Effect of specificity on ligand conformation in acyl-chymotrypsins

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I.r. difference spectroscopy combined with ^{13}C and ^{18}O double-isotope substitution was used to examine the ester acyl carbonyl stretching vibration of hydrocinnamoyl-chymotrypsin. A single acyl carbonyl stretching band was observed at 1731 cm^{-1} . This contrasts with previous i.r. and resonance Raman spectroscopic studies of a number of *trans*-3-arylacryloyl-chymotrypsins which showed two acyl carbonyl stretching bands in the region of 1700 cm^{-1} , which were proposed to represent productive and non-productive conformations of the acyl-enzyme. The single acyl carbonyl band for hydrocinnamoyl-chymotrypsin suggests only a single conformation, and the comparatively high frequency of this band implies little or no hydrogen-bonding to this carbonyl group. Enzymic hydrogen-bonding to the acyl carbonyl is believed to give bond polarization and thereby catalytic-rate acceleration. Thus, in view of the apparent lack of such hydrogen-bonding in hydrocinnamoyl-chymotrypsin, it should be the case that this acyl-chymotrypsin is less specific than *trans*-3-arylacryloyl-chymotrypsins, whereas the opposite is true. It is therefore proposed that there may be a productive acyl carbonyl

population of lower stretching frequency for hydrocinnamoyl-chymotrypsin, but that this is too small to be discerned because of either a relatively high deacylation rate or an unfavourable conformational equilibrium. The single acyl carbonyl band for hydrocinnamoyl-chymotrypsin is significantly broader than those for *trans*-3-arylacryloyl-chymotrypsins, indicating that this group is more conformationally mobile and dispersed in the former. This can be correlated with the absence of acyl carbonyl hydrogen-bonding in hydrocinnamoyl-chymotrypsin, and with the much greater flexibility of the saturated hydrocinnamoyl group than unsaturated *trans*-3-arylacryloyl. This flexibility is presumably the reason why hydrocinnamoyl-chymotrypsin is more specific than *trans*-3-arylacryloyl-chymotrypsins. Resonance Raman spectroscopy is limited to the non-specific *trans*-3-arylacryloyl-chymotrypsins because of its chromophoric requirement, whereas i.r. may be used to examine non-chromophoric more specific acyl-enzymes such as hydrocinnamoyl-chymotrypsin. The results presented in this paper suggest that *trans*-3-arylacryloyl-chymotrypsins are atypical.

INTRODUCTION

Chymotrypsin-catalysed hydrolysis of amides and esters proceeds through acylation of the enzyme. The unique ester acyl carbonyl of the acyl-enzyme is hydrogen-bonded via its oxygen lone pairs to two enzymic peptide amides. This interaction is called the oxyanion hole, and there is good evidence from structural (Steitz et al., 1969; Robertus et al., 1972; Alber et al., 1976), modelling and computational (Weiner et al., 1986) and site-directed mutagenesis (Bryan et al., 1986; Wells et al., 1986) studies for the involvement of this interaction in the generation of catalytic-rate enhancement. Although this has largely been interpreted in terms of stabilization of the oxyanionic transition state (Weiner et al., 1986; Wells and Estell, 1988), the contention has been that ground-state electronic strain in the form of acyl carbonyl polarization in the ground states is a further important factor (White and Wharton, 1990).

The stretching vibrational mode of the acyl carbonyl may be studied by both i.r. (Wharton, 1986) and resonance Raman (rR) (Carey and Tonge, 1990) spectroscopies. Analysis of such data ought to give information relating to the bond enthalpies of both the acyl carbonyl and the oxyanion hole hydrogen bonds, and should hence allow ground-state electronic strain to be demonstrated and quantified.

A number of *trans*-3-arylacryloyl-chymotrypsins (aryl- $\text{CH}=\text{CH}-\text{C}(=\text{O})-\text{O}-\text{Chy}$) (Figure 1a) have been examined using rR spectroscopy (Tonge and Carey, 1989, 1990, 1992). These include (3-nitro-4-amino)cinnamoyl-, 3-(5-methylthien-2-yl)acryloyl-, and 3-(indol-3-yl)acryloyl-chymotrypsins. These

acyl ligands are chromophoric with a λ_{max} at approx. 350 nm, whereas the enzyme has a λ_{max} at approx. 280 nm. Excitation at about 350 nm with suitable lasers thus gives selective resonance enhancement of ligand absorbances over enzymic absorbances. Such rR studies have proven quite effective, but have nevertheless been shown to be subject to an artefact caused by laser-induced *trans* to *cis* isomerization about the acrylic double bond (Tonge et al., 1991).

