Hydrogen-Bonding in 2-Aminobenzoyl-α-chymotrypsin Formed by Acylation of the Enzyme with Isatoic Anhydride: IR and Mass Spectroscopic Studies

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The acyl-enzyme formed upon acylation of α-chymotrypsin with isatoic anhydride has been characterised by infrared spectroscopy. Acylation at pH 7 to yield the 2-aminobenzoyl-enzyme is rapid ($k = 5.57 \times 10^{-2}\ s^{-1}$), while deacylation is much slower ($k = 3.7 \times 10^{-5}\ s^{-1}$). The [^{13}C=O]-labelled form of isatoic anhydride has been synthesised, to allow construction of [^{12}C=O]- minus [^{13}C=O]-difference spectra; these highlight the carbonyl absorbance of the ligand and eliminate spectral effects that arise from protein perturbation. The ester carbonyl band of the acyl-enzyme absorbs at a wavenumber of 1695 cm^{-1} and has been shown by deconvolution analysis to represent a single, well-defined conformation. Model studies of ethyl 2-aminobenzoate in a range of solvents show that its carbonyl group is in a hexane-like environment (that is, very nonpolar). It is proposed that the low wavenumber of the carbonyl absorbance arises from the presence of an internal hydrogen bond between the 2-amino group and the ester carbonyl oxygen; this leads to polarisation of the carbonyl group both in the enzyme and in nonpolar solvents. However, in view of the slow deacylation, it is clear that the acyl group is in a nonproductive conformation, with no interaction with the oxyanion hole, and that deacylation occurs from this form or from a minor, invisible form. The infrared data have been supported by kinetic electrospray mass spectroscopic measurements, which demonstrate that the acyl-enzyme is that previously anticipated, and by molecular modelling of 2-aminobenzoyl-α-chymotrypsin. It is concluded from pH-dependence measurements that general base catalysis by the 2-amino group is not involved in deacylation.

KEYWORDS:

acylation · chymotrypsin · enzymes · hydrogen bonds

Introduction

The serine proteinase, α-chymotrypsin (α-CT), is one of the most studied and best understood of all enzymes. Many of the ways in which it achieves a catalytic rate enhancement have been described. It has been comprehensively reviewed[1–3] and this knowledge about its structure and mechanism makes it an important model enzyme for mechanistic studies. Structural studies, especially the elucidation of the crystal structure,[4] backed up by many years of kinetic investigation have shown that α-CT achieves catalysis by an exquisitely fine-tuned acyl-enzyme mechanism.[1, 5] It was shown by Moorman and Abeles that α-CT can be efficiently acylated by isatoic anhydride, in a stoichiometric fashion, to form a stable acyl-enzyme, which is inactive against the substrate;[6] the mechanism is shown in Scheme 1. The importance of studying isatoic anhydride as an acylating agent is that it also acylates some serine-containing β-lactamases,[7] a very important group of enzymes involved in bacterial resistance. These enzymes confer resistance on bacteria by hydrolysing β-lactam antibiotics, preventing their action on the target molecules. The two groups of enzymes share some mechanistic similarities,[8, 9] and for this reason α-CT represents a good model for the β-lactamases, since its mechanism is, by comparison, relatively simple and well understood. The action of the serine proteinases occurs through an acyl-enzyme intermediate[10, 11] as does that of the β-lactamases.[12–13] In both enzymes there is an extensive hydrogen-bonding network with general base activity attached to the serine residue, which acts as the attacking nucleophile,[1, 14] and both contain an oxyanion hole.[15, 16] For these reasons it is pertinent to investigate α-CT as a putative model for some aspects of β-lactamase catalysis.

Isatoic anhydride is a possible precursor to the design of an inhibitor of the β-lactamases. However, it is too reactive and insufficiently specific to be useful as it stands. Investigation of the interactions of this compound with α-CT, to produce a stable acyl-enzyme, might indicate ways in which the compound can

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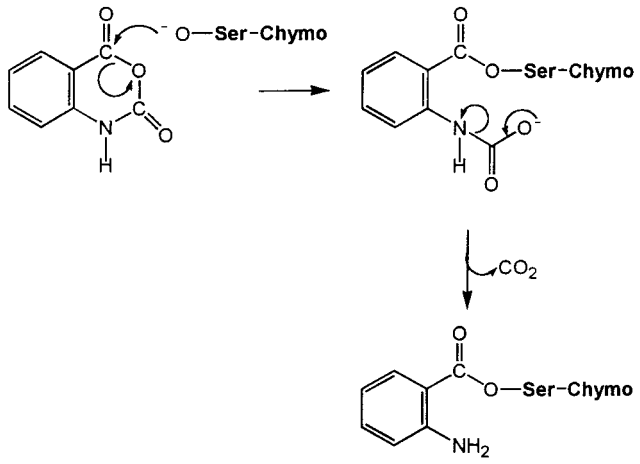

Scheme 1. *The mechanism of the reaction between isatoic anhydride and α-chymotrypsin as proposed by Moorman and Abeles.[6] The reaction produces a stable acyl-enzyme that subsequently deacylates to release 2-aminobenzoate at a rate of 3.76(±0.87) × 10⁻⁵ s⁻¹ at pH 7 and 37 °C. Chymo = chymotrypsin.*

be tailored to afford a more effective, better-targeted and clinically useful inhibitor of the β-lactamases. It is also interesting to study isatoic anhydride by infrared (IR) spectroscopy because of the relative ease of synthesis of the [^{13}C=O]-labelled form, compared with the considerable difficulty involved in the synthesis of such labelled forms of β-lactam antibiotics. Labelling of isatoic anhydride in this way facilitates the assignment of the ester carbonyl group and allows isotope-edited infrared difference spectroscopy.

