, $s = 0.13 \pm 0.01$ and $r = 0.99$. The substituents are F_3C– (○, ●), H– (□, ■), H_3C–O– (△, ▲), and $(CH_3)_2N$– (◇, ◆) where open symbols refer to the σ values and the closed symbols refer to the deacylation rate. The deacylation rates are from Caplow & Jencks (1962) except for the $(CH_3)_2N$– substituent, which is our data.

values plotted in Figure 2 for the unfolded acyl enzyme represent a value that is probably that for a partially renatured acyl enzyme. As was mentioned under Materials and Methods, it was impossible to obtain the Raman spectrum of the unfolded acyl enzyme at 60 °C. We could only obtain the Raman spectrum of the unfolded acyl enzyme after it was cooled and concentrated. During this process, the absorption maximum changed measurably from 318 to 322 nm. The latter value is plotted in Figure 2 for the unfolded acyl enzyme. It is reasonable that this small red shift in the absorption maximum upon cooling is due to partial refolding of the acyl enzyme and that further unfolding of the acyl enzyme would shift the spectroscopic parameters even closer to the line for the methyl ester. For example, if we take the value for the absorption maximum at high temperature (318 nm) as that for the denatured enzyme, the resulting coordinate is nearly on the line for that of the methyl ester. Thus, we conclude that it is the specific conformation of the native enzyme and not solvent effects that changes the spectroscopic properties of the chromophore. It is reasonable to conclude that these changes arise from the same forces that cause the catalytic events.

In addition to the relation between the spectroscopic parameters shown in Figure 2, we have also searched for a relation between the spectroscopic parameters and the rate of deacylation. We have used the work of Caplow & Jencks (1962) to compare the rate of deacylation of para-substituted benzoyl acyl enzymes with the spectroscopic parameters of the corresponding methyl esters. Changing the substituent on the para position of the benzoyl esters will predictably change their absorption maxima for a complex variety of reasons. But the (substituent) effect on the carbonyl vibrational frequency is not so clear. Figure 3 shows a plot of the log of the rate of deacylation of benzoyl-α-chymotrypsins vs. the frequency of the carbonyl vibration of the corresponding para-substituted methyl benzoates. This linear plot extends over almost 4 orders of magnitude in rate constant. We ascribe this effect to the change in bond order that occurs when the para substituent

Table IV: Variation of Carbonyl Frequency with Substituent for Various Benzaldehydes and Methyl Benzoates[a]

substituent (X)	Hammett σ value	k ($\times 10^{-3}$ min^{-1})	X-benzaldehyde in methylene chloride [$\nu_{C=O}$ (cm^{-1})]	X-methyl benzoate in diethyl ether [$\nu_{C=O}$ (cm^{-1})]
p-$N(CH_3)_2$	−0.6	0.16	1667	1719
p-OCH_3	−0.27	2.43	1686/1699	1727
p-CH_3	−0.17	3.24	1690/1705	
H	0	12.3	1704	1733
p-Cl	0.23	19.7	1705	
p-Br	0.23		1703/1712	
m-F	0.34	53.3	1698/1716	
p-CF_3	0.54	122	1712	1741
p-NO_2	0.78	21.7	1713	

[a] k is the rate of deacylation of the corresponding substituted benzoylchymotrypsin from Caplow & Jencks (1962).

withdraws more electrons from the ring and concomitantly from the carbonyl. This reduction in the bond order of the carbonyl makes the carbon atom at the carbonyl less susceptible to attack by the hydroxide ion of the water, which is a necessary step in deacylation. This effect appears related to the Hammett σ values that were previously shown to be linearly related to the log of the rate constant (Caplow & Jencks, 1962). Figure 3 also shows a plot of the carbonyl frequency of the methyl benzoates vs. the corresponding Hammett σ factor taken from the review by Jaffe (1953). This linear free-energy effect appears to be due to the ease of polarization of the carbonyl. The higher the double-bond character, the higher the carbonyl frequency and the greater the polarizability and ease of attack by the hydroxyl of the water to promote a more rapid deacylation. A linear correlation with the carbonyl frequency is also obtained for the corresponding benzaldehydes. These data are given in Table IV.