The *trans*-3-arylacryloyl-chymotrypsins given by cinnamoyl- ($\Phi-\text{CH}=\text{CH}-\text{C}(=\text{O})-\text{O}-\text{Chy}$), 3-(indol-3-yl)acryloyl- and 3-(5-methylthien-2-yl)acryloyl- have also been examined using i.r. spectroscopy (White and Wharton, 1990). Selection of ligand absorbances over enzyme absorbances was achieved here by employing difference spectra of acyl-enzyme minus free enzyme. These difference spectroscopic data have also been shown to be subject to an artefact, which arises from perturbation of enzymic amide and carboxyl groups on binding of ligand. These groups absorb in the same frequency region as the acyl carbonyl, and, when perturbed, will therefore contribute features to the acyl carbonyl group profile of the difference spectra (White et al., 1991, 1992). This perturbation problem was successfully overcome by isotopic substitution at the acyl carbonyl group of $^{13}\text{C}=^{16}\text{O}$ for $^{12}\text{C}=^{16}\text{O}$ and determination of $[^{13}\text{C}=^{16}\text{O}]\text{acyl-enzyme}$ minus $[^{13}\text{C}=^{16}\text{O}]\text{acyl-enzyme}$ i.r. difference spectra. As like is compared with like in these spectra, perturbation is thereby eliminated, and two acyl carbonyl profiles are observed, a positive profile for $^{12}\text{C}=^{16}\text{O}$ and a negative profile for $^{13}\text{C}=^{16}\text{O}$ seen some 37 cm^{-1} down frequency from the $^{12}\text{C}=^{16}\text{O}$ profile (White et al., 1991, 1992).

Abbreviations used: rR, resonance Raman; cm^{-1} , wavenumber; pH*, pH-meter reading in $^2\text{H}_2\text{O}$ using a glass combination electrode; FWHH, full band width at half maximum band height; ν_p , productive conformation acyl carbonyl stretching frequency; k_3 , acyl-chymotrypsin deacylation rate constant; Chy, chymotrypsin; Φ , phenyl; mAU, milli-absorbance unit.

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Figure 1 Structures of cinnamoyl- and hydrocinnamoyl-chymotrypsins

(a) Structure of cinnamoyl-chymotrypsin or *trans*-3-phenylacryloyl-chymotrypsin. Other *trans*-3-arylacryloyl-chymotrypsins are formed by substitution of another aromatic ring group for the phenyl. (b) Structure of hydrocinnamoyl-chymotrypsin.

According to rR studies, disregarding the band arising from laser-induced *trans* to *cis* isomerization, *trans*-3-arylacryloyl-chymotrypsins give two acyl carbonyl bands at approx. 1700 cm^{-1} with approx. $5\text{--}15\text{ cm}^{-1}$ separation (Tonge and Carey, 1989, 1990, 1992). The exception is 3-(indol-3-yl)acryloyl-chymotrypsin which gives only a single band at 1702 cm^{-1} . In i.r. studies, $^{12}\text{C}^{16}\text{O}$ - minus $^{13}\text{C}^{16}\text{O}$ -cinnamoyl-chymotrypsin spectra also give two acyl carbonyl bands at 1709 cm^{-1} and 1699 cm^{-1} (White et al., 1991, 1992).

The structure of 3-(indol-3-yl)acryloyl-chymotrypsin at pH 4.0 has been crystallographically determined (Henderson, 1970), and the acyl carbonyl observed not to be bound in the oxyanion hole but rather hydrogen-bonded to a water molecule, which is in turn hydrogen-bonded to $\epsilon\text{N-H}$ of the imidazolium of enzymic His-57. The acyl carbonyl hydrogen-bonding in this case is believed to be weaker than with the oxyanion hole, on grounds of order, distance and alignment, and furthermore, the acyl-carbonyl-group geometry is incorrect for deacylation. Thus, this structure of 3-(indol-3-yl)acryloyl-chymotrypsin may be conjectured to be non-productive because of the less strong acyl-carbonyl-group hydrogen-bonding and the incorrect geometry, and the oxyanion-hole-bound structure may conversely be considered productive.

The two acyl carbonyl bands observed with rR and i.r. spectroscopy for *trans*-3-arylacryloyl-chymotrypsins are thus respectively assigned to a productive 'oxyanion hole structure and a non-productive 'Henderson'-type structure (White and Wharton, 1990). Vibrational frequencies are known to be inversely related to hydrogen-bonding strength (Badger and Bauer, 1939; Abraham et al., 1989; White and Wharton, 1990), and so, for each *trans*-3-arylacryloyl-chymotrypsin, the lower-frequency acyl carbonyl band is assigned to the productive oxyanion-hole-bound structure and the higher frequency band to the non-productive 'Henderson' structure. It has proved possible to calculate acyl carbonyl bond enthalpies from acyl carbonyl stretching i.r. frequencies, and to relate this information to catalysis on the basis of ground-state electronic strain (White and Wharton, 1990). The exception to this assignment regime seems to be 3-(indol-3-yl)acryloyl-chymotrypsin which gives only a single acyl carbonyl band (Tonge and Carey, 1989) and a clearly defined non-productive crystallographic structure (Henderson, 1970). It is therefore proposed that 3-(indol-3-yl)acryloyl-chymotrypsin must be overwhelmingly present in the non-productive 'Henderson' form in solution as well as in the crystal, and, in keeping with this, 3-(indol-3-yl)acryloyl-chymotrypsin has a deacylation rate that is atypically low compared with other *trans*-3-arylacryloyl-chymotrypsins (White and Wharton, 1990).

