Hydrogen-bonding in enzyme catalysis

Fourier-transform infrared detection of ground-state electronic strain in acyl-chymotrypsins and analysis of the kinetic consequences

Andrew J. WHITE and Christopher W. WHARTON
Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

I.r. difference spectra are presented for 3-(indol-3-yl)acryloyl-, cinnamoyl-, 3-(5-methylthien-2-yl)acryloyl-, dehydrocinnamoyl- and dihydrocinnamoyl-chymotrypsins at low pH, where the acyl-enzymes are catalytically inactive. At least two absorption bands are seen in each case in the ester carbonyl stretching region of the spectrum. Cinnamoyl-chymotrypsin substituted at the carbonyl carbon atom with ^{13}C was prepared. A difference spectrum in which ^{13}C-substituted acyl-enzyme was subtracted from [^{12}C]acyl-enzyme shows two bands in the ester carbonyl region and thus confirms the assignment of the features to the single ester carbonyl group. The frequencies of the ester carbonyl bands are interpreted in terms of differential hydrogen-bonding. In each case a lower-frequency relatively narrow band is assigned to a productive potentially reactive binding mode in which the carbonyl oxygen atom is inserted in the oxyanion hole of the enzyme active centre. The higher-frequency band, which is broader, is assigned to a non-productive binding mode in each case, where a water molecule bridges from the carbonyl oxygen atom to His-57; this mode is equivalent to the crystallographically determined structure of 3-(indol-3-yl)acryloyl-chymotrypsin, i.e. the Henderson structure. A difference spectrum of dihydrocinnamoyl-chymotrypsin taken at higher pH shows resolution of a feature centred upon 1731 cm^{-1}, which is assigned to a non-bonded conformer in which the carbonyl oxygen atom is not hydrogen-bonded. Perturbation of the protein spectrum in the presence of acyl groups is interpreted in terms of enhanced structural rigidity. It is reported that the ester carbonyl region of the difference spectrum of cinnamoyl-subtilisin is complicated by overlap of features that arise from protein perturbation. Measurements of carbonyl absorption frequencies in a number of solvents of the methyl esters of the acyl groups used to make acyl-enzymes have permitted determination of the apparent dielectric constants experienced by carbonyl groups in the enzyme active centre as well as a discussion of the effects of polarity. The ester carbonyl bond strengths of the various conformations were estimated by using simple harmonic oscillator theory and an empirical relation between the force constants and bond strengths. The fractional bond breaking induced by hydrogen-bonding was used to calculate rate enhancement factors by using absolute reaction rate theory. Thus ground-state electronic strain induced by hydrogen-bonding in the oxyanion hole is calculated to yield a factor of 5.0×10^4 in rate enhancement of the deacylation of dihydrocinnamoyl-chymotrypsin. Hydrogen-bonding in aniline–ethyl acetate complexes was used to allow a correlation between the carbonyl frequency shift and the enthalpy of hydrogen-bond formation to be determined, and this is evaluated in terms of interaction in acyl-enzymes. Linear correlations (Badger–Bauer relationships) between the carbonyl frequency shift and enthalpy of hydrogen-bond formation were used to analyse hydrogen-bond strength in acyl-enzymes. It is proposed that ground-state hydrogen-bond strength is related to the attainable hydrogen-bonding in the transition state and thus is a measure of potential transition-state stabilization. A spectroscopic–kinetic correlation is presented that lends support to this hypothesis. Our conclusion is that ground-state electronic strain plays an important role in chymotrypsin catalysis and supplies perhaps half of the overall rate enhancement. The distribution, energy balances and the dynamics of exchange of the conformers are discussed in terms of hydrogen-bonding and steric strain. An apparent paradox that arises in the interpretation of results from experiments in which the oxyanion hole of subtilisin was partially deleted by site-specific mutagenesis is discussed in the context of the main conclusions of this paper.