Upon acylation of α-CT by isatoic anhydride, a unique ester carbonyl bond forms between the ligand and the enzyme (Scheme 1). This unique ester carbonyl group can be observed directly by infrared spectroscopy, because it absorbs at a characteristic frequency away from strong protein absorbances and arises from a local stretching mode that absorbs strongly and as a well-defined band. Measurement of the frequency of the ester carbonyl band in a number of acyl-enzymes has been shown to provide useful information on the conformation of the ligand and its interaction with the oxyanion hole of the enzyme.[17]

To complement the infrared spectroscopic study, this reaction between isatoic anhydride and α-CT has also been studied by electrospray ionisation mass spectrometry (ESIMS). The use of ESIMS allows observation of the mass increase of the enzyme upon formation of the acyl-enzyme and gives confidence that the acyl-enzyme formed is the one expected. It also shows that there are no extra adducts formed with the enzyme through nonspecific reactions with residues other than the catalytic serine 195 moiety. ESIMS is a powerful technique for verifying the identity of species within a reaction and confirming the proposed mechanism.

This paper presents an infrared spectroscopic study of the acyl-enzyme formed upon acylation of α-chymotrypsin by isatoic anhydride. In conjunction with data obtained by other techniques, it is suggested that hydrogen bonding within the ligand together with poor binding of the ligand in the active site results in the low deacylation rate.

Results and Discussion

Electrospray ionisation mass spectrometry

In an attempt to confirm the structure of the acyl-enzyme species (that is, to confirm that decarboxylation has occurred in the first observable species), and that the mechanism is that first hypothesised by Moorman and Abeles,[6] ESIMS was carried out. The data (see Figure 1) show the reaction process in a clear qualitative fashion. It can be seen that the peak at 25 233 Da for the free enzyme,[18] decreases and the peak at 25 352 Da increases. This shows that the acylated species is the one expected (Scheme 1), since the mass difference is exactly that of the 2-aminobenzoyl moiety (120 Da). There is no evidence of a species containing a carboxy group attached to the amine of the

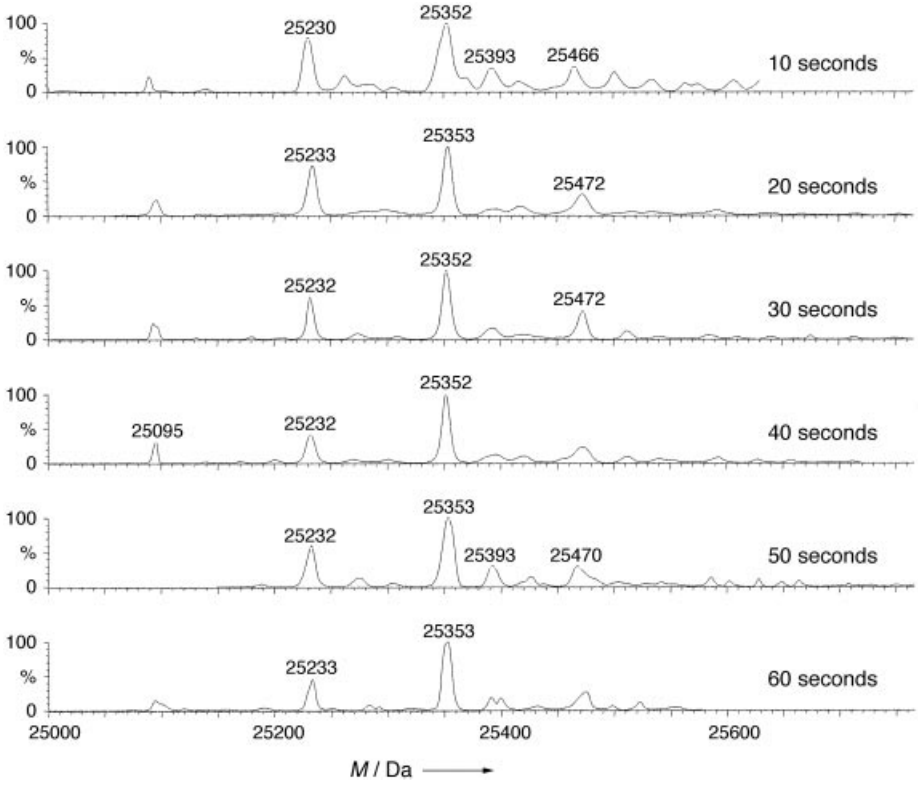

Figure 1. *Electrospray ionisation mass spectra of the acylation of α-chymotrypsin by isatoic anhydride at pH 7 and 37 °C. The spectra were determined on samples quenched at various time intervals (shown on the right-hand side) after addition of isatoic anhydride to α-chymotrypsin.*

2-aminobenzoyl moiety. Decarboxylation of the ligand with concomitant release of carbon dioxide occurs rapidly relative to the development of the stable acyl-enzyme, and it may be concluded that release of carbon dioxide provides an entropic driving force which aids the acylation process.

The deacylation process was also followed by ESIMS. The acyl-enzyme peak at 25 353 Da decreased while the peak for the free enzyme at 25 233 Da increased, and deacylation was estimated to occur with a $t_{1/2}$ of approximately 5 hours. It was not possible to measure the rate of deacylation of 2-aminobenzoyl-α-CT by infrared spectroscopy because of autolysis and aggregation of α-CT in the infrared cuvette, which caused large changes in the amide I band over the long time period required for observation of deacylation. These changes obscured loss of the ester carbonyl band and formation of product, although the amount of small peptide fragments observed at low molecular mass in ESIMS was minimal. Hence, it was necessary to follow the rate by measuring the recovery of activity upon deacylation by assay with succinyl-phenylalanine-*p*-nitro-anilide (Succ-Phe-PNA). This gave a rate for the deacylation of 2-aminobenzoyl-α-CT of $3.76(\pm 0.87) \times 10^{-5}\,s^{-1}$ at pH 7 and 37 °C and, thus, a half time for deacylation of approximately 5.1 hours.

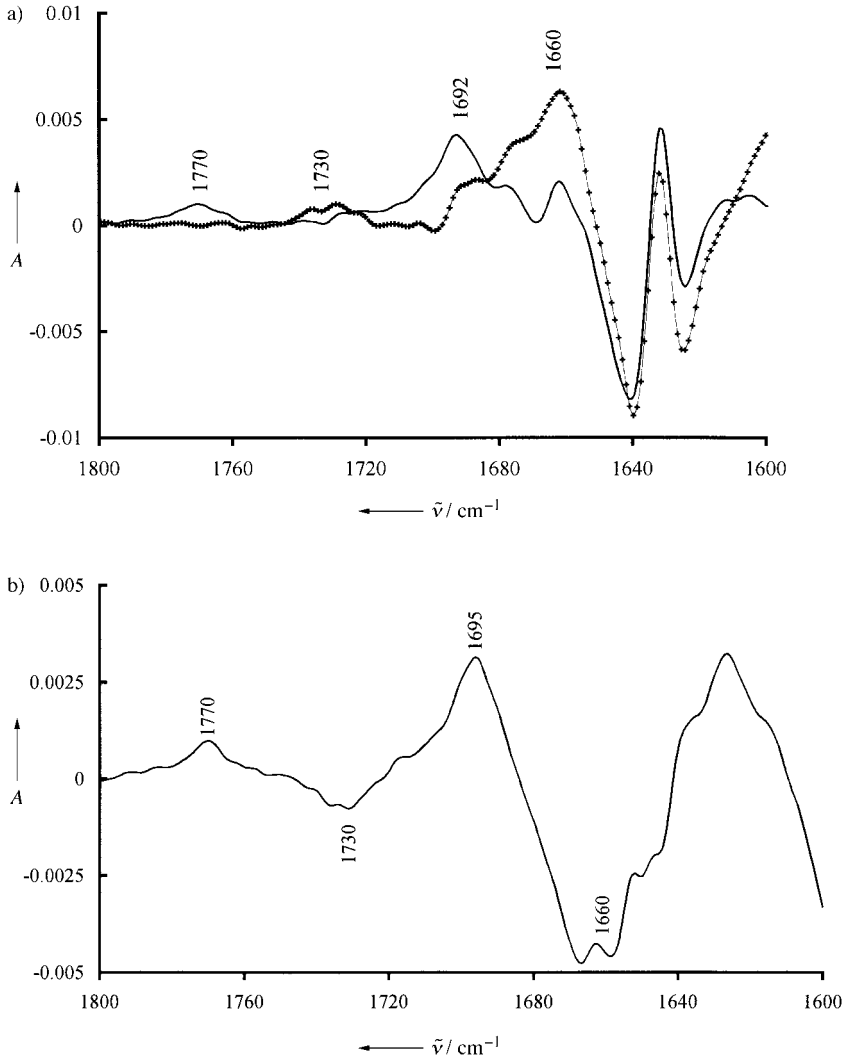

Figure 2. *Isotope-edited infrared difference spectra of 2-aminobenzoyl-α-chymotrypsin at pM 7 in deuterated phosphate buffer (0.1 M). a) IR difference spectra of [^{12}C=O]-2-aminobenzoyl-α-CT minus free α-CT (solid line) and [^{13}C=O]-2-aminobenzoyl-α-CT minus free α-CT (+ −+−+). b) IR difference spectrum of [^{12}C=O]-2-aminobenzoyl-α-CT minus [^{13}C=O]-2-aminobenzoyl-α-CT. All spectra were collected at 23 °C with 4 cm^{-1} resolution over 1024 scans and with a 100 μm spacer and CaF_2 cell windows. α-CT and 2-aminobenzoyl-α-CT solutions were at a concentration of 2 mM in phosphate buffer (pM 7, 0.1 M), made up in deuterium oxide. The pM value is the pH meter reading measured in ^2H_2O with a glass combination electrode.*

Infrared spectroscopic studies of 2-aminobenzoyl-α-chymotrypsin

[^{12}C=O]-2-Aminobenzoyl-α-chymotrypsin minus free enzyme spectrum: In the [^{12}C=O]-2-aminobenzoyl-α-CT minus free enzyme spectrum (Figure 2a), there are very small perturbation features caused by acylation of α-CT by isatoic anhydride, and the overall change in the amide I (protein amide C=O stretch) band upon acylation is only 0.6%. These small changes are probably in the β structure of the enzyme, and the bands at 1640 and 1624 cm^{-1} are associated with this change in the protein amide spectrum upon interaction of the enzyme and the ligand. The band that appears at 1692 cm^{-1} in the [^{12}C=O]-2-aminobenzoyl-α-CT spectrum is in the ester region of the spectrum as expected for the acyl-enzyme carbonyl band. This band is shifted in the [^{13}C=O]-2-aminobenzoyl-α-CT spectrum into a region of strong protein perturbation within the spectrum and so the exact shift in frequency is difficult to quantify.

Underlying protein perturbations make assignment of the ester carbonyl band difficult in the [^{12}C=O]-2-aminobenzoyl-α-CT minus α-CT spectrum and measurement of the acylation rate from this band impossible. Isotope-edited infrared difference spectroscopy (see below) is required for the unambiguous assignment of the ester carbonyl band.