The major advantage of i.r. over rR spectroscopy for these studies is that acyl ligands need not be chromophoric for i.r.

spectroscopy. It is hence possible to use i.r. spectroscopy to examine acyl-enzymes of non-chromophoric acyl ligands such as hydrocinnamoyl-chymotrypsin ($\Phi\text{-CH}_2\text{-CH}_2\text{-C(=O)-O-Chy}$) (White and Wharton, 1990; White et al., 1992) (Figure 1b). Such ligands are often considerably more specific than most *trans*-3-arylacryloyl ligands, especially when intrinsic reactivity is corrected for. The present paper reports i.r. difference spectra for hydrocinnamoyl-chymotrypsin obtained using a double-isotope substitution method. The 37 cm^{-1} isotopic frequency shift between $^{12}\text{C}^{16}\text{O}$ and $^{13}\text{C}^{16}\text{O}$ was sufficient for clear separation and definition of the acyl carbonyl profiles in $^{12}\text{C}^{16}\text{O}$ - minus $^{13}\text{C}^{16}\text{O}$ -cinnamoyl-chymotrypsin difference spectra, but is insufficient for clear separation and definition of the acyl carbonyl profiles in equivalent spectra for hydrocinnamoyl-chymotrypsin because of overlap and interference between the acyl carbonyl profiles (White et al., 1992). A further isotopic substitution to give $^{13}\text{C}^{18}\text{O}$ was therefore employed. The calculated isotopic frequency shift for the double-isotope substitution from $^{12}\text{C}^{16}\text{O}$ to $^{13}\text{C}^{18}\text{O}$ is 80 cm^{-1} , which is expected to be sufficient for clear separation and presentation of the acyl carbonyl profiles in $^{12}\text{C}^{16}\text{O}$ - minus $^{13}\text{C}^{18}\text{O}$ -hydrocinnamoyl-chymotrypsin difference spectra.

EXPERIMENTAL

Materials

H_2^{18}O (95 % enriched) was obtained from Aldrich Chemical Co., and Type II α -chymotrypsin (lot 71H7110) was obtained from Sigma Chemical Co. $^{13}\text{C}^{16}\text{O}$ Hydrocinnamic acid was synthesized as outlined in White et al. (1992).

Synthesis of $^{13}\text{C}^{18}\text{O}$ hydrocinnamoyl-imidazole

$^{13}\text{C}^{16}\text{O}$ Hydrocinnamic acid (83 mg) was incubated in an air-tight container at 37°C with H_2^{18}O (2.5 ml), dried acetonitrile (1.25 ml) and 10 M HCl (35 μl in H_2^{16}O) for 1 week. This allowed exchange of ^{16}O for ^{18}O atoms in the carboxylic acid group. Solvent was then removed by evaporation under a stream of dry nitrogen, and $^{13}\text{C}^{18}\text{O}$ hydrocinnamoyl-imidazole was synthesized as outlined in White et al. (1992).

The $^{13}\text{C}^{18}\text{O}$ hydrocinnamoyl-imidazole product gave only one spot on t.l.c. corresponding to a $^{12}\text{C}^{16}\text{O}$ hydrocinnamoyl-imidazole reference spot. The $^{13}\text{C}^{18}\text{O}$ hydrocinnamoyl-imidazole was also shown to acylate chymotrypsin, as measured by active-site titration (Schonbaum et al., 1961), to the same extent as $^{12}\text{C}^{16}\text{O}$ hydrocinnamoyl-imidazole.

Incorporation of ^{18}O at the carbonyl oxygen of hydrocinnamoyl-imidazole was confirmed by i.r. spectroscopy (Figure 2a). The carbonyl region of the $^{13}\text{C}^{18}\text{O}$ hydrocinnamoyl-imidazole showed two bands, at 1691 cm^{-1} and 1656 cm^{-1} . The less intense 1691 cm^{-1} band was assigned to $^{13}\text{C}^{16}\text{O}$ hydrocinnamoyl-imidazole, and the much more intense 1656 cm^{-1} band was assigned to $^{13}\text{C}^{18}\text{O}$ hydrocinnamoyl-imidazole. Comparison with a spectrum of $^{12}\text{C}^{16}\text{O}$ hydrocinnamoyl-imidazole gives a nearly matching fingerprint region suggesting structural identity. Comparison of the carbonyl profiles shows an isotopic frequency shift of 75 cm^{-1} between the $^{12}\text{C}^{16}\text{O}$ and $^{13}\text{C}^{18}\text{O}$ bands, which is close to the calculated value of 80 cm^{-1} . Both spectra show the minor carbonyl band at about 1690 cm^{-1} for $^{13}\text{C}^{16}\text{O}$ hydrocinnamoyl-imidazole. In the case of the $^{12}\text{C}^{16}\text{O}$ hydrocinnamoyl-imidazole spectrum this is due to the natural abundance of ^{13}C .