INTRODUCTION

In order that we may be able to manipulate enzymic catalysis to serve new or alternative purposes not provided for by evolution, we need a much more fundamental knowledge of protein structure and folding, and of the functioning of catalytic devices used by enzymes. It has proved easy to damage enzyme activity by altering their natural structure by means of site-specific mutagenesis, although in one case specificity has been successfully changed while the specific activity has been retained [1]. Despite the above, site-specific mutagenesis has proved incisive in terms of the analysis of interactions in enzyme active sites. Knowles [2] has rehearsed the main factors that prejudice experimental intervention in protein structures (and mechanisms) and has provided pointers to the knowledge required to allow us to make changes with the confidence that activity and stability will be retained. We are here concerned with dissection of catalytic mechanism, and have focused upon the 'oxyanion hole' rate-enhancement device (shown in Fig. 1) used by the serine proteinases [3,4]. Evidence that this device is important in catalysis by providing transition-state stabilization comes from several sources. X-ray crystallography of formyltryptophan bound to α-chymotrypsin shows bifurcated hydrogen bonds from the acidic carbonyl oxygen atom to two backbone amide protons (Gly-193 and Ser-195) [5]. An acyl-enzyme of elastase shows a similar interaction [6]. Although the results must as yet be accepted with caution, computational analysis of the trypsin mechanism has led to the proposal that the 'oxyanion hole' interaction is of comparable importance with the charge-relay system and performs a vital role in transition-state stabilization,

Abbreviation used: pH*, pH-meter reading in ^2H_2O.

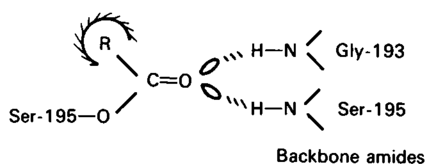

Fig. 1. The oxyanion hole interaction in the chymotrypsin active centre

and molecular-modelling studies support this role [7]. Transition-state stabilization is seen as being achieved by optimal hydrogen-bonding in this state, which results from the accumulation of negative charge on the oxygen atom and shortening of the hydrogen bonds as the bond order of the carbonyl bond decreases. Although site-specific mutagenesis of the backbone α-chymotrypsin residues that participate in the oxyanion hole interaction is not possible, subtilisin in which one element of the hole is the side chain of Asn-155 can be successfully mutagenized [8]. When Asn-155 was changed to Ala-155 the K_m was almost unchanged while $k_{cat.}$ was decreased 400-fold. This result has been presented as clear evidence of the role of the oxyanion hole interaction in transition-state stabilization; we address the conclusiveness of this interpretation in the present paper. Vibrational spectroscopy provides excellent methods for the study of hydrogen-bonding in enzyme catalytic intermediates provided that the feature of interest can be seen against the strong background of the protein itself. Resonance Raman spectroscopy has been used to very good effect by Carey and co-workers in the study of chymotrypsin acyl-enzyme intermediates, albeit of a non-specific nature [9–11]. Recently the ester carbonyl stretch vibration, although not particularly strong in Raman spectra, has been studied in reactive conditions [12,13]. The method relies upon use of conjugated acyl groups, which show near-u.v. absorption and thus provide resonant enhancement of the acyl group against the non-resonant background of the enzyme.

Use of i.r. difference spectroscopy relies upon the low-noise properties of the Fourier-transform method and observation of bands that are clear of the strong background absorbance of the enzyme. Thus ester, aldehyde and ketone carbonyl groups generally absorb at frequencies above 1700 cm^{-1}, whereas the strong amide 1 absorption of proteins is centred at approx. 1640 cm^{-1} but extends to approx. 1680 cm^{-1} [14]. The carbonyl stretch vibration is very strong in the i.r., and a single group in a protein complex of M_r 20000–30000 can, under certain circumstances, be observed [15–17]. Because of the relatively low energy of the vibrational transition, the frequencies of these bands are sensitive to hydrogen-bonding and shift to lower frequency; this has been exploited in studies of enzyme–substrate complexes [15–17] and intermediates [18–20]. Thus substrate carbonyl interactions with triose-phosphate isomerase [15], fructose-bisphosphate aldolase [16] and citrate synthase [17] have been observed. In all cases a significant shift to lower frequency on complex-formation has been accompanied by a decrease in bandwidth, and in the case of citrate synthase by an increase in absorption coefficient. We have previously reported the observation of spectra of non-specific acyl-chymotrypsins that show multiple ester carbonyl bands indicative of the coexistence of multiple conformations [18–20].