[^{12}C=O]- minus [^{13}C=O]-2-aminobenzoyl-α-chymotrypsin spectrum: The ester carbonyl band of the acyl-enzyme, 2-aminobenzoyl-α-CT, clearly seen at 1695 cm^{-1} in the [^{12}C=O]-minus [^{13}C=O]-2-aminobenzoyl-α-CT spectrum (Figure 2b), has been assigned to the stable ester carbonyl group formed upon acylation of α-CT by isatoic anhydride. There are no other acyl bands visible in the ester carbonyl region of the infrared spectrum of 2-aminobenzoyl-α-CT. Hence, this band can definitively be assigned as the ester carbonyl band and is confirmed by [^{13}C=O] isotope-edited infrared difference spectroscopy, which eliminates protein perturbation from the infrared spectrum. The [^{13}C=O] ester carbonyl band is more difficult to assign

than the equivalent [$^{12}C{=}O$] band, because it appears in a region of intense absorption by the protein. This band is centred around 1660 cm^{-1} and forms a complicated absorbance peak. In order to resolve this band conclusively, isatoic anhydride with a [$^{13}C{=}^{18}O$]-isotope label would be required, to shift the ester carbonyl band away (approximately 80 cm^{-1}) from this intense protein absorbance region. The small positive band seen at 1770 cm^{-1} and the negative band at 1730 cm^{-1} observed in the isotope-edited infrared difference spectrum are due to a slight excess of isatoic anhydride, the spectrum of which is shown in Figure 3.

From the assignment of the ester carbonyl band at 1695 cm^{-1}, it is possible to deduce the environment experienced by the ester carbonyl within the active site of the enzyme. In a separate experiment, the carbonyl stretching frequency of a model compound, ethyl 2-aminobenzoate, was measured in a variety of solvents with different dielectric constants, from hexane to D_2O (Figure 4). A nearly linear relationship exists between the shift in carbonyl frequency and the dielectric constant of the solvent, as has been observed for other model esters.[19, 20] This shows that the ester carbonyl of the acyl-enzyme experiences a dielectric constant close to that of hexane, that is, a nonpolar environment. This in turn implies that there is little, if any, hydrogen bonding between the ester carbonyl group and the oxyanion hole. Hence it is proposed that the acyl band at 1695 cm^{-1} is a nonproductive conformation of the enzyme–ligand complex. Since no other bands are visible in this region, it is apparent that deacylation occurs inefficiently from this conformer as suggested below or through a very sparsely populated conformer. The latter pathway would imply a band that had an intensity too small to measure and that would be spectroscopically invisible. The band assigned as the acyl-enzyme is symmetrical, and deconvolution followed by bandfitting of the spectrum confirmed that the acyl-enzyme apparently exists as a single conformational species. The full width at half maximum band height (FWHH) of the acyl band is approximately 19.3 cm^{-1} at pM 7, whereas the FWHH for the ester band of the model, ethyl 2-aminobenzoate, is 10 or 32 cm^{-1} in hexane or D_2O, respectively. As the polarity of the environment increases, the bandwidth of the carbonyl band and the dispersion of the solvated species also increase.

A possible explanation for the lack of hydrogen bonding to the oxyanion hole is that isatoic anhydride is a relatively "short" molecule and does not extend to fit fully into the aromatic binding pocket of the enzyme's active site, tuned to phenylalanine. This may induce a constrained conformation of the ligand, in which the carbonyl is not planar with the benzene ring and, thus, hydrogen bonding to the oxyanion hole is prevented. A similar distortion has been postulated in *p*-(dimethylamino)benzoyl-α-CT.[21] Alternatively, the 2-aminobenzoyl moiety could form an intramolecular hydrogen bond with itself due to the proximity of the amino group to the carbonyl, which could out-compete potential hydrogen bonds donated by the enzyme oxyanion hole, since these would be sterically unfavourable. The position of the amino group relative to the carbonyl is shown diagrammatically in Scheme 1. The intramolecular hydrogen bonding between the amino group and the carbonyl moiety should polarise the C=O bond, making the carbonyl carbon more δ+ and hence more prone to attack, as does the oxyanion hole with good substrates. This also accounts in part for the low wavenumber of the ester carbonyl band at 1695 cm^{-1} in comparison to that of other acyl-enzymes (for example, the ester carbonyl group of 2-phenylpropanoyl-α-CT absorbs at 1731 cm^{-1}[22]), since the polarised C=O bond is weakened and hence absorbs at a lower frequency. The other factor is the electron-donating character of the amino substituent, seen in both the 2- and the 4-amino isomers (see below).

To show that this proposed hydrogen bonding occurs within the 2-aminobenzoyl

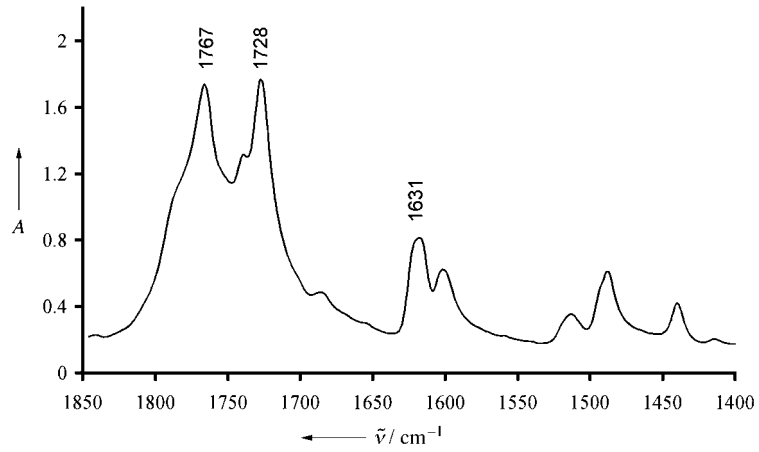

Figure 3. *The infrared spectrum of isatoic anhydride determined from a KBr disc. The prominent bands at 1767 and 1728 cm^{-1} represent the carbonyl stretching frequencies of the anhydride moiety.*