Mass spectroscopy also verified the isotopic substitution. Owing to lability of the imidazole esters, useful mass spectra of hydrocinnamoyl-imidazoles could not be collected. However,

Figure 2 Confirmation of $^{16}\text{O}/^{18}\text{O}$ exchange

(a) I.r. spectra of $^{12}\text{C}=^{16}\text{O}$ hydrocinnamoyl-imidazole (—) and $^{13}\text{C}=^{18}\text{O}$ hydrocinnamoyl-imidazole (---) in KBr discs collected at 2 cm^{-1} resolution using 64 scans. (b) Mass spectrum of hydrocinnamic acid after $^{16}\text{O}/^{18}\text{O}$ exchange.

mass spectra of the acid product showed 78% abundance of $^{13}\text{C}=^{18}\text{O}$, $^{13}\text{C}-^{18}\text{OH}$ hydrocinnamic acid and 12% abundance of $^{13}\text{C}=^{18}\text{O}$, $^{13}\text{C}-^{16}\text{OH}/^{13}\text{C}=^{16}\text{O}$, $^{13}\text{C}-^{18}\text{OH}$ hydrocinnamic acid (Figure 2b). An abundance of $^{13}\text{C}=^{18}\text{O}$ hydrocinnamoyl-imidazole of 84% is thus predicted theoretically provided that ^{18}O does not discriminate between the double and single bond of the carboxylic group.

I.r. spectroscopy

Hydrocinnamoyl-chymotrypsin was synthesized and i.r. difference spectra were collected as described in White and Wharton (1990) and White et al. (1992).

RESULTS

Difference i.r. spectra at pH* (pH-meter reading in $^2\text{H}_2\text{O}$) 4.0 and 6.0 were collected (Figures 3 and 4). At pH* 4.0 (Figure 3), the $^{12}\text{C}=^{16}\text{O}$ hydrocinnamoyl-chymotrypsin minus chymotrypsin i.r. difference spectrum shows three positive bands in the acyl carbonyl region, two broader bands at 1726 cm^{-1} and 1704 cm^{-1} , and a sharper band at 1692 cm^{-1} . The corresponding $^{13}\text{C}=^{18}\text{O}$ hydrocinnamoyl-chymotrypsin minus chymotrypsin i.r. difference spectrum shows a broader negative band at 1736 cm^{-1} , a broader positive band at 1703 cm^{-1} and a sharper positive band at 1692 cm^{-1} . The $^{13}\text{C}=^{18}\text{O}$ acyl carbonyl stretching absorbance can be seen at about 1650 cm^{-1} . Exact band locations and

Figure 3 I.r. difference spectra of hydrocinnamoyl-chymotrypsin at pH* 4.0

(a) I.r. difference spectra in $^2\text{H}_2\text{O}$ at pH* 4.0 of $^{12}\text{C}=^{16}\text{O}$ hydrocinnamoyl-chymotrypsin minus free chymotrypsin (—) and $^{13}\text{C}=^{18}\text{O}$ hydrocinnamoyl-chymotrypsin minus free chymotrypsin (---). (b) I.r. difference spectrum in $^2\text{H}_2\text{O}$ at pH* 4.0 of $^{12}\text{C}=^{16}\text{O}$ hydrocinnamoyl-chymotrypsin minus $^{13}\text{C}=^{18}\text{O}$ hydrocinnamoyl-chymotrypsin. All spectra were collected at 2 cm^{-1} resolution using 512 scans and a $50\text{ }\mu\text{m}$ pathlength transmission cell with CaF_2 windows. The proportion of the chymotrypsin that was acylated, as measured by active-site titration (Schonbaum et al., 1961), was over 94% at the time of data collection.

characteristics are difficult to determine for the latter because of the presence of enzymic amide perturbation features in this region. The $^{12}\text{C}=^{16}\text{O}$ - minus $^{13}\text{C}=^{18}\text{O}$ -hydrocinnamoyl-chymotrypsin i.r. difference spectrum shows a single positive band in the $^{12}\text{C}=^{16}\text{O}$ acyl carbonyl region at 1731 cm^{-1} with a full width of half height (FWHH) of 20 cm^{-1} . This band is essentially symmetrical, reinforcing the assignment as a single band. A single negative band in the $^{13}\text{C}=^{18}\text{O}$ acyl carbonyl region is present at 1650 cm^{-1} with a FWHH of 12 cm^{-1} .

At pH* 6.0 (Figure 4), the signal-to-noise ratio decreases. The $^{12}\text{C}=^{16}\text{O}$ hydrocinnamoyl-chymotrypsin minus chymotrypsin i.r. difference spectrum again shows three positive bands at 1731 cm^{-1} , 1705 cm^{-1} and 1692 cm^{-1} . However, the $^{13}\text{C}=^{18}\text{O}$ hydrocinnamoyl-chymotrypsin minus chymotrypsin i.r. difference spectrum only shows two features in the $^{12}\text{C}=^{16}\text{O}$ acyl carbonyl region, a broader positive band at 1702 cm^{-1} and sharper positive band at 1692 cm^{-1} . The negative band at 1736 cm^{-1} present in the $^{13}\text{C}=^{18}\text{O}$ hydrocinnamoyl-chymotrypsin minus chymotrypsin spectrum at pH* 4.0 is absent. This supports previous claims (White et al., 1992) that this negative band arises as a result of the absorbance of a carboxylic group which ionizes on going from pH* 4 to 6. The $^{13}\text{C}=^{18}\text{O}$ acyl carbonyl absorbance again manifests itself as increased absorbance at about 1650 cm^{-1} . The $^{12}\text{C}=^{16}\text{O}$ - minus $^{13}\text{C}=^{18}\text{O}$ -hydrocinnamoyl-chymotrypsin spectrum again shows two bands, an essentially symmetrical positive band at 1731 cm^{-1} and a negative band at 1650 cm^{-1} , with respective FWHH of 20 cm^{-1} and 12 cm^{-1} . The latter band