In the present paper we report the i.r.-spectroscopic properties of acyl-enzyme carbonyl bands, confirmation of the assignment of the bands by ^{13}C substitution and some model studies that enable us to interpret the magnitude of the observed frequencies. The effect of carbonyl hydrogen-bonding is analysed in terms of harmonic oscillation, force constants, bond energies and the kinetic consequences of this interaction.

MATERIALS AND METHODS

Materials

Cinnamoyl-imidazole, 3-(indol-3-yl)acrylic acid, cinnamic acid, dihydrocinnamic acid and dehydrocinnamic acid were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.) and were used as such. ^2H_2O was obtained from Aldrich Chemical Co. (Gillingham, Dorset, U.K.).

Acylimidazoles were prepared by the method of MacClement *et al.* [21], and were used as 60 mM solutions in dried acetonitrile for acylation of the enzyme with the exception of [3-(indol-3-yl)acryloyl]imidazole, which was used as a 20 mM solution owing to lower solubility. Carbonyl-^{13}C-substituted cinnamic acid was prepared by condensation of 90%-enriched 2,3-^{13}C-disubstituted malonate, obtained from Merck, Sharpe and Dohme (Rahway, NJ, U.S.A.), with benzaldehyde by the method of MacClement *et al.* [21]. Methyl esters were prepared via the acid chlorides by reaction first with thionyl chloride followed by methanol/triethylamine. [3-(5-Methylthien-2-yl)acryloyl]imidazole was a gift from Dr. P. J. Tonge and Dr. P. R. Carey (National Research Council, Ottawa, Ont., Canada).

α-Chymotrypsin was obtained from Sigma Chemical Co. as type 2 three-times-crystallized enzyme and was generally used as such. A few experiments used enzyme that had been passed through a Sepharose–ovomucoid column to remove inactive enzyme as described in ref. [22]. The fraction of enzyme with a variable active centre was measured by titration with cinnamoylimidazole [23].

I.r.-spectroscopic experiments

Unless otherwise stated, i.r. spectra of acyl-enzymes and non-acylated enzyme were collected with a Nicolet Instruments MX-1 Fourier-transform instrument. Scans (32) were taken for 1 min at 2 cm^{-1} resolution. Latterly this instrument has been interfaced with an IBM-compatible personal computer running Nicolet PC/IR software, which has facilitated data analysis and display. Spectra were scanned in 50–75 μm-pathlength CaF_2 demountable liquid cells with Teflon spacers. We have found that close attention has to be paid to water vapour in the instrument; if there is any imbalance, small-amplitude features in difference spectra are obscured by the spectra of the water vapour.

The instrument is equipped with a small sample chamber (approx. 250 ml) with pneumatically operated doors that isolate the sample chamber from the main body of the spectrometer and allow the purging period required to re-establish dry air conditions after opening of the instrument to be much shorter (approx. 1 min) than would otherwise be the case. More recently we have used an '*in situ*' cell connected by tubing to the exterior through which the cell can be filled and washed. This has the great advantage that it is not necessary to break the dry air purge on the instrument and so eliminates the period required to re-establish dry conditions. The main problem in obtaining good-quality balanced difference spectra is the presence of micro-bubbles in the lightpath; carefully cleaned cells and gentle pressure on loading are necessary to ensure that these do not become a major nuisance.

A typical acylation experiment is conducted as follows. α-Chymotrypsin (50 mg; 80–95% active) is dissolved in 1 ml of ^2H_2O and left at room temperature for 18–24 h to allow protein protons to exchange with solvent deuterons. The pH* is adjusted to the experimental value with 30% (w/v) NaO^2H with a Hamilton microsyringe, and two samples (0.4 ml each) were removed from the stock solution. To one of these is added 10 μl of 60 mM-acylimidazole in acetonitrile. To the other sample is added 10 μl of 60 mM-imidazole in acetonitrile. The acyl-enzyme sample is loaded into the cell and scanned for 1 min after purging

of the instrument. The spectrum is stored, and the sample is removed from the cell, washed dry and replaced by the control sample, which is, in turn, scanned and stored. At the same time as the i.r. spectra are scanned a sample (0.1 ml) of acyl-enzyme or enzyme is titrated with cinnamoylimidazole to measure the extent of acylation [23], which in the experiments reported in the present paper was always essentially quantitative. The difference spectrum is obtained by subtracting the control spectrum from the acyl-enzyme spectrum. It has proved necessary always to use a subtraction factor of unity; unbalanced spectra have not proved to be correctable by altering the amount of control subtracted. We have found that about one in five difference spectra fail owing to imbalance, despite very careful handling of materials; these spectra must be discarded. This is not surprising when it is considered that the difference observed is 1×10^{-3}–5×10^{-3} absorbance units (in the ester C=O region, $> 1700\ cm^{-1}$) against a background of 1–1.5 absorbance units (at $1640\ cm^{-1}$). In all experiments the enzyme was acylated rapidly (very much faster than spectral acquisition). The concentrated (approx. 2 mM) enzyme solution has proved to be an excellent buffer in that the pH* change measured upon addition of acylating agent or imidazole has never exceeded 0.1 unit.

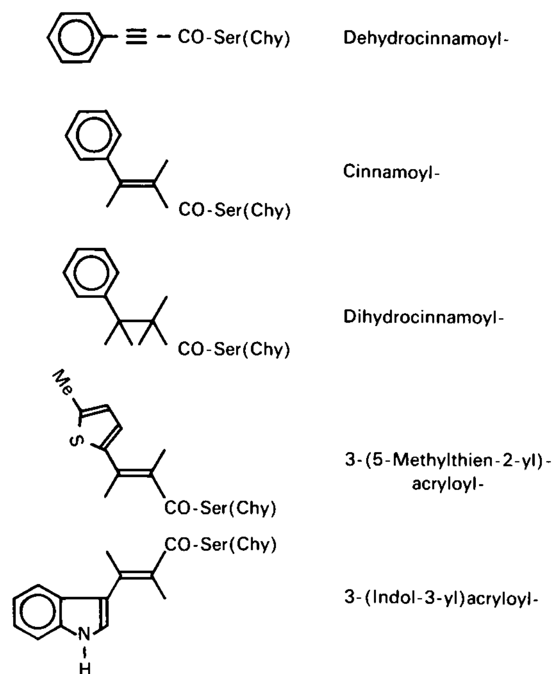

Fig. 2. Acyl groups used in the form of acylimidazoles to prepare acyl-chymotrypsins

Abbreviation: Chy, chymotrypsin.

RESULTS AND DISCUSSION

Spectroscopic properties of acyl-enzymes

The structures of all the acylating groups reported in this study are shown in Fig. 2. The i.r. difference spectra of cinnamoyl- and dihydrocinnamoyl-chymotrypsins at approx. pH* 4.0 have been published previously, although not discussed in detail [18,19]. The main features comprise more than one band in the ester carbonyl region in each case, together with marked excursions in the amide 1 region (1680–$1600\ cm^{-1}$) ascribed to structural perturbation of the protein in the presence of the acyl group relative to the free enzyme. Difference spectra of the acyl-enzymes are shown in Fig. 3, and the frequency maxima in the ester carbonyl region of acyl-enzymes together with estimated bandwidths are collected in Table 1.

All the difference spectra show some common features in the ester carbonyl region, namely a relatively narrow band at low frequency and a broader band at higher frequency. Manual deconvolution by curve-tracing reveals that the lower frequency band in cinnamoyl-chymotrypsin has a bandwidth (approx. $10\ cm^{-1}$) approximately one-third that of the higher-frequency band. Since hydrogen-bonding is well known to cause a decrease in frequency, in this case due to a weakening of the C=O stretching vibration, we ascribe these lower-frequency bands to 'productive' conformers in which the carbonyl oxygen atom is inserted in the oxyanion hole and that should lead to deacylation at higher pH, where the enzyme is catalytically active. Although hydrogen-bonding in model systems always leads to a decrease in frequency, it also usually has a band-broadening effect (which may often be partly due to the effect of a change in the medium dielectric constant resulting from the presence of polar hydrogen-bonding material) [25].

There is, however, good precedent for a narrowing effect in carbonyl bands of hydrogen-bonded enzyme–substrate complexes. Dihydroxyacetone binding to triose-phosphate isomerase [15], fructose 1,6-bisphosphate binding to fructose-bisphosphate aldolase [16] and oxaloacetate binding to citrate synthase [17] all show such an effect relative to free solution, the decrease in bandwidth on binding in the latter case being 2-fold. The decrease in bandwidth is seen as resulting from reduced conformational dispersity and/or mobility in the enzyme-bound species, where the number of accessible rotameric states will be decreased relative to free solution. The higher-frequency bands are accordingly assigned to 'non-productive' conformers in which the carbonyl oxygen atom is not inserted in the oxyanion hole.

It is notable that 3-(indol-3-yl)acryloyl-chymotrypsin shows two bands in the ester carbonyl region. The X-ray structure at low pH of this acyl-enzyme has been determined by Henderson [24], and the acyl group is seen to be in a non-productive conformation in which the carbonyl oxygen atom interacts with a water molecule that forms a bridge to His-57 rather than being inserted in the oxyanion hole. Thus it seems that a single conformation is 'frozen out' in crystals, whereas in solution two conformers coexist despite the fact that deacylation is apparently identical in crystal and solution forms [26]. We deduce, as do Tonge & Carey [13] on the basis of resonance Raman spectra, that the high-frequency bands assigned to non-productive conformers generally arise from structures that are related to the Henderson structure of 3-(indol-3-yl)acryoyl-chymotrypsin. Alone of the acyl-enzymes, dihydrocinnamoyl-chymotrypsin shows a resolution of the broad $1710\ cm^{-1}$ band at higher pH*. Fig. 4 shows a difference spectrum taken at pH* 5.6 in which a band centred upon $1731\ cm^{-1}$ is seen. We propose that this band represents a non-bonded conformer in which there are no hydrogen bonds to the carbonyl oxygen atom. The Henderson conformation almost disappears, and the acyl group thus distributes itself between non-bonded conformer and the productive one as the pH region where the enzyme has catalytic activity is approached. The proposed conformations are illustrated in Fig. 5.

Whereas the acyl groups that have double or triple bonds show high-frequency 'non-productive' bands that are some 2–3-fold broader than the productive band, the more flexible dihydrocinnamoyl group shows a very broad band centred at $1710\ cm^{-1}$. This is interpreted to mean that the rigid conjugated acyl groups adopt two relatively well-defined conformations and may be able to 'flip' between these, whereas the flexible saturated group is able to adopt a wide range of conformations when the carbonyl oxygen atom is not inserted in the oxyanion hole.

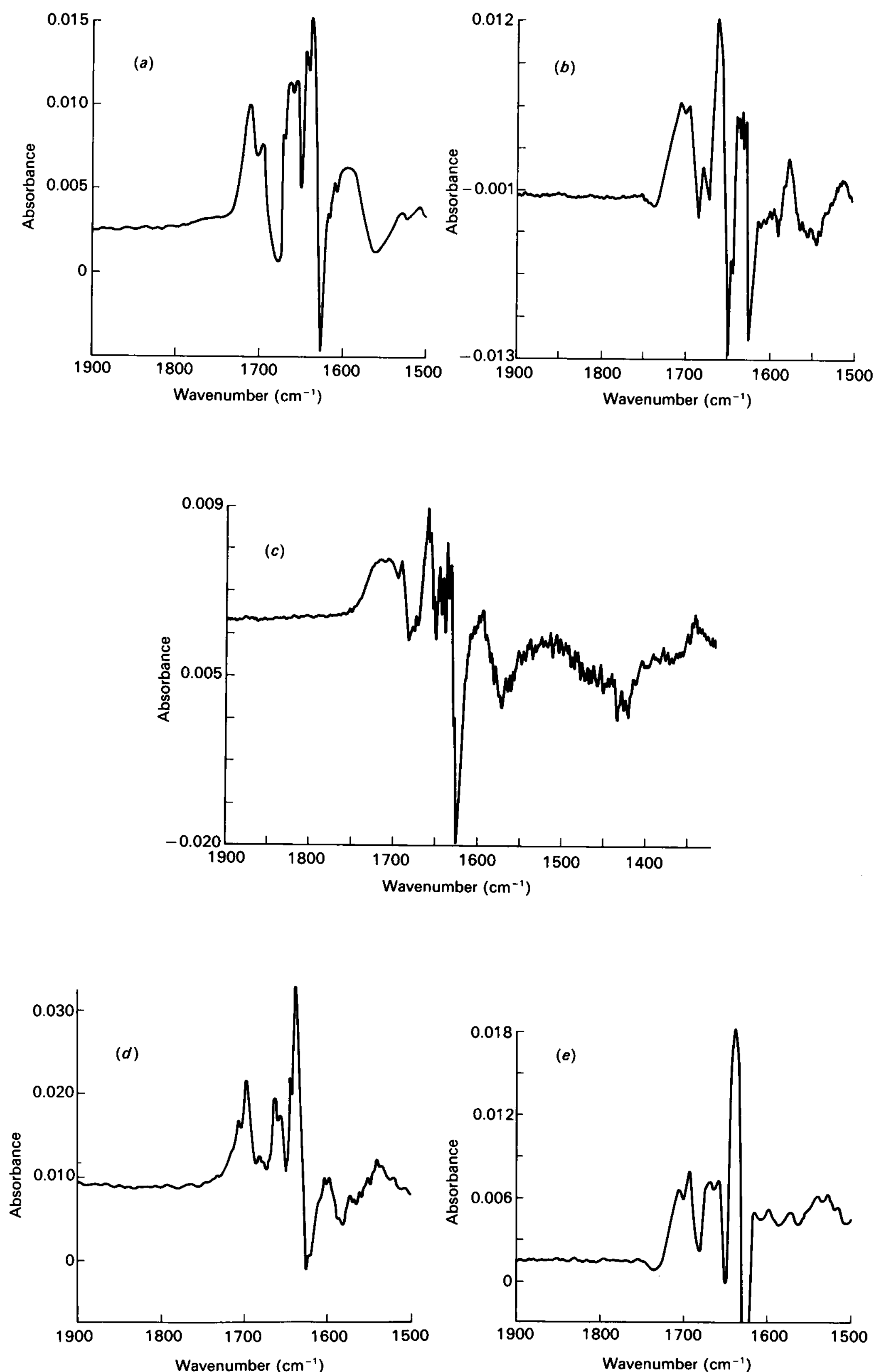

Fig. 3. I.r. difference spectra of acyl-chymotrypsins

The structures of the acyl groups are shown in Fig. 2. (*a*) Dehydrocinnamoyl-; (*b*) cinnamoyl-; (*c*) dihydrocinnamoyl-; (*d*) 3-(5-methylthien-2-yl)acryloyl-; (*e*) 3-(indol-3-yl)acryloyl-. α-Chymotrypsin (50 mg/ml) in ^2H_2O was acylated as described in the Materials and methods section. Samples of acyl-enzyme and non-acylated enzyme were scanned at pH* 4.0 [3-(5-methylthien-2-yl)acryloyl-chymotrypsin at pH* 6.1] for 1 min before subtraction in a 75 μm-pathlength cell with CaF_2 windows. The degree of acylation was determined by titration with cinnamoylimidazole [23] at the same time as the spectra were scanned and was always between 85% and 100% of the active enzyme, which was 80–95% of total enzyme.

Table 1. Frequencies and estimated bandwidths of i.r.-absorption bands in difference spectra of acyl-chymotrypsins

Experimental details as described in the Materials and methods section. Frequencies are pH-independent with the exception of the 1710 cm^{-1} band of dihydrocinnamoyl-chymotrypsin, which moves to 1731 cm^{-1} at pH* 5.6. Bandwidths are full widths at half height and were estimated by measuring half widths at half height on the side away from the overlap in the spectra (see Fig. 3). Non-productive conformers are proposed to be similar to the structure determined by Henderson [24] for 3-(indol-3-yl)acryoyl-chymotrypsin (see the text).

	Frequency (cm^{-1})		Bandwidth (cm^{-1})	
Acyl group	Non-productive conformer	Productive conformer	Non-productive conformer	Productive conformer
3-(Indol-3-yl)acryloyl*	1702	1693	31	13
Cinnamoyl	1705	1695	30	10
3-(5-Methylthien-2-yl)acryloyl*	1706	1695	18	7
Dehydrocinnamoyl	1708	1695	20	10
Dihydrocinnamoyl	1710 (1731†)	1692	~ 45 (23†)	7

* pH* 6.1.
† This band measured at pH* 5.6.

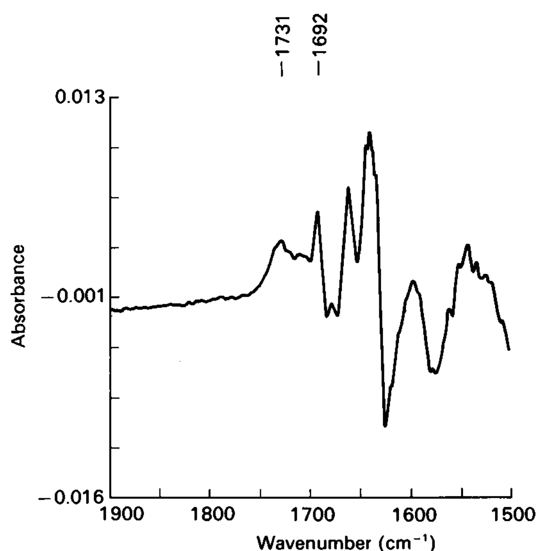

Fig. 4. I.r. difference spectrum of dihydrocinnamoyl-chymotrypsin at pH* 5.6

Conditions were as described in the legend to Fig. 3.

Substitution of ^{13}C at the carbonyl carbon atom of cinnamoyl-chymotrypsin

It is possible that the bands in the ester carbonyl region of the difference spectra might arise from perturbation of carboxy groups in the enzyme consequent upon acylation; indeed, a negative excursion seen at low pH and centred upon 1737 cm^{-1} in the cinnamoyl-enzyme spectrum has been interpreted in terms of an enzyme carboxy group whose p*K* is increased in the acyl-enzyme [18]. That the positive peaks result from the ester carbonyl group is clearly demonstrated by inspection of Fig. 6, which shows a difference spectrum where 90%-enriched [^{13}C]cinnamoyl-enzyme is substracted from the ^{12}C variant. The two ester carbonyl features remain, whilst most of the excursions that result from protein perturbation have been cancelled. The $^{13}C=O$ stretch frequencies are not seen, since they are expected to be shifted some 38 cm^{-1} to lower frequency (shift $^{12}C \rightarrow ^{13}C$ for ethyl cinnamate in acetonitrile equals 37.6 cm^{-1}), a region where the protein absorbance is very strong (approx. 1.5 at 1640 cm^{-1}) and subtraction leads to noisy spectra. That the lower-frequency band occurs at 1700 cm^{-1} rather than 1695 cm^{-1} as in difference spectra versus free enzyme might mean that subtraction is unreliable below 1700 cm^{-1}. We consider this unlikely, since the difference spectra are highly repeatable in this region. Difference spectra of the [^{13}C]acyl-enzyme versus free enzyme (not shown) show small peaks at 1705 cm^{-1} and 1695 cm^{-1} resulting from the 10% ^{12}C material present; it is notable that the intensity of the 1695 cm^{-1} band is some 25% more intense that the 1705 cm^{-1} peak. This suggests that there is some feature that arises from protein perturbation underlying the lower-frequency band and, although not large, this may distort the difference spectra somewhat in which free enzyme is subtracted and may explain why the lower-frequency maximum is seen at 1700 cm^{-1} in Fig. 6 rather than 1695 cm^{-1}, since any such underlying effect will be eliminated.

I.r. difference spectra of cinnamoyl-subtilisin

The ester carbonyl region in cinnamoyl-subtilisin shows a single feature centred on approx. 1702 cm^{-1} at pH* 5.5 (results not shown); there is thus no evidence for more than one conformation of the acyl group. The band is, however, significantly distorted by features that result from protein perturbation in the presence of the acyl group. That such features should extend to higher frequency than in chymotrypsin spectra reflects the significantly different structure of subtilisin. For example, subtilisin has more α-helix in its structure (approx. 31%) than does chymotrypsin (approx. 11%). Deconvolution will be required for the resolution and interpretation of difference spectra of acyl-subtilisins.

Protein perturbation by acyl groups

All acyl-enzyme difference spectra show striking features between 1680 and 1600 cm^{-1} that arise from protein perturbation (see Fig. 3). These features, although sometimes somewhat noisy, since they occur in a region of high background absorbance, are repeatable. The appearance for cinnamoyl- and dihydrocinnamoyl-enzymes is similar to that expected if a relatively broad band was being subtracted from a narrower one, i.e. not unlike a second differential of a Gaussian peak. It is well known that enzyme–inhibitor complexes show diminished rates of deuterium exchange relative to free enzyme [27], and so it is proposed that the structure is generally tightened up in enzyme–inhibitor complexes. It seems reasonable to assume that a similar effect occurs in acyl-enzymes, and this, in turn, would be predicted to lead to a narrowing of the amide 1 band owing to reduced

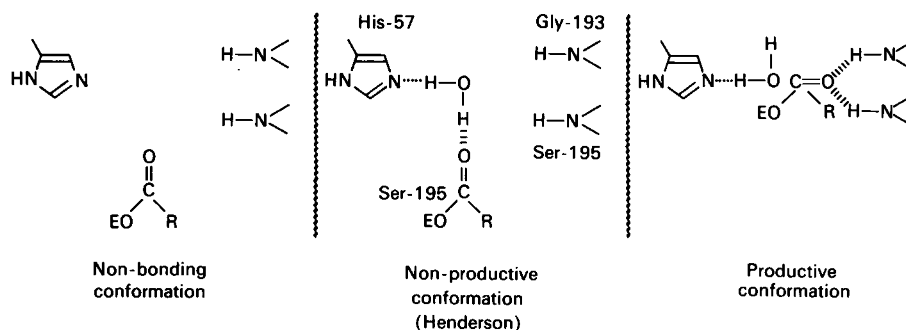

Fig. 5. The three conformations of dihydrocinnamoyl-chymotrypsin deduced to exist from i.r. difference spectra

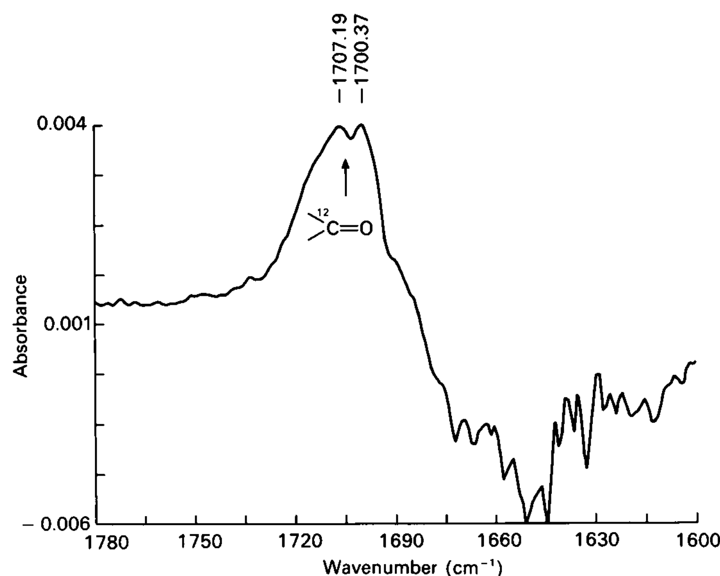

Fig. 6. Difference spectrum of [carbonyl-^{12}C]cinnamoyl-chymotrypsin minus [carbonyl-^{13}C]cinnamoyl-chymotrypsin

Acyl-enzymes were prepared as described in the Materials and methods section. Spectra were collected as described in the legend to Fig. 3.

dispersion relative to the free enzyme. By contrast, a simple spectral shift in difference spectra gives rise to a sinusoidal curve similar to the first differential of a Gaussian band, as is seen for the other acyl-enzymes.

Model studies

Table 2 gives the i.r.-spectral properties of some of the esters of the acyl groups shown in Fig. 2. As the polarity of the solvent increases the frequency decreases, while the bandwidth increases. It is apparent that all frequencies of non-productive acyl-enzyme carbonyl groups lie within the ranges defined by the model compounds in hexane and ^2H_2O. The non-productive band in cinnamoyl-chymotrypsin is 6 cm^{-1} below that of methyl cinnamate in acetonitrile, and the 1710 cm^{-1} band is well below 1732 cm^{-1}, the frequency of methyl dihydrocinnamate in this solvent. The productive band at 1695 cm^{-1} in cinnamoyