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Analysis and elimination of protein perturbation in infrared difference spectra of acyl-chymotrypsin ester carbonyl groups by using ^{13}C isotopic substitution

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I.r. spectroscopy has been applied to the study of hydrogen-bonding of the unique ester carbonyl group of acyl-chymotrypsins in the oxyanion hole of the enzyme. This catalytic device provides electrophilic stabilization of negative charge in the transition states and tetrahedral intermediates along the reaction pathway. The use of ^{13}C isotope substitution of the ester carbonyl group reinforces the previous observation [White & Wharton (1990) Biochem. J. **270**, 627–637] that the ester carbonyl group is significantly polarized in the ground state by hydrogen bonding in the oxyanion hole. I.r. difference spectra of [*carbonyl*-^{12}C]- minus [*carbonyl*-^{13}C]-cinnamoyl-chymotrypsin as well as each of these acyl-enzymes minus free enzyme are reported. These spectra show that the contribution of protein perturbation (i.e. spectral features that arise from the enzyme which is distorted on acylation) in [*carbonyl*-^{12}C]cinnamoyl-chymotrypsin minus free enzyme spectra is significant. The contribution of the perturbation components of the spectra is pH-dependent and can represent up to 50% of the total absorbance in the spectral region from 1690 to 1740 cm^{-1}. Use of the isotopic difference method has allowed problems associated with protein perturbation to be eliminated. Similar difference spectra are presented for dihydrocinnamoyl-chymotrypsin. In this case the effect of perturbation is very marked and leads to the cancellation of the band assigned to the non-bonded conformation of the acyl group which has previously only been observed at higher pH. The isotopic difference method again proves reliable and shows that the frequency difference previously used to calculate the ground-state electronic strain induced by the oxyanion-hole catalytic device is not affected by the perturbation, although the amplitudes of the spectral features are different. A study of the deacylation of cinnamoyl-chymotrypsin in water and deuterium oxide using both u.v. and i.r. spectroscopies has confirmed that the use of deuterium oxide as solvent has no serious effect on the deacylation behaviour of the enzyme. I.r. bands assigned to non-productive and productive conformers decline identically during deacylation, which shows that the conformers are in dynamic exchange on the reaction time-scale.

INTRODUCTION

Vibrational spectroscopy has proved to be a powerful method for the characterization of hydrogen-bonding of the ester carbonyl group in acyl-chymotrypsins [1–6]. These are metastable ester intermediates which lie on the catalytic pathway of chymotrypsin catalysis. Other enzyme mechanisms, such as that of triose phosphate isomerase, where carbonyl groups are involved at the reaction centre, may be studied using this methodology [7–9]. By observation of the vibrational frequencies of the unique ester carbonyl group in acyl-chymotrypsins, it has been shown that the acyl group exists in more than one conformation [2–5]. Three conformations have been assigned to the dihydrocinnamoyl group in the active site of the enzyme (Figs. 1 and 2). These comprise a non-bonded (NB) conformation, in which the carbonyl group is not hydrogen-bonded into the oxyanion hole of the enzyme, a conformation known as non-productive (NP), in which the conformation involves a bridging water molecule from the acyl carbonyl group to His-57 [10], and a productive catalytically active (P) conformation in which the carbonyl oxygen is bound in the oxyanion hole of the enzyme.

Hydrogen-bonding can be detected with high sensitivity by using vibrational spectroscopy, since a characteristic down-frequency shift occurs as a result of weakening of the bond (such as a carbonyl bond) on formation of a hydrogen bond. Comparison of the frequencies observed for acyl carbonyl groups in enzyme active sites with those of model compounds in a variety of solvents which cover a range of dielectric constants allows the presence and strength of hydrogen-bonding to be deduced [2].

Quantification of the strength of hydrogen-bonding is not easy and can be approached in several ways [2]. The aim is to estimate the extent to which hydrogen-bonding in the oxyanion hole in these acyl-enzymes provides ground-state electronic strain which can be used to promote catalysis. Model studies and calculations based on simple harmonic motion [2] as well as empirical estimates of carbonyl bond breaking [9,11] can be used for this, but at present these methods are difficult to drive to a secure and robust conclusion. Application of simple-harmonic-motion theory to the difference in the vibration frequencies of the NB and P conformations (i.e. the maximal extent of hydrogen-bonding) of dihydrocinnamoyl-chymotrypsin has been used to estimate the decrease in strength of the carbonyl bond that occurs on hydrogen-bonding; this has been estimated as approx. 15–25 kJ/mol [2].

Significant perturbation of vibrational spectroscopic features which arise from distortion of the enzyme, rather that from the acyl group, can occur when the enzyme is acylated [1,2,12]. Such perturbations are evident in difference spectra in which the spectrum of the free enzyme is subtracted from a spectrum of the acyl-enzyme, since any structural change in the enzyme that occurs on acylation is not compensated in these spectra. An example of this occurs in the cinnamoyl-chymotrypsin difference spectrum at low pH, where a negative feature is seen at 1738 cm^{-1} [12]. This was ascribed to a decrease in the pK_a of a carboxy

Abbreviations used: NP, non-productive conformation; NB, non-bonding conformation; P, productive conformation (of acyl-chymotrypsin); rR, resonance Raman; FTIR, Fourier transform infrared; pH*, pH-meter reading in ^2H_2O.