In view of the kinetic stability of the DAB–acyl enzyme bond, it was possible to study the thermal unfolding of the acyl enzyme. Changing the conformation of the protein from the

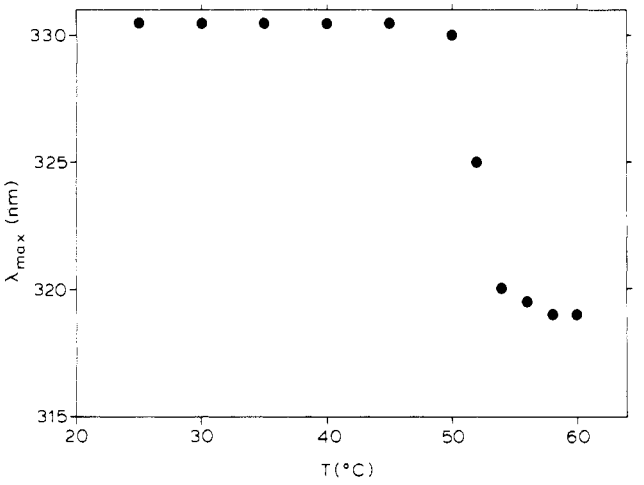

FIGURE 4: Thermal denaturation profile of DAB-α-chymotrypsin at pH 4.0 as followed by the change in wavelength of maximum absorption. The transition temperature is 52 °C.

active form to the inactive unfolded form changes the absorption spectrum in a dramatic way. Figure 4 shows the melting curve as monitored by a plot of the absorption maximum vs. temperature. The unfolding as monitored by the absorption maxima shows a rather sharp change with temperature. This transition is sharper and occurs at a lower temperature (52 °C) than that monitored on the whole protein by calorimetric methods (at 56 °C; Privalov, 1974). Thus, the environment at the active site appears to become unfolded at a temperature lower than the protein as a whole (Privalov, 1974). Apparently, it is not necessary to completely unfold the protein for the active site to lose those elements of its environment that change so completely the spectral parameters of the bound chromophore. During this transition between native and denatured acyl enzyme, a single isosbestic point was observed. This indicates that there are only two spectrally distinct species.

Although α-chymotrypsin can be thermally unfolded and refolded repeatedly with only slight loss in enzymatic activity, the same is not true with the acyl enzyme. As described above, the absorption maximum of the unfolded acyl enzyme does not return to the value of the native acyl enzyme upon cooling but stays close to the high-temperature value. On the other hand, a sample of α-chymotrypsin, which is used as a blank in the spectroscopic measurement and heated along with the acyl enzyme, shows normal activity upon cooling to room temperature. From these experiments, we conclude that the refolding pathway may be blocked by the presence of an acyl group on the serine at the active site.

In summary, there is a linear correlation between the absorption maxima and the carbonyl vibrational frequencies for several derivatives of DAB acid. This allows one to determine whether the DAB acyl enzyme possesses properties of a typical small molecule of the same chemical structure in a special solvent environment or whether other structural or environmental forces are involved. We found that the native acyl enzyme, DAB-α-chymotrypsin, does not behave spectrally like a normal ester in any known solvent. Thus, other structural or induced electronic effects, completely different from ordinary solvent effects, must be at work in the environment of the active site. These conformationally dependent forces induce values for the spectroscopic parameters of the acyl enzyme that are close to that of the corresponding model aldehyde. But these forces are relaxed when the acyl enzyme is thermally unfolded, and the spectroscopic parameters approach the values expected from the linear correlation of absorption maximum and carbonyl frequency of an ordinary ester. Therefore, it is the conformation of the native enzyme and not solvent effects that change the spectroscopic properties of the acyl chromophore. These changes presumably reflect the increased nucleophilic susceptibility of the acyl enzyme carbonyl, which results in increased catalytic efficiency. This conclusion is supported by the linear correlations between the carbonyl frequency and the Hammett σ factor and the carbonyl frequency and the deacylation rate of acyl enzyme.

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Registry No. DABIm, 87970-46-5; DAB-O-Me, 1202-25-1; DABA, 100-10-7; DAB acid, 619-84-1; *p*-methoxybenzaldehyde, 123-11-5; *p*-methylbenzaldehyde, 104-87-0; benzaldehyde, 100-52-7; *p*-chlorobenzaldehyde, 104-88-1; *p*-bromobenzaldehyde, 1122-91-4; *m*-fluorobenzaldehyde, 456-48-4; *p*-(trifluoromethyl)benzaldehyde, 455-19-6; *p*-nitrobenzaldehyde, 555-16-8; methyl *p*-methoxybenzoate, 121-98-2; methyl benzoate, 93-58-3; methyl *p*-(trifluoromethyl)-benzoate, 2967-66-0; chymotrypsin, 9004-07-3.

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