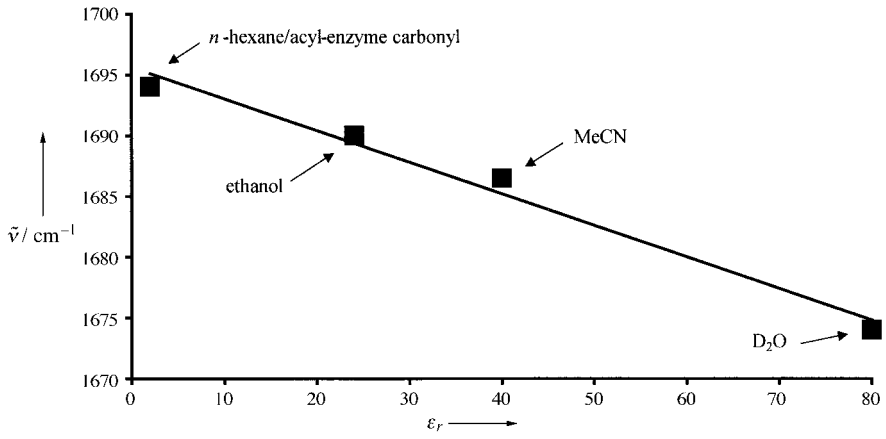

Figure 4. *Ester carbonyl frequencies of ethyl 2-aminobenzoate as a function of solvent dielectric constant. Spectra of ethyl 2-aminobenzoate (1%) dissolved in the various solvents were collected.*

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moiety, a further model study was carried out. The ester carbonyl stretching wavenumbers (in hexane) of ethyl aminobenzoates with the amino group in the 2, 3 and 4 positions were measured as 1697, 1728 and 1720 cm^{-1}, respectively, with the unsubstituted ester having a wavenumber of 1730 cm^{-1}. This provides further evidence for the existence of an internal hydrogen bond between the amino group and the carbonyl moiety of ethyl 2-aminobenzoate, since its ester carbonyl absorbance frequency is 23 cm^{-1} lower than that of ethyl 4-aminobenzoate, despite the fact that electronically they should be similar. That the 2-amino isomer shows an abnormally low carbonyl wavenumber, even allowing for inductive and resonance effects, is demonstrated by a study of the carbonyl absorption wavenumbers of methyl 2-, 3- and 4-methoxybenzoates in a variety of solvents. In these esters, hydrogen bonding cannot intervene, although the IR spectra of the 2-position isomer are complicated by steric effects involving the rotation (s-*cis* or s-*trans* with respect to the methoxy group) of the ester carbonyl group.[23] The frequency shift between the 3- and 4-position isomers in non-hydrogen-bonding solvents is $-5(\pm 1)$ cm^{-1}, while that between the 4- and 2-position isomers is $-6(\pm 2)$ cm^{-1}. In hydrogen-bonding solvents such as ethanol or deuterium oxide, the carbonyl wavenumbers (of the hydrogen-bonded forms) are shifted down by more than -20 cm^{-1}, relative to their values in non-hydrogen-bonding solvents. Therefore, the shift of -23 cm^{-1} seen between the 4- and 2-position isomers of ethyl aminobenzoate can conclusively be ascribed to intramolecular hydrogen bonding in the 2-position isomer and not to other influences such as induction or resonance.

Kinetic analysis

Table 1 gives rate constants for the hydrolysis of some model esters and deacylation rate constants for related acyl-enzymes. The ester hydrolysis rate constants show a trend that differs sharply from that of the IR absorption wavenumbers of the carbonyl groups. The amino substituents are strongly electron releasing, which slows the hydrolytic rates, but the largest effect is seen in the 4-amino, rather than the 2-amino, isomer. Thus the 4-amino substituent is the most effective in electron donation to the ring. The 2-position isomer will not be intramolecularly hydrogen bonded in aqueous solution, owing to the overwhelming competition by water, and so this effect is removed. This strongly reinforces our interpretation of the IR spectrum of 2-aminobenzoyl-α-CT in terms of an intramolecular hydrogen bond.

Table 1. *Rate constants for the hydrolysis of model esters and deacylation of acyl-enzymes.*[a]

Ester/acyl-enzyme	Measurement wavelength [nm]	Rate constant for hydrolysis [min^{-1}]	Ratio of deacylation/hydrolysis rate constants
ethyl benzoate	275	0.7 ($\pm$0.02)	
ethyl 2-aminobenzoate	340	0.092($\pm$0.003)	
ethyl 3-aminobenzoate	320	0.55($\pm$0.005)	
ethyl 4-aminobenzoate	295	0.036($\pm$0.001)	
methyl 4-methoxybenzoate	260	0.41($\pm$0.008)	
benzoyl-α-CT		0.012	0.0175
2-aminobenzoyl-α-CT		0.0023	0.025
4-methoxybenzoyl-α-CT		0.0024	0.006

[a] Ester hydrolyses were measured at 25 °C with 0.1 mM solutions in 1.0 M KOH and 20% acetonitrile; reaction progress was measured by the decrease in absorbance at the wavelengths given in the table. Deacylation rate constants of benzoyl- and 4-methoxybenzoyl-α-CT, taken from the results of Caplow and Jencks, were measured in 0.1 M phosphate buffer (pH 7.0).[24] The rate constant for 2-aminobenzoyl-α-CT was measured at pH 7.0 as described in the Materials and Methods section.