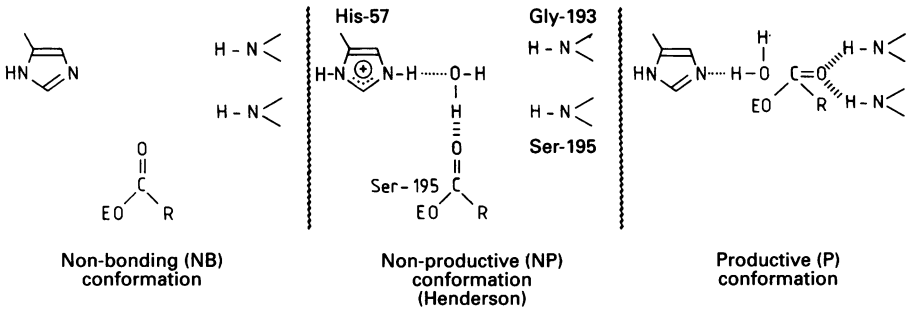

Fig. 1. The three conformations of the bound acyl group in dihydrocinnamoyl-chymotrypsin deduced from FTIR spectroscopy [2]

E represents chymotrypsin, with the oxygen being that of Ser-195. R is the phenylethyl moiety of dihydrocinnamate. It is postulated that only the productive conformer can deacylate. The hydrogen-bonding interaction of the non-productive conformer is deduced from the X-ray crystallographic structure of indolylacryloyl-chymotrypsin as determined by Henderson [10].

group in the enzyme consequent on acylation. Much larger features in difference spectra occur at lower frequencies, in the region where the protein amide carbonyl groups show strong absorbance (1600–1690 cm^{-1}), and these can also interfere with analysis of the acyl-enzyme ester carbonyl absorbance.

It is clearly necessary to ensure that there are no underlying protein-based spectral perturbations in the region of the acyl-enzyme ester carbonyl group absorption, since these would cause distortion of the acyl-group carbonyl band profile and render deductions based on an analysis of the spectra unreliable. Substitution of ^{13}C for ^{12}C in a carbonyl group has the effect of shifting the carbonyl stretch vibration some 38 cm^{-1} to lower frequency. This means that a spectrum determined with $^{13}C{=}O$ in the acyl carbonyl group can be subtracted from an equivalent $^{12}C{=}O$ spectrum in order to give a difference spectrum in which the acyl carbonyl vibrations are unperturbed by any features which may arise from enzyme perturbation, since this cancels out in the difference spectrum. We have used ^{13}C substitution in the ester carbonyl group of cinnamoyl-chymotrypsin and determined [*carbonyl*-^{12}C]- minus [*carbonyl*-^{13}C]-acyl-enzyme difference spectra in order to eliminate such structural perturbations, and have deduced that some perturbation is present in spectra where the free enzyme is used for subtraction [2].

Recently Tonge *et al.* [5] have made a detailed comparison of resonance Raman (rR) and Fourier transform i.r. (FTIR) difference spectroscopies in the study of 3-(5-methylthien-2-yl)acryloyl-chymotrypsin. In rR spectra a band is apparent with a frequency of 1727 cm^{-1}, characteristic of a non-bonded conformer (see Fig. 1). A band that might be ascribed to such a conformer is not seen in equivalent FTIR difference spectra. This feature in the rR spectrum has been related to a laser-induced conformation, and this assignment has been confirmed by irradiation of a sample of acyl-enzyme before FTIR spectroscopy whereupon a band at 1727 cm^{-1} appears in the FTIR spectrum. It has been proposed that this effect may be explained by a laser-induced *trans* → *cis* isomerization about the acryloyl double bond of the 3-(5-methylthien-2-yl)acryloyl group. This is a plausible explanation, since such a *trans* → *cis* isomerization is a well-known photochemical process [13–15].

It was also observed that the frequencies of the other two features seen in the ester C=O region of rR spectra did not match those of either of the two frequencies observed in this region in FTIR difference spectra. Analysis of the bands in the ester carbonyl region showed that approx. 50% of the intensity in FTIR difference spectra (acyl-enzyme minus enzyme) could be ascribed to protein perturbation features [6].

In this paper we report a study of the origin of perturbation in

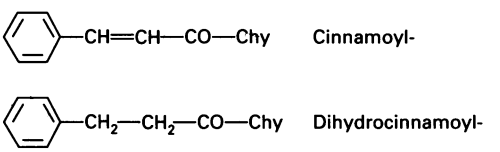

Fig. 2. Structures of the acyl groups used to prepare the acyl-chymotrypsins described in this paper

Abbreviation: Chy, chymotrypsin.

FTIR difference spectra of cinnamoyl-chymotrypsin and a more preliminary study of dihydrocinnamoyl-chymotrypsin, neither of which have been studied by rR spectroscopy. Each has been substituted with ^{13}C at the ester carbonyl carbon in order to allow distinction between absorbances which arise from protein perturbation and those that arise from the ester carbonyl bond. A direct comparison between FTIR and rR spectroscopies cannot always be made, since suitable electronic transitions which may be exploited to give resonance enhancement in rR spectra may not be available. Thus, although cinnamoyl-chymotrypsin, the acyl linkage of which absorbs maximally at approx. 290 nm, might just be accessible to study by rR spectroscopy without protein absorption becoming problematical, the dihydrocinnamoyl derivative cannot be studied in this way, since there is no accessible electronic transition. This also applies to the natural and semisynthetic but highly specific substrates of the enzyme such as acetyl-L-tyrosyl derivatives.

Transmission FTIR spectra can be measured in water if very-short-path-length (5 μm) cells are used. Such cells have to be dismantled for filling and cleaning, which makes the determination of precisely balanced difference spectra difficult. When ^2H_2O is used as solvent, longer (50 μm) pathlength cells can be used, which allows use of the '*in situ*' cell [2]. The use of ^2H_2O as solvent could introduce a perturbation of the system, over and above the kinetic isotope effect, in that incomplete exchange of enzyme protons with the solvent might affect the spectral, conformational or kinetic properties of acyl-enzymes. In previous publications we have examined the nature and balance of the various conformations of the acyl groups in acyl-enzymes [2–4]. Such analysis, which may often make use of data from experiments done in water (e.g. X-ray structures), would be of limited interest, even when protein-perturbation features were eliminated by using the isotopic difference method, if it was found that the isotopic nature of the solvent affects the spectra and/or kinetics. Accordingly we have measured the deacylation kinetics of

cinnamoyl-chymotrypsin in both solvents using u.v. spectroscopy and in ^2H_2O using FTIR spectroscopy.

MATERIALS AND METHODS

α-Chymotrypsin was obtained from Sigma as type 2, multiply recrystallized and was used as supplied. ^2H_2O was 99.9% enriched, and was obtained from Aldrich. Sodium [^{13}C]cyanide, 99% enriched in ^{13}C, was obtained from MSD Isotopes (Montreal, Canada). Buffers were 0.1 M and were adjusted to the same pH-meter reading for both water and ^2H_2O solvents. [*carbonyl*-^{12}C]Acylimidazoles and [*carbonyl*-^{13}C]cinnamoylimidazole were prepared as previously described [2].

Synthesis of [*carbonyl*-^{13}C]dihydrocinnamoylimidazole

2-Phenylethyl bromide (1.4 ml) was refluxed with sodium cyanide 99% enriched in ^{13}C (0.5 g) in 20 ml of 50% ethanol/water for 4 h. Ethanol was removed by rotary evaporation and the 2-phenylethyl nitrile product extracted into chloroform (3 × 15 ml). The combined chloroform extracts were dried with anhydrous Na_2SO_4 and rotary-evaporated to give the nitrile product. This was characterized by the presence in the i.r. spectrum of a strong C≡N stretching vibration at 2187 cm^{-1}. The nitrile was hydrolysed to the carboxylic acid by refluxing for 1 h with conc. H_2SO_4 (2.5 ml) in 50% acetic acid/water (5 ml). Acetic acid and water were removed by rotary evaporation, 5 ml of water was added, and the aqueous solution was extracted with chloroform (3 × 20 ml). The chloroform extracts were combined and dried with anhydrous Na_2SO_4, and the acid product was recovered by rotary evaporation. The acid was purified by recrystallization from water (yield 0.36 g, 24%) and was identified as the correct product by thin-layer co-chromatography with an authentic sample of the [*carbonyl*-^{12}C]acid. [*carbonyl*-^{13}C]Dihydrocinnamic acid (0.2 g) was refluxed with thionyl chloride (2 ml) for 15 min and excess thionyl chloride removed by rotary evaporation. The acid chloride was dissolved in acetonitrile (2 ml) and to this stirred and cooled solution (4 °C) was added imidazole (0.18 g, 2 equiv.) dissolved in acetonitrile (2 ml) over a period of 15 min. After further stirring at room temperature (1 h), the solvent was removed by rotary evaporation and the product purified by recrystallization from cyclohexane/di-isopropyl ether. The [*carbonyl*-^{13}C]dihydrocinnamoylimidazole product gave a single spot on t.l.c. and was shown to acylate chymotrypsin at pH 3 to exactly the same extent as the ^{12}C=O compound.

Acylation of chymotrypsin

The enzyme, 2 mM in protein, made up in ^2H_2O and adjusted to the appropriate pH* value with 5 M-^{2}HCl or NaO^{2}H, was acylated for i.r. experiments by using 60 mM solutions of the acylmidazoles in acetonitrile as previously described [2]. Deacylation kinetics were measured in the u.v. at 310 and 335 nm using Hepes and carbonate buffers (0.1 M). A 0.1 ml sample of the 50 mg/ml acyl-enzyme solution used for i.r. spectroscopy was added to 2.9 ml of the appropriate ^1H_2O or ^2H_2O buffer thermostatically controlled at 25 °C. The pH-meter was calibrated using 'Colourkey' standard buffers (BDH) at pH 4.0 and pH 7.0. Buffers made up in either ^1H_2O or ^2H_2O were adjusted to the same pH-meter reading.

I.r. spectroscopy

All i.r. spectra were determined in ^2H_2O as solvent. I.r. spectra were obtained by using the '*in situ*' cell as previously described [2]. The time-course of deacylation was followed in the i.r. by scanning spectra (against an open-beam background) at intervals and subtracting the first spectrum from those obtained after various time-intervals.

RESULTS AND DISCUSSION

I.r. difference spectra of [*carbonyl*-^{12}C]cinnamoyl-chymotrypsin minus [*carbonyl*-^{13}C]cinnamoyl-chymotrypsin

We have previously presented a difference spectrum in which 90%-enriched [*carbonyl*-^{13}C]cinnamoyl-chymotrypsin was subtracted from the [*carbonyl*-^{12}C] version [2]. We underestimated the contribution of the perturbation components in the ester carbonyl region of [*carbonyl*-^{12}C]acyl-enzyme minus free enzyme spectra. This was because we ascribed the decrease in intensity in the [*carbonyl*-^{12}C] minus [*carbonyl*-^{13}C] spectrum, relative to that seen in [*carbonyl*-^{12}C]acyl-enzyme minus free enzyme spectra, to subtraction of the 10% ^{12}C=O component in the ^{13}C=O spectrum

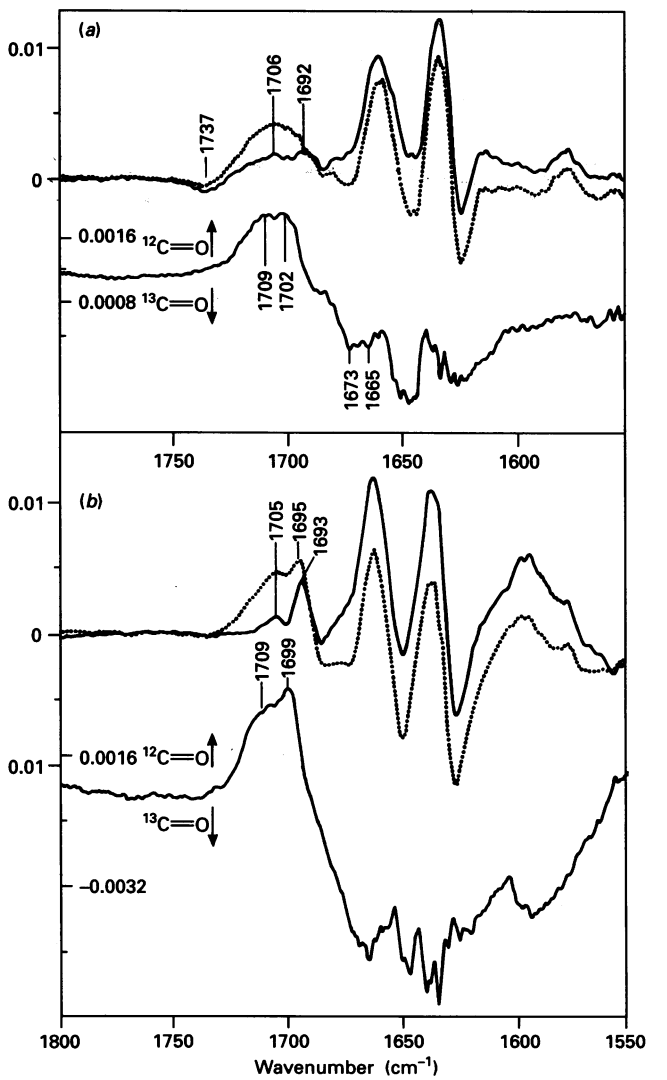

Fig. 3. [*carbonyl*-^{12}C]Cinnamoyl- and [*carbonyl*-^{13}C]cinnamoyl-chymotrypsin i.r. difference spectra at pH* 3.0 and pH* 6.0

(*a*) Difference spectra measured at pH* 3, upper lines [*carbonyl*-^{12}C]cinnamoyl-chymotrypsin minus free enzyme (dotted line) and [*carbonyl*-^{13}C]cinnamoyl-chymotrypsin minus free enzyme (continuous line). Lower line, [*carbonyl*-^{12}C]cinnamoyl- minus [*carbonyl*-^{13}C]cinnamoyl-chymotrypsin. (*b*) As (*a*) but spectra taken at pH* 6.0. The spectra were measured in ^2H_2O at room temperature, using 32 scans for each spectrum.

Table 1. I.r. absorption frequencies of acyl carbonyl and protein-perturbation components in difference spectra of cinnamoyl-chymotrypsin

The frequencies reported here are those presented in Fig. 3. All bands represent positive absorbance unless specifically stated.

Spectrum	pH*	Frequencies (cm^{-1})	Assignments
[*carbonyl*-^{12}C]Cinnamoyl-chymotrypsin minus free enzyme (see ref. [2])	4.0	1705	Mixed perturbation and acyl carbonyl components
		1695	
		1738 (−ve)	Enzyme carboxy group
	3.0	1706	Unresolved band, mixed perturbation and acyl carbonyl components
		1737 (−ve)	Enzyme carboxy group
	6.0	1705	Mixed perturbation and acyl carbonyl components
		1695	
[*carbonyl*-^{12}C]Cinnamoyl-chymotrypsin minus 90%-enriched [*carbonyl*-^{13}C]cinnamoyl-chymotrypsin (see ref. [2])	4.0	1707	^{12}C=O NP conformation
		1700	^{12}C=O P conformation
[*carbonyl*-^{12}C]Cinnamoyl-chymotrypsin minus 99%-enriched [*carbonyl*-^{13}C]cinnamoyl-chymotrypsin	3.0	1709	^{12}C=O NP conformation
		1702	^{12}C=O P conformation
		1673 (−ve)	^{13}C=O NP conformation
		1665 (−ve)	^{13}C=O P conformation
	6.0	1709	^{12}C=O NP conformation
		1699	^{12}C=O P conformation
[*carbonyl*-^{13}C]Cinnamoyl-chymotrypsin minus free enzyme	3.0	1706	Perturbation component
		1692	Perturbation component
		1737 (−ve)	Enzyme carboxy group
	6.0	1705	Perturbation component
		1693	Perturbation component

used as a 'blank' to obtain the difference. In fact, most of the decrease seen in the [*carbonyl*-^{12}C]acyl group intensity was the result of the removal of perturbation components. Fig. 3 shows FTIR difference spectra of [*carbonyl*-^{12}C]cinnamoyl-chymotrypsin and 99%-enriched [*carbonyl*-^{13}C]cinnamoyl-chymotrypsin relative to both free enzyme and to each other at pH* values of 3.0 and 6.0. The frequencies of interest and their assignments are given in Table 1. At pH* values of 3.0 and 6.0 the [*carbonyl*-^{13}C]acyl-enzyme minus free enzyme spectra show considerable absorbance in the range where the ester carbonyl group would be expected to absorb. The ester carbonyl vibration is moved down-frequency some 39 cm^{-1} in ^{13}C=O, clear of the normal ^{12}C=O ester carbonyl absorption region, so the remaining absorption in the ^{12}C=O region must result from protein perturbation. At pH* 3.0 the perturbation spectrum is not well resolved, but at pH* 6.0 the perturbation component resolves into two bands with frequencies of 1705 and 1693 cm^{-1}. These frequencies are almost coincidental with those previously reported for the ester carbonyl group on the basis of [*carbonyl*-^{12}C]cinnamoyl-chymotrypsin minus free enzyme spectra at pH* 4.0 [1,2,4] (see Table 1). Both at pH* 3.0 and at pH* 6.0 the [*carbonyl*-^{12}C]- minus [*carbonyl*-^{13}C]-acyl-enzyme spectra show resolution of two bands that can be ascribed to the ^{12}C=O vibration of the cinnamoyl-enzyme. At pH* 3.0, these frequencies occur at 1709 and 1702 cm^{-1} and compare with previously reported values of 1705 and 1695 cm^{-1} for [*carbonyl*-^{12}C]cinnamoyl-chymotrypsin minus free enzyme and 1707 and 1700 cm^{-1} for ^{12}C=O minus ^{13}C=O at pH* 4.0. At pH* 3.0 in the [*carbonyl*-^{12}C]- minus [*carbonyl*-^{13}C]-acyl-enzyme spectrum, two rather indistinct negative features at 1673 and 1665 cm^{-1} in a region of strong amide protein absorbance can be ascribed to the ^{13}C=O absorptions, since they are shifted 37 cm^{-1} down-frequency relative to the ^{12}C=O bands.

At pH* 3.0 a negative feature is seen at 1737 cm^{-1} in the spectra of both the [*carbonyl*-^{12}C]- and [*carbonyl*-^{13}C]-cinnamoyl-chymotrypsins minus free enzyme, but not in the ^{12}C=O minus ^{13}C=O spectrum. This, as previously assigned in spectra of the cinnamoyl-enzyme [4] and also in the equivalent spectra of 3-(5-methylthien-2-yl)acryloyl-chymotrypsin [5], is ascribed to a carboxy group which has a lowered pK_a or is subject to a frequency shift (to a lower value) in the acyl-enzyme. This is now supported by the observation that the negative feature is not seen in spectra taken at pH* 6.0; this shows that the group deprotonates below pH* 6.0. Protonated (or deuterated) carboxy groups have carbonyl stretching frequencies in this region, albeit usually at somewhat lower frequencies, e.g. acetic acid 1710 cm^{-1} and fully protonated lysine 1727 cm^{-1} [16]. The small magnitude of the negative feature probably means that there is some cancellation with positive perturbation features and/or the acyl carbonyl absorbance. This group is unlikely to be either the side chain of Asp-102 of the catalytic relay system or Asp-194 of the conformationally important salt bridge, since the pK_a values of these groups have been reported to be less than 3.0 [17,18].

pH*-dependence of the intensity of the carbonyl stretching bands in cinnamoyl-chymotrypsin

A comparison of the spectra at pM 3.0 and 6.0 reveals a pH*-dependent intensity change in the perturbation components and in the ester carbonyl components. For both the acyl carbonyl and perturbation components of the spectra the intensity of the lower frequency band increases as the pH* is increased, with the acyl carbonyl conformations being approximately equally populated at pM 4.0 [2]. The lower-frequency feature in the ^{12}C=O minus ^{13}C=O spectra represents the P conformer, which is hydrogen-bonded and inserted in the oxyanion hole. It is reasonable (see Fig. 1) that this conformation should be favoured and that the NP form be disfavoured as His-57 is deprotonated to give active enzyme, since neutral His-57 will hydrogen-bond to a water proton rather than to an oxygen. Perusal of the frequencies given in Table 1 shows that the influence of protein perturbation has a relatively small effect on the apparent values of the acyl carbonyl group frequencies.

[*carbonyl*-^{13}C]Dihydrocinnamoyl-chymotrypsin

The effect of protein perturbation on the frequencies of the ester carbonyl groups of cinnamoyl-chymotrypsin as determined from [*carbonyl*-^{12}C]acyl-enzyme minus free enzyme spectra noted above is not large. The errors in the ester carbonyl frequencies introduced by such perturbation could, however, be important if estimates of carbonyl polarization and ground-state electronic strain were to be made from measurements of frequency differences. Such estimates, briefly described in the Introduction and in detail in ref. [2], have been made for dihydrocinnamoyl-chymotrypsin on the basis of difference in frequency between the NB and P conformers. Accordingly we have prepared the

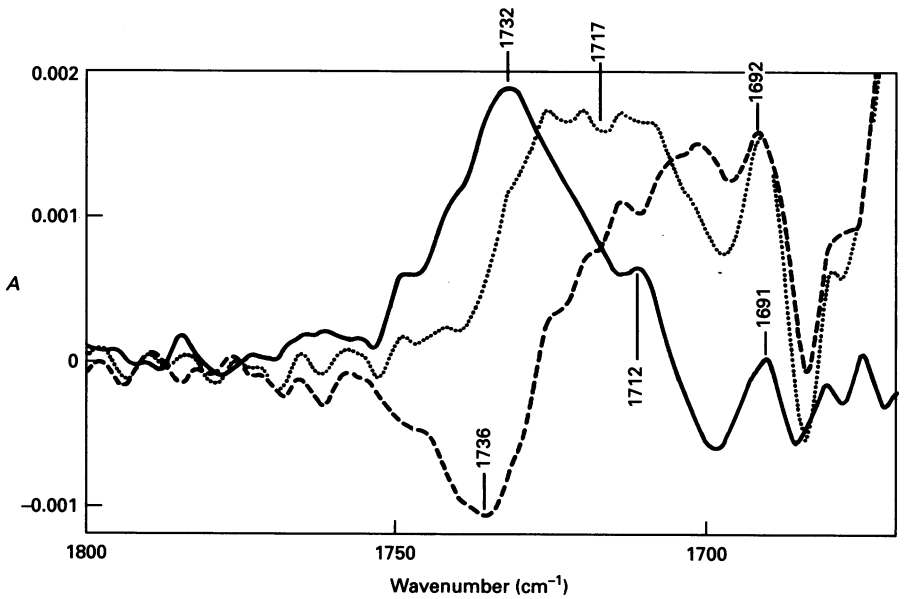

Fig. 4. I.r. difference spectra of dihydrocinnamoyl-chymotrypsin determined at pH* 4.0

[*carbonyl*-^{12}C]Dihydrocinnamoyl-chymotrypsin minus free enzyme (dotted line); [*carbonyl*-^{13}C]dihydrocinnamoyl-chymotrypsin minus free enzyme (dashed line); [*carbonyl*-^{12}C]- minus [*carbonyl*-^{13}C]-dihydrocinnamoyl-chymotrypsin (continuous line). Experimental details were as for Fig. 3.

Table 2. I.r. absorption frequencies of acyl carbonyl and protein-perturbation components in difference spectra of dihydrocinnamoyl-chymotrypsin at pH* 4.0

The frequencies reported here refer to the features presented in Fig. 4. All bands represent positive absorbance unless specifically stated.

Spectrum	Frequency (cm^{-1})	Assignment
[*carbonyl*-^{12}C]Dihydrocinnamoyl-chymotrypsin minus free enzyme	1717 (centre)	Broad band(s), NP and perturbation components
	1692	P conformation
[*carbonyl*-^{12}C]Dihydrocinnamoyl-chymotrypsin minus [*carbonyl*-^{13}C]dihydrocinnamoyl-chymotrypsin	1732	NB conformation
	1712	NP conformation
	1691	P conformation
	1699* (−ve)	^{13}C=O NB conformation?
[*carbonyl*-^{13}C]Dihydrocinnamoyl-chymotrypsin minus free enzyme	1736 (−ve)	Large perturbation component, carboxyl group, pK_a change or frequency shift
	1692	Sharp perturbation component, protein amide band?

* This frequency is 33 cm^{-1} down frequency from the *carbonyl*-^{12}C NB band. A shift of 38 cm^{-1} would be predicted to arise from a change from ^{12}C to ^{13}C. This may be the result of overlap between the *carbonyl*-^{13}C band and the *carbonyl*-^{12}C P band at 1691 cm^{-1}.

carbonyl-^{13}C-labelled derivative of dihydrocinnamoylimidazole and used this together with the *carbonyl*-^{12}C form to estimate the influence of protein perturbation in previously published [*carbonyl*-^{12}C]acyl-enzyme minus free enzyme difference spectra. Fig. 4 shows a set of spectra of dihydrocinnamoyl-chymotrypsin, similar to those shown for cinnamoyl-chymotrypsin in Fig. 3. Table 2 gives the frequencies and assignments. A broad band centred on 1717 cm^{-1} seen in the [*carbonyl*-^{12}C]acyl-enzyme minus enzyme spectrum has been assigned to the NP conformation, but this largely disappeared at higher pH* (5.6), to be replaced by a band at higher frequency (1732 cm^{-1}) assigned to an NB conformer [2]. It was previously unclear why this apparent exchange of conformers should occur in this pH* region, but fortunately the spectra shown in Fig. 4 allow the various factors involved in this behaviour to be disentangled. At pH* 4.0 there is a large compensating negative excursion in the perturbation component in [*carbonyl*-^{12}C]acyl-enzyme minus enzyme spectra that obscures the NB component. Indeed the NB component forms by far the major component at pH* 4. The negative feature seen at 1736 cm^{-1} in the [*carbonyl*-^{13}C]acyl-enzyme minus enzyme spectrum may be explained in terms of a carboxy group of perturbed pK_a or a frequency shift of a carboxy group as in the case of cinnamoyl-chymotrypsin, although experiments at higher pH* have not yet been possible owing to the relatively high reactivity of this acyl-enzyme. The band at 1691 cm^{-1}, which corresponds to the P conformer, is quite small relative to the NB band at 1732 cm^{-1}, but has been observed with a similar intensity in four experiments. It is necessary to ensure that this band, which is nearly coincident with a perturbation feature (see Fig. 4 and Table 2), is real and does not result from a subtraction artefact or concentration mismatch. Active-site titration of the [*carbonyl*-^{12}C]- and [*carbonyl*-^{13}C]-acyl-enzymes with cinnamoylimidazole showed that both were 68±2% acylated. We thus believe that the feature at 1691 cm^{-1} in [*carbonyl*-^{12}C]- minus [*carbonyl*-^{13}C]-acyl-enzyme difference spectra does not arise from a subtraction mismatch. Similar considerations also apply to the small feature at 1712 cm^{-1}.

The important point that emerges from examination of these spectra is that the frequency difference between the bands assigned to the NB and P conformers is the same as (or slightly larger than) that used previously to calculate the loss of bond enthalpy in the carbonyl bond. This loss results from hydrogen-bonding in the oxyanion hole and is responsible for the rate enhancement that arises from the induced ground-state electronic strain [2].

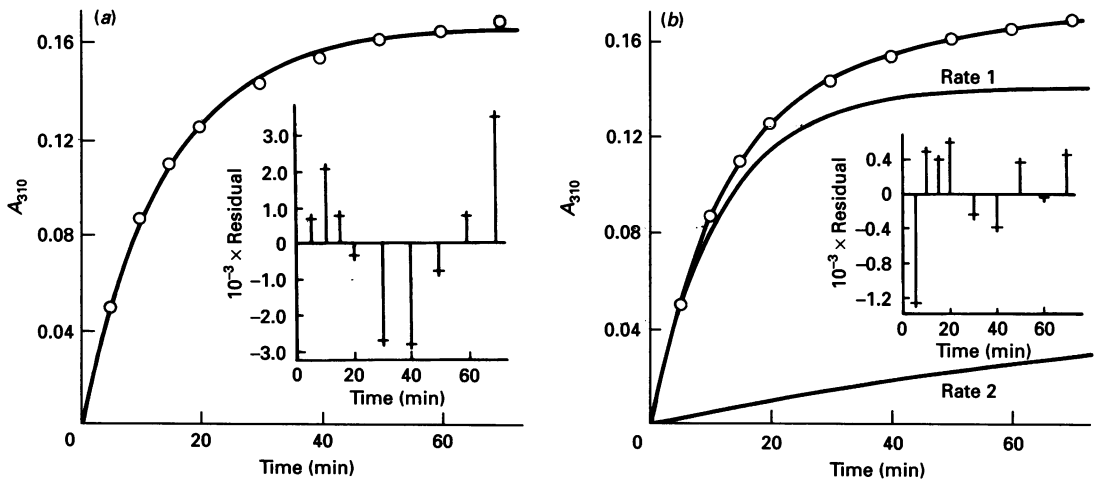

Fig. 5. Deacylation of cinnamoyl-chymotrypsin followed by u.v. spectroscopy at 310 nm and pH* 7.0 in ^2H_2O at 25 °C

(*a*) Lack of fit to a first-order equation, clearly shown by the systematic deviation of the residuals. (*b*) A double-exponential fit to the deacylation data. The enzyme was incubated at pH* 4.0, 25 °C, with ^2H_2O for 4 h before acylation. The enzyme was acylated by adding 10 μl of 60 mM-cinnamoylimidazole to 0.4 ml of 2 mM-enzyme (approx. 70% active by cinnamoylimidazole titration), which was adjusted to pH* 7.0 with conc. NaOH using a Hamilton microsyringe immediately before acylation. To follow deacylation 0.1 ml of acylated enzyme was added to 2.9 ml of 100 mM-sodium phosphate buffer, pH* 7.0, in a 1 cm-path-length cuvette which had been equilibrated to 25 °C. Note that the observed absorbance decreases with time, and the data as presented in the Figure, have been inverted with respect to the absorbance axes and normalized to zero at zero time.

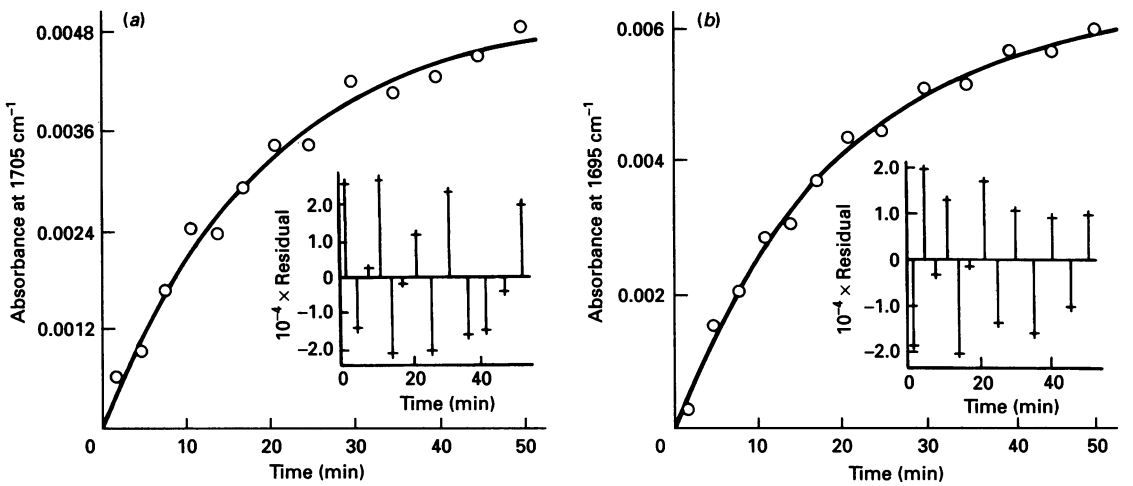

Fig. 6. Deacylation of cinnamoyl-chymotrypsin at pH* 7.0 and 25 °C, determined (in ^2H_2O) by i.r. spectroscopy

(*a*) Deacylation determined by measurement of the absorbance at 1705 cm^{-1}. (*b*) Deacylation determined by measurement of the absorbance at 1695 cm^{-1}. The acyl-enzyme was prepared as described in the Materials and methods section. Note that the observed absorbances decrease with time, and the data in the Figure have been inverted with respect to the absorbance axes and normalized to zero at zero time.

Effect of ^2H_2O as solvent and of deuteration of the enzyme on the kinetic behaviour of cinnamoyl-chymotrypsin

All of our studies of acyl-chymotrypsins using FTIR have been done with ^2H_2O as solvent. Water has a strong absorption at 1640 cm^{-1} which makes experiments with transmitted i.r. light difficult, as explained in the Introduction. Since we observe multiple spectroscopic features, which are assigned to a single acyl carbonyl group, it is important to ensure that neither the solvent itself nor incomplete ^2H exchange of the enzyme protons causes these observations. To address this issue we have made a comparison of the kinetic behaviour of cinnamoyl-chymotrypsin in water and ^2H_2O by using u.v. spectroscopy, since this, unlike FTIR, can be used with both solvents.

Measurements of deacylation in water using u.v. spectroscopy

At pH 7.0 and pH 10.5 the deacylation of cinnamoyl-chymotrypsin, when followed at both 310 nm and 335 nm, was accurately first order to more than 99% completion; see also ref. [19]. Methanol can compete in the deacylation process with water and is somewhat more effective as a nucleophile [20]. Added methanol (0–100 μl/3 ml) gave, at each pH value, a linear increase in deacylation rate constant (results not shown). Thus in our hands the deacylation of cinnamoyl-chymotrypsin is well behaved in water as solvent.

Measurements of deacylation in ^2H_2O using u.v. spectroscopy

In ^2H_2O the deacylation behaviour was slightly more complex.

When measured at pH* 10.5, after 24 h incubation with ^2H_2O at pH* 3.0 to allow ^2H exchange of the protein, the deacylation when monitored at 310 nm or 335 nm was accurately first order. When the enzyme was dissolved in ^2H_2O immediately before measurement of deacylation, the process showed systematic deviation from a first-order fit and could only be fitted by using a double exponential at either pH* 7.0 or 10.5. After 24 h incubation at pH* 3.0, the deacylation time-course was still biphasic when assayed at pH* 7.0 and only became first order after 5 days incubation with ^2H_2O. Fig. 5 shows an example of a time-course of deacylation measured at pH* 7.0 that is biphasic, together with a double-exponential fit and the deconvoluted exponentials. The fast process ($k = 0.085\ s^{-1}$) accounts for 60% of the overall change in u.v. absorbance. In an attempt to determine whether both of these processes represent deacylation, methanol was added. Whereas the fast phase responded linearly to methanol addition, the slower phase was not affected. This implies that the slower process does not represent deacylation and probably relates to ^2H exchange of the acyl-enzyme or to turbidity formation (if this is the correct explanation, the turbidity was too slight to be visible). That the u.v. absorbance of the free enzyme does not show any such change indicates that the slow phase is a property of the acyl-enzyme. Bender and co-workers found cinnamoyl-chymotrypsin to deacylate up to approx. 75% according to first-order kinetics in ^2H_2O with a normal isotope effect of 2.5 [21].

Measurements of the rate of deacylation of cinnamoyl-chymotrypsin in ^2H_2O by using FTIR

Preliminary experiments indicated that the spectroscopic bands assigned to carbonyl groups decay with similar rates when cinnamoyl-chymotrypsin deacylates [2]. We have measured the rate of deacylation using FTIR by following the decrease in the absorbance at 1695 and 1705 cm^{-1} at pH* 7.0 in acyl-enzyme minus enzyme spectra. A good first-order dependence was obtained at both frequencies regardless of the time for which the enzyme was preincubated with ^2H_2O. Examples of first-order plots for such a deacylation are shown in Fig. 6. The rate constants of these processes were determined as $(8.7 \pm 0.8) \times 10^{-4}\ s^{-1}$ when measured at 1705 cm^{-1} and $(8.3 \pm 0.5) \times 10^{-4}\ s^{-1}$ at 1695 cm^{-1}. Thus, within experimental error, the absorbances decay identically. The NP and P conformations are therefore in dynamic exchange on the reaction time-scale if it is assumed, as seems reasonable, that deacylation can occur only from the P conformation. These studies demonstrate that the deacylation of cinnamoyl-chymotrypsin measured by FTIR follows first-order kinetics in ^2H_2O. In view of the results presented above, we feel reasonably confident that no gross perturbations of the enzyme's mechanism or kinetics occur as a consequence of the use of ^2H_2O as solvent. It is possible that the distribution of conformations of bound ligands will be somewhat different as compared with that in water, but this is unlikely to affect the main conclusions that have been drawn concerning the nature of the conformations. Many n.m.r. studies of proteins make use of ^2H_2O as solvent, but, as far as we are aware, no serious problems are known to have arisen from this, although perhaps the full story may not yet have emerged, since a comparison with studies in water cannot easily be made.

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