Figure 4 I.r. difference spectra of hydrocinnamoyl-chymotrypsin at pH* 6.0

(a) I.r. difference spectra in $^2\text{H}_2\text{O}$ at pH* 6.0 of $^{12}\text{C}=^{16}\text{O}$ hydrocinnamoyl-chymotrypsin minus free chymotrypsin (—) and $^{13}\text{C}=^{18}\text{O}$ hydrocinnamoyl-chymotrypsin minus free chymotrypsin (---). (b) I.r. difference spectrum in $^2\text{H}_2\text{O}$ at pH* 6.0 of $^{12}\text{C}=^{16}\text{O}$ hydrocinnamoyl-chymotrypsin minus $^{13}\text{C}=^{18}\text{O}$ hydrocinnamoyl-chymotrypsin. All spectra were collected at 2 cm^{-1} resolution using 64 scans and a $50\text{ }\mu\text{m}$ pathlength transmission cell with CaF_2 windows. The proportion of the chymotrypsin that was acylated, as measured by active-site titration (Schonbaum et al., 1961), was over 80% at the time of data collection.

is subject to increased noise because of difficulties in subtraction of the enzymic amide absorbance in that frequency region.

DISCUSSION

The double-isotope substitution technique has permitted thorough analysis of hydrocinnamoyl-chymotrypsin by i.r. difference spectroscopy. Normal spectra of $^{12}\text{C}=^{16}\text{O}$ hydrocinnamoyl-chymotrypsin minus chymotrypsin (Figures 3a and 4a, solid lines), i.e. with non-substituted acyl-enzyme, give three bands, at about 1731 cm^{-1} , about 1705 cm^{-1} and 1692 cm^{-1} . These have previously been assigned to acyl carbonyl (White and Wharton, 1990). However, this spectral profile could also potentially include contributions arising from perturbation of enzymic groups between free enzyme and acyl-enzyme (Tonge et al., 1991; White et al., 1991, 1992). Spectra of substituted $^{13}\text{C}=^{18}\text{O}$ hydrocinnamoyl-chymotrypsin minus chymotrypsin (Figures 3a and 4a, dashed lines) should disclose any enzymic perturbation features in the approx. 1700 cm^{-1} region, as acyl carbonyl bands in this region will be shifted down about 80 cm^{-1} to about 1620 cm^{-1} by the isotope editing. The spectra obtained in this study show that both the approx. 1705 cm^{-1} band and the 1692 cm^{-1} band of the normal $^{12}\text{C}=^{16}\text{O}$ hydrocinnamoyl-chymotrypsin minus chymotrypsin spectra clearly correspond to enzymic perturbation features and hence not, as formerly

thought, to acyl carbonyl absorbance. These substituted spectra also show a negative enzymic perturbation feature at approx. 1736 cm^{-1} , at lower pH*. This feature was observed at lower pH* in acyl-enzyme minus enzyme spectra for all of the *trans*-3-arylacryloyl-chymotrypsins (White and Wharton, 1990; Tonge et al., 1991; White et al., 1991, 1992), but was not previously observed for hydrocinnamoyl-chymotrypsin (White and Wharton, 1990). The origin of these enzymic perturbation features has been ascribed to perturbation of enzymic carboxyl group(s) for those perturbation effects evident only at lower pH*, and to perturbation of enzymic amide group(s) for those perturbation effects evident over the whole pH* range studied (White and Wharton, 1990; White et al., 1992).

Spectra of normal $^{12}\text{C}=^{16}\text{O}$ hydrocinnamoyl-chymotrypsin minus substituted $^{13}\text{C}=^{18}\text{O}$ hydrocinnamoyl-chymotrypsin (Figures 3b and 4b) should show only acyl carbonyl bands, unadulterated by enzymic perturbation contribution, as the only difference between the two components of this subtraction is the isotope editing of the acyl carbonyl group. In such spectra, one would expect to observe a positive set of bands for normal $^{12}\text{C}=^{16}\text{O}$ acyl carbonyl and a corresponding negative set of bands for substituted $^{13}\text{C}=^{18}\text{O}$ acyl carbonyl, approx. 80 cm^{-1} down frequency. Examination of these spectra for hydrocinnamoyl-chymotrypsin, at both lower and higher pH*, obtained in this study, seems to clearly show a single $^{12}\text{C}=^{16}\text{O}$ acyl carbonyl band at 1731 cm^{-1} . This corresponds to the approx. 1731 cm^{-1} band of the normal $^{12}\text{C}=^{16}\text{O}$ hydrocinnamoyl-chymotrypsin minus chymotrypsin spectra. There is a single negative band at approx. 1650 cm^{-1} , which is about 80 cm^{-1} down frequency from the 1731 cm^{-1} band, and is assigned to $^{13}\text{C}=^{18}\text{O}$ acyl carbonyl. The 1731 cm^{-1} acyl carbonyl band is fairly broad with a FWHH of 20 cm^{-1} , but is also quite symmetrical, which verifies that this band profile is unlikely to include more than one band. This is further reinforced by the narrower profile of the $^{13}\text{C}=^{18}\text{O}$ acyl carbonyl band. For normal $^{12}\text{C}=^{16}\text{O}$ hydrocinnamoyl-chymotrypsin minus chymotrypsin spectra, the single broad acyl carbonyl band at 1731 cm^{-1} will heavily overlap with the broad negative enzymic perturbation feature present at about 1736 cm^{-1} at lower pH*, and these two features will extensively cancel one another. This cancellation explains therefore both the absence of the approx. 1736 cm^{-1} negative feature and the reduction in intensity of the 1731 cm^{-1} band at lower pH* in the normal $^{12}\text{C}=^{16}\text{O}$ hydrocinnamoyl-chymotrypsin minus chymotrypsin spectra (White and Wharton, 1990).

Summarizing therefore, the three bands in the approx. 1700 cm^{-1} region of the normal $^{12}\text{C}=^{16}\text{O}$ hydrocinnamoyl-chymotrypsin minus chymotrypsin spectra (about 1731 cm^{-1} , about 1705 cm^{-1} and 1692 cm^{-1}) may be assigned as follows. The 1731 cm^{-1} band is acyl carbonyl stretching, and is the only such band. The approx. 1705 cm^{-1} and 1692 cm^{-1} bands are both enzymic perturbation features. The 1731 cm^{-1} acyl carbonyl band is cancelled at lower pH* by a negative enzymic perturbation feature at about 1736 cm^{-1} .

This i.r. study of hydrocinnamoyl-chymotrypsin, made possible by the double-isotope substitution technique, clearly then shows a single acyl carbonyl band and thence a single conformation for the ester acyl carbonyl group of hydrocinnamoyl-chymotrypsin, in contrast with the two bands and two conformations seen for *trans*-3-arylacryloyl-chymotrypsins. The observation of a single conformation has very important repercussions for analyses previously applied to this sort of vibrational spectroscopic data where the two acyl carbonyl conformations were rationalized in terms of non-productive and productive conformations.

Figure 5 Linear regression line (—) calculated from the data of Tonge and Carey (1992) with original data points not shown and the limits of 1 S.D. indicated (---)

The points for hydrocinnamoyl-chymotrypsin (○) (Results section; White and Wharton, 1990) and cinnamoyl-chymotrypsin (△) (White et al., 1992; White and Wharton, 1990) are plotted but are not included in the regression calculation.

The original analysis of White and Wharton (1990) was based on the application of vibrational theory to estimate the acyl carbonyl bond enthalpy from the acyl carbonyl stretching frequency. Unfortunately, this analysis was constructed with respect to a comparison of the different conformations for each acyl-chymotrypsin, and obviously cannot be utilized in this way for a single conformation of hydrocinnamoyl-chymotrypsin. Nevertheless, the basic principle of this analysis is sound, and ought to be applicable to the single-conformation case through adoption of an amended analytical strategy.

The later analysis of Tonge and Carey (1992) was based simply on a proposed inverse linear relationship between the productive acyl carbonyl band frequencies (ν_p) and the logarithms of acyl-chymotrypsin deacylation rates ($\log_{10} k_3$). This analysis should be applicable to all acyl-chymotrypsins if the productive frequency is used where there are two bands and the frequency of the single band is used where there is one band, as in the case of hydrocinnamoyl-chymotrypsin, i.e. considering the single band to represent the productive conformation. According to Tonge and Carey (1992), the greater the k_3 , the lower the ν_p , this demonstrating ground-state electronic strain, in accordance with the proposals of White and Wharton (1990). Utilizing ν_p values from isotopic substitution i.r. data and corresponding values of $\log_{10} k_3$ gives $\nu_p = 1699 \text{ cm}^{-1}$ (White et al., 1992) and $\log_{10} k_3 = -1.7$ (White and Wharton, 1990) for cinnamoyl-chymotrypsin, and, $\nu_p = 1731 \text{ cm}^{-1}$ (from the Results section) and $\log_{10} k_3 = -0.6$ (White and Wharton, 1990) for hydrocinnamoyl-chymotrypsin. The values for cinnamoyl-chymotrypsin give a point close to the linear regression line of Tonge and Carey (1992), but the values for hydrocinnamoyl-chymotrypsin give a point that clearly and strikingly does not correspond at all to the reported trend (Tonge and Carey, 1992) (Figure 5). The term $R_k = 1 + \log_{10}(k_3/k_{OH})$, where k_{OH} is the rate of hydrolysis for the corresponding acyl-imidazole at pH 10.5, accounts for both catalytic and intrinsic reactivities (White and Wharton, 1990). R_k is 0.8 for cinnamoyl-chymotrypsin and 3.2 for hydrocinnamoyl-chymotrypsin (White and Wharton, 1990). Thus, even with the corrected R_k term, the ν_p value of 1731 cm^{-1} for hydrocinnamoyl-chymotrypsin is contrary to the expected trend of increased deacylation rate correlating with decreased ν_p .

The ν_p , $\log_{10} k_3$ and R_k data for hydrocinnamoyl-chymotrypsin conspicuously violate therefore the trend proposed for *trans*-3-arylacryloyl-chymotrypsins from both rR and isotopic-substitution i.r. spectroscopic evidence. The ν_p value for hydrocinnamoyl-chymotrypsin of 1731 cm^{-1} may be assessed with respect to previous studies of methyl hydrocinnamate in a range of solvents (White and Wharton, 1990). On this basis, the apparent dielectric constant experienced by the acyl carbonyl of hydrocinnamoyl-chymotrypsin may be estimated as 40, which is roughly that for methyl hydrocinnamate in acetonitrile. In other words, the ν_p for hydrocinnamoyl-chymotrypsin corresponds to acyl carbonyl in a relatively non-polar environment, one where there is quite possibly little or no hydrogen-bonding to that acyl carbonyl. This is contrary to expectations where the elevated $\log_{10} k_3$ and R_k for hydrocinnamoyl-chymotrypsin should be associated with stronger oxyanion-hole hydrogen-bonding and thus a higher apparent dielectric constant and a lower ν_p , this stronger oxyanion-hole hydrogen-bonding would be expected to provide greater ground-state electronic strain and hence greater rate acceleration.

The implications of this apparent incongruity for hydrocinnamoyl-chymotrypsin are not clear. Either the notion of the oxyanion-hole and associated ground-state electronic strain is brought into doubt or else there is a productive acyl carbonyl population with a lower stretching frequency which is present but is too small to generate a significant band in these i.r. spectra. It could thus be that most of the hydrocinnamoyl-chymotrypsin is in a non-productive conformation in which there is little or no hydrogen-bonding, with a small amount of productive oxyanion-hole conformation present. There are two plausible models that could explain this situation. Firstly, that the deacylation rate from the productive conformation is fast compared with the rate of exchange between the two conformations, leading to depletion of the productive conformation. Secondly, that the conformation exchange rate is fast compared with the deacylation rate but the conformational equilibrium strongly disfavors the productive conformation. Distinction between these two alternative models is not possible at present, but as the hydrocinnamoyl-chymotrypsin deacylation half-life at pH* 6.0 is about 1 min in $^2\text{H}_2\text{O}$, the conformation exchange rate would have to be very slow for the first model to apply.

At both pH* 4.0 and 6.0, His-57 will predominantly be in the ionized imidazolium form. It may be that at higher pH* at which His-57 becomes deprotonated to imidazole, and the enzyme becomes fully active, the productive conformation may accumulate in larger amounts and so be observable in the i.r. experiments. Attempts to collect difference spectra at pH* 8.0 using a stopped-flow and rapid spectral scanning methodology have so far proved unsuccessful owing to the reduced half-life of the acyl-enzyme.

The single acyl carbonyl band observed for hydrocinnamoyl-chymotrypsin at 1731 cm^{-1} is broader than either of the pair of acyl carbonyl bands for cinnamoyl-chymotrypsin at 1709 cm^{-1} and 1699 cm^{-1} by about 2-fold with respect to the former non-productive band and by about 3-fold with respect to the latter productive band (White et al., 1991, 1992), although the width of the overall acyl carbonyl profile is similar. The width of bands in vibrational spectra may be directly, albeit crudely, correlated with the conformational mobility and dispersion of the absorbing moiety. It therefore seems that the acyl carbonyl of the single conformation of hydrocinnamoyl-chymotrypsin has a greater degree of mobility and dispersion than the acyl carbonyls of either of the conformations for cinnamoyl-chymotrypsin. This is as might be expected given the notion that the non-productive acyl carbonyl band of hydrocinnamoyl-chymotrypsin represents

a conformation with little or no hydrogen-bonding to the acyl carbonyl and the acyl carbonyl bands of cinnamoyl-chymotrypsin represent conformations with hydrogen bonds to the acyl carbonyl which would restrict mobility and dispersion. Also, the ligand structure of hydrocinnamoyl-chymotrypsin is saturated with free C–C rotation and the ligand structure of cinnamoyl-chymotrypsin is α,β -unsaturated with C–C rotation strictly limited by extensive π conjugation. The open-chain flexibility of the hydrocinnamoyl group compared with the rigid planarity of the *trans*-3-arylacryloyl groups is presumably a major factor in giving the greater specificity of the former than the latter, and also in giving only one acyl carbonyl conformation in hydrocinnamoyl-chymotrypsin and two acyl carbonyl conformations in cinnamoyl-chymotrypsin. *S-cis* and *S-trans* rotational isomerism in the *trans*-3-arylacryloyl ligands may have a crucial role in the generation of two acyl carbonyl conformations for acyl-enzymes with these ligands. It is worth noting that in all i.r. spectra collected the $^{13}\text{C}=^{18}\text{O}$ bands were always approximately half the width of the $^{12}\text{C}=^{16}\text{O}$ bands. As this is true for both the acyl-enzyme difference spectra and the free ligand spectra (see the Results section), one may conclude that this band narrowing is a direct consequence of the double-isotope substitution itself.

One interesting idea is to project the value of $\log_{10} k_3$ for hydrocinnamoyl-chymotrypsin on to the linear regression line of the Tonge and Carey (1992) $\log_{10} k_3$ versus ν_p plot (Figure 5), in order to obtain a corresponding estimate of ν_p for hydrocinnamoyl-chymotrypsin calculated on the basis of the regression. The ν_p estimate for hydrocinnamoyl-chymotrypsin obtained using this procedure is 1672 cm^{-1} . Closer scrutiny of the normal [$^{12}\text{C}=^{16}\text{O}$]hydrocinnamoyl-chymotrypsin minus substituted [$^{13}\text{C}=^{18}\text{O}$]hydrocinnamoyl-chymotrypsin spectra at pH* 4.0 and 6.0 obtained in this study (Figures 3b and 4b) does indeed show some evidence for a small positive feature at about 1670 cm^{-1} . However, there are a number of important observations to be made regarding this apparent feature. Firstly, it is rather small with an intensity of about 1 mAU. It appears in only about half of the spectra collected overall (results not shown). It does not seem to give a corresponding negative [$^{13}\text{C}=^{18}\text{O}$] band at approx. 1590 cm^{-1} (results not shown). It is quite close to, and so subject to interference from, the approx. 1650 cm^{-1} negative [$^{13}\text{C}=^{18}\text{O}$]acyl carbonyl band. It is in the region of raised noise in the difference spectrum arising as a result of intense underlying enzymic amide I absorbance. It is in a position corresponding to the site of an amide perturbation feature and will thus be particularly sensitive to slight imbalances in enzymic concentration. As a result of these factors, the signal-to-noise ratio of this potential band is low, low enough in fact to make even assignment as a genuine band dubious. Moreover, it is probable that the approx. 1670 cm^{-1} feature, if indeed genuine, arises from imbalance with respect to the enzymic amide I absorbance that underlies the subtraction in this region. Nevertheless, the idea of this feature being the productive acyl carbonyl band certainly cannot be ignored without further investigation.

There is, then, the intriguing but apparently slight possibility that the small approx. 1670 cm^{-1} potential feature could represent the acyl carbonyl stretching band of a productive oxyanion-hole conformation for hydrocinnamoyl-chymotrypsin. This conformation would be sparsely populated, as discussed earlier. It must be stressed that this situation, even if true, is very different from that of the *trans*-3-arylacryloyl-chymotrypsins. In the latter case, there are two approximately equalled sized narrow acyl carbonyl bands at about 1700 cm^{-1} with approx. 10 cm^{-1} separation, whereas in the hydrocinnamoyl-chymotrypsin case being considered here, there are two acyl carbonyl bands, with one (at 1731 cm^{-1}) being much larger than the other. They are quite

broad, and one band is found at 1731 cm^{-1} and the other at 1670 cm^{-1} with about 60 cm^{-1} separation.

One may summarize as follows. Studies of acyl-chymotrypsins by rR are restricted by the u.v./visible chromophoric requirement to non-specific *trans*-3-arylacryloyl-chymotrypsins. These give two conformational populations with respect to the acyl carbonyl group, which can also be examined and evaluated by difference i.r. spectroscopy with single-isotope substitution. Moreover, i.r. difference spectroscopy can, in principle, be used to examine any acyl-chymotrypsin, as there is no chromophoric necessity for this method. Isotopic substitution in the ligand is, however, nearly always essential to ensure elimination of enzymic perturbation contributions to the i.r. difference spectra. The more specific hydrocinnamoyl-chymotrypsin has been studied here by i.r. difference spectroscopy using a double-isotope substitution in order to allow clear definition of the acyl carbonyl profile, and only a single population with respect to the acyl carbonyl was observed at both of two different pH* values. This particular acyl carbonyl population, on the basis of its stretching band frequency and width, appears to be subject to little or no hydrogen-bonding. An oxyanion-hole-bound productive acyl carbonyl group population is proposed which is too small to be discerned in these i.r. spectra because of either a relatively fast deacylation rate or an unfavourable conformation equilibrium. This overall description requires extension and reinforcement through examination of further more specific acyl-chymotrypsins, and given the inadequacy of rR for the task, the only suitable means of achieving this will be i.r. difference spectroscopy with double-isotope substitution.

A final point is that although rR spectroscopy does seem to have reached its limit as far as study of acyl-chymotrypsins is concerned, it may be possible to apply Raman spectroscopy without the resonance effect and thus without the chromophoric requirement. This can be achieved through difference Raman spectroscopy employing intense laser sources and highly sensitive multichannel detectors. Amide groups give a much weaker signal in Raman than i.r., and so perturbations are somewhat less of a problem. Nevertheless, isotopic substitution is still necessary. Highly significant advances in such Raman difference techniques for the study of enzyme–ligand complexes have been made by Callender and co-workers (Deng et al., 1989, 1992a,b; Yue et al., 1989).

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