The rate constants for deacylation also show the effect of the electron-releasing nature of the amino group (Hammett 4-amino substituent: $\sigma = -0.66$) as well as the methoxy substituent ($\sigma = -0.27$). Although more electron releasing than the methoxy group, since it also readily participates in resonance ($\sigma^+ = -1.27$), the 2-amino derivative has the same deacylation rate constant as the methoxy derivative; again, intramolecular hydrogen bonding is the only reasonable explanation for this.

Finally, when the ratios of the rate constants for deacylation to those of aqueous hydrolysis are considered, it is found that the 2-amino derivative has the largest value. This means that the intramolecular hydrogen bond more than compensates for the potent electron-donating potential of the amino group.

Another way of analysing the kinetic implications of the proposed intramolecular hydrogen bond is to relate our result with the inverse linear correlation, established by Tonge and Carey by Raman spectroscopy in 1992, between the log of the deacylation rate constant and the acyl-enzyme ester carbonyl absorption frequency for a wide range of arylacryloyl derivatives.[22, 25] Before the comparison can be made it is necessary to correct the acyl-enzyme carbonyl frequency to allow for the presence of the 2-amino group. The correction is deduced to be 35 cm^{-1}, the difference between the frequencies observed for ethyl cinnamate and ethyl 2-aminobenzoate, both measured in hexane. This gives a "corrected" acyl-enzyme frequency of 1730 cm^{-1} and places the 2-aminobenzoyl derivative very close to the trend line, which gives a predicted deacylation rate constant slightly higher than, but within a factor of four of, the predicted value. This is closer to the predicted value than the values found in our previous work for cinnamoyl-α-CT (ninefold difference) and hydrocinnamoyl-α-CT (4×10^4-fold!).[22] 2-Aminobenzoyl-α-CT falls in the region of the plot (high frequency, low rate constant) where oxyanion hole interaction would, at best, be predicted to be very poor. The 2-amino intramolecular hydrogen bond may thus be regarded as resembling a (very) poor oxyanion hole.

Molecular modelling of 2-aminobenzoyl-α-chymotrypsin

The acyl-enzyme model was constructed by using the Sybyl molecular modelling program and minimised with a Powell minimisation to produce the conformation with the lowest conformation. The minimisation was conducted from several starting points and always converged upon a singular solution; the lowest energy model produced is shown in Figure 5.

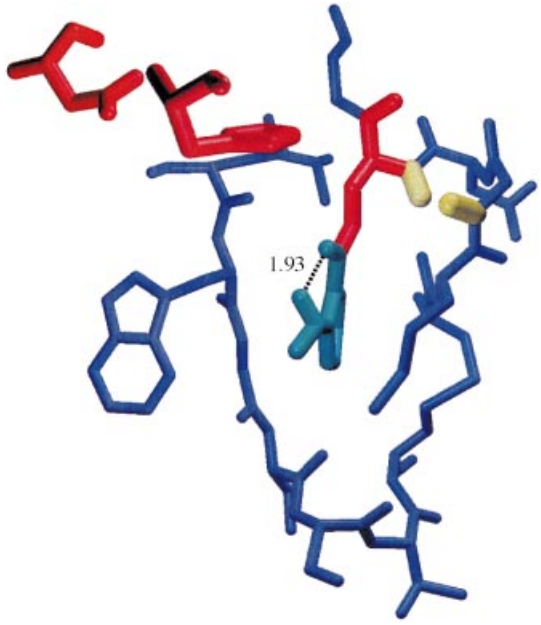

Figure 5. *Molecular model of the acyl-enzyme (2-aminobenzoyl-α-chymotrypsin) derived by using the Sybyl molecular modelling program and the X-ray structure of tosylated bovine α-chymotrypsin. The aromatic ring of isatoic anhydride is shown residing in the P1 aromatic binding pocket of α-CT. The Asp 102 (top left), His 57 and Ser 195 relay system is shown in red. The oxyanion hole ⟩N–H (Ser 195, Gly 193) groups are shown in yellow and the 2-aminobenzoyl ligand is shown in green.*

It is observed that the distance between the amino group hydrogen and the carbonyl oxygen atoms in this acyl-enzyme conformation (1.93 Å) is much less than the distance between the oxyanion hole and the carbonyl oxygen atom (4.26 and 4.42 Å). This makes it far more likely that the ester carbonyl moiety will form an intramolecular hydrogen bond rather than a hydrogen bond with the oxyanion hole. The hydrogens on the amino group do not point directly at the oxygen of the carbonyl group. However, they are sufficiently close that hydrogen bonding can occur between them, since the specificities of hydrogen bonds are far more dependent on the bond length than on the orientation.[26] It is possible that the energy minimum discovered by this minimisation is not the global minimum, but a local minimum. However, even if this is the case, the hydrogen-bonding distances proposed by this model provide convincing support for the proposed intramolecular hydrogen bonding within the 2-aminobenzoyl moiety.

General base catalysis by the 2-amino group?

It is conceivable that the deacylation of 2-aminobenzoyl-α-CT might arise from intramolecular general base catalysis by the 2-amino group, since this has been observed to occur in some alicylic esters.[27] If this were the case, then the pH dependence of deacylation should reflect the pK_a of the amino group, and not that of the enzyme, which falls between 6.0 – 7.0 for a wide range of acyl groups. The pK_a of ethyl 2-aminobenzoate is 2.36, while the pK_a determined from the study of the pH dependence of deacylation (Figure 6) is 6.43(±0.05). This clearly shows that the rate effect arises from electrophilic assistance by the 2-amino group acting on the carbonyl group and not from general base catalysis.

Concluding Remarks

The reaction between isatoic anhydride and α-CT forms a stable acyl-enzyme. A single spectroscopically visible conformation of the acyl-enzyme can be observed by infrared spectroscopy and has been assigned to a band absorbing at 1695 cm^{-1}. The low deacylation rate and model studies with ethyl 2-aminobenzoate indicate that the acyl-enzyme represents a nonproductive conformation, in which the ester carbonyl exhibits essentially no hydrogen bonding with the oxyanion hole of the enzyme. This can be explained by the ligands being "short" and hence forming a constrained conformation, unable to form hydrogen bonds with the oxyanion hole and thus inhibiting attack of the hydrolytic water molecule. However 2-aminobenzoyl-α-CT is also prevented from hydrogen bonding with the oxyanion hole by an intramolecular hydrogen bond between its amino group and its carbonyl moiety; this bond has clearly been demonstrated by molecular modelling of the 2-aminobenzoyl-α-CT molecule. Although this intramolecular hydrogen bonding seems likely to be responsible for an enhancement of the deacylation rate, it is possible that compounds with groups specifically placed to outcompete hydrogen bonds from the oxyanion hole may provide a mechanism for controlling the properties of serine proteinase inhibitors and possibly the β-lactamases, some of which are also acylated by isatoic anhydride.[7] It is hoped that these studies will form a platform for future work with the β-lactamases and compounds related to isatoic anhydride that may prove more clinically useful.

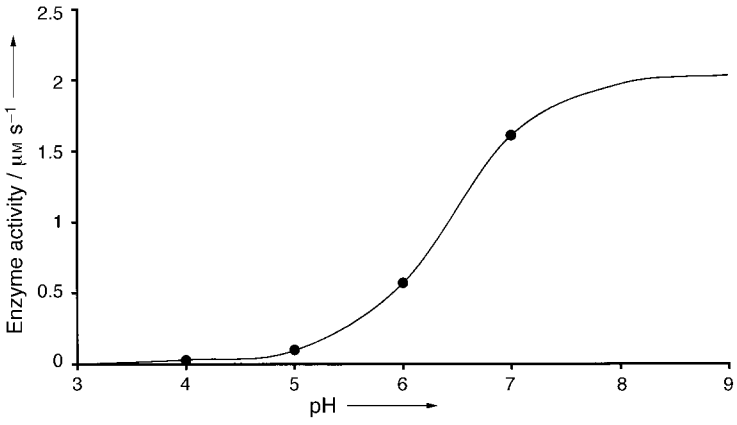

Figure 6. *The pH dependence of the deacylation of 2-aminobenzoyl-α-CT. Enzyme activity regenerated upon deacylation was measured by using Succ-Phe-PNA as described in the Materials and Methods section. The study was conducted at 25 °C in the presence of 0.2 M KCl, since the acyl-enzyme has a tendency to precipitate below pH 7 at 37 °C and at low salt concentrations. The enzyme activity regained 200 h after initiation of deacylation was used to fit the pH-dependence curve, since autolysis of the free enzyme became significant after this time and the reactions at pH values of 5 and 4 were too slow to determine accurate rate constants.*

Materials and Methods

Materials: α-CT was supplied from Sigma as type 2, multiply recrystallised, and was used as supplied. For ESIMS, a purer form of α-CT with a minimum of additionally charged species was required. For this, α-CT recrystallised three times and supplied by Worthington was used as supplied. ^2H_2O was 99.9% enriched, obtained from Aldrich. All samples were prepared in 0.1 M phosphate buffer and adjusted to the required pH value, or pM in ^2H_2O, by addition of NaOH or NaO^{2}H. [^{12}C=O]-Isatoic anhydride was supplied by Aldrich and 1,2-[^{13}COOH]-phthalic acid (99% enriched in ^{13}C) was obtained from Isotec, Miamisburg (USA).

Synthesis of [^{13}C=O]-labelled isatoic anhydride: [^{13}C=O]-labelled isatoic anhydride was prepared from 1,2-[^{13}COOH]-phthalic acid. The phthalic acid (0.1 g) was heated under reflux with acetic anhydride (1 ml) for 2 h. After the mixture had cooled to 4 °C, crystals of phthalic anhydride were filtered off and dried in a vacuum desiccator over P_2O_5 and NaOH. The yield of phthalic anhydride was effectively quantitative with respect to the phthalic acid. Phthalic anhydride (0.1 g), azidotrimethylsilane (0.4 mL) and chloroform (1 mL) were heated under reflux for 2 h. After rotary evaporation and redissolution of the residue in acetone (1 – 2 mL), the *N*-trimethylsilane derivative of isatoic anhydride was hydrolysed by addition of a stoichiometric quantity of ethanol (0.03 mL). The acetone was removed by rotary evaporation and the residue was treated with chloroform (1 ml). The isatoic anhydride was recovered by filtration. The yield was 48 – 52% and the product was shown to be pure by thin layer co-chromatography with the [^{12}C=O] form.

Infrared spectroscopic experiments: All infrared spectra were collected on a Bruker IFS66 Fourier transform infrared spectrometer with ^2H_2O as the solvent. A mercury cadmium telluride detector cooled by liquid nitrogen was used. IR spectra were accumulated by taking 1024 scans at 4 cm^{-1} resolution and a scan rate of 16 s^{-1} with a scanner modulation frequency of 180 kHz. The absence of water bands in the spectra was ascertained by overlaying the spectra with that of a water vapour spectrum to look for coincident bands; none were found.

Samples were prepared by mixing α-CT, dissolved in deuterated phosphate buffer and adjusted to the required pM value (2 mM, 200 μL) with isatoic anhydride in dry acetonitrile (60 mM, 5 μL). The samples (≈60 μL) were injected into an "in situ" IR cuvette with a syringe (100 μL volume). The cuvette had CaF_2 windows and a path length of 100 μm. This arrangement allows the cuvette to be filled, cleaned and dried without the need to dismantle the cuvette or to break the dry air purge of the spectrometer.

α-CT and 2-aminobenzoyl-α-CT spectra were generated by subtraction of a ^2H_2O spectrum. To measure the structural changes upon acylation, the spectrum of the free enzyme was subtracted from that of 2-aminobenzoyl-α-CT. In order to assign the peak position of the acyl band unequivocally, isotope-edited difference infrared spectroscopy was used. Isotope-edited difference spectra were obtained by subtraction of the spectrum of the [^{13}C=O] form of the acyl-enzyme from that of the [^{12}C=O] form. This technique ensures assignment of the ester carbonyl acyl band free from protein perturbation, although unreacted reagent and/or product features may be present.

Electrospray ionisation mass spectrometry: The reaction between α-CT and isatoic anhydride was followed by ESIMS. α-CT (4 mM) in H_2O as the solvent was used to follow the time course of deacylation at pH 8. To lower the acylation rate so that it could be partially observed, the enzyme was used at a concentration of 0.04 mM at pH 5. At various time points, an aliquot of enzyme (10 nmoles) was removed and quenched in formic acid (0.1 M) to lower the pH value to 3 and to stop the reaction. The samples were then freeze dried and analysed by ESIMS.

Each sample was treated for mass spectrometric study in the same way: A portion (ca. 10%) of the solid was removed and dissolved in acetonitrile/0.1% aqueous formic acid (1:1; 100 μL). In some of the spectra, salt-related ions obscured the acyl-enzyme. To eliminate this problem, samples were passed through a desalting cartridge arranged in line with the mass spectrometer. After injection of the protein sample into the cartridge, the cartridge was washed with water (2 × 100 μL) and then eluted into the mass spectrometer with acetonitrile/0.1% aqueous formic acid (1:1) at a flow rate of 50 μL min^{-1}. The resulting solutions were analysed at the School of Biochemistry and Molecular Biology, The University of Leeds (UK), by positive ionisation electrospray on a Platform II single quadrupole mass spectrometer (Micromass UK Ltd.; purchased with funds from the Wellcome Trust). The system was calibrated by separate injection of horse heart myoglobin (molecular mass: 16951 Da) and the mass accuracy is predicted to be ±0.01%.

Measurement of the rate of deacylation of 2-aminobenzoyl-α-chymotrypsin: The rate of deacylation of 2-aminobenzoyl-α-CT was measured by assay with Succ-Phe-PNA at pH 7 and 37 °C. 2-Aminobenzoyl-α-CT (0.2 mM) was synthesised by treatment of α-CT (0.2 mM, 10 mL) in phosphate buffer (pH 7, 0.1 M) with isatoic anhydride in dry MeCN (6 mM, 330 μL). The solution of 2-aminobenzoyl-α-CT was then placed in a water bath at 37 °C alongside a solution of α-CT (0.2 mM, 10 mL) in phosphate buffer (pH 7, 0.1 M) to measure the rate of autolysis of α-CT over time at this pH value and temperature. At various time points, aliquots (100 μL) of the 2-aminobenzoyl-α-CT solution and of the α-CT solution were removed from the water bath and assayed to measure their activity. The rate of hydrolysis of Succ-Phe-PNA is proportional to the free-enzyme concentration. The assay solution was made up of phosphate buffer (pH 7, 0.1 M, 870 μL), Succ-Phe-PNA in dry dimethylsulfoxide (100 mM, 20 μL) and the reaction mixture (100 μL), which was either 2-aminobenzoyl-α-CT or α-CT. The assays were carried out over 68 h.

Measurements of the pH dependence of deacylation were made at 25 °C, by using the same general procedure above. Phosphate buffer (0.1 M, as above but also containing 0.2 M KCl) was used at pH 7.0, while 0.01 M buffer was used for incubation at pH 6. Acetate buffer (0.01 M) containing 0.2 M KCl was used at pH values of 5.0 and 4.0. The more dilute buffers were used at the lower pH values to ensure that the pH value of the assays (7.0) was unaffected by addition of acyl-enzyme that had been incubated at lower pH values. The pH-dependence studies were conducted at 25 °C to obviate precipitation of the acyl-enzyme, which occurred below pH 7.0 at 37 °C. The reactions were followed for 240 h.

Molecular Modelling: Molecular modelling of 2-aminobenzoyl-α-CT was carried out with the general molecular modelling program Sybyl, written by Tripos. The model was created by superimposing a minimised structure of 2-aminobenzoate over the *p*-toluenesulfonyl (tosyl) moiety of a 2.0 Å structure determined from tosylated bovine α-CT (E.C. 3.4.21.1)[28] by X-ray diffraction and then removing the tosyl moiety. Once this had been carried out, the whole of the new molecule was minimised by a Powell minimisation,[29] a member of the conjugate gradient family of minimisation.

We thank Dr. M. G. P. Page for helpful discussions. This work was supported by a studentship award to J.J.G from F. Hoffmann – La-Roche Ltd., Basel (Switzerland).

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Received: May 15, 2001 [F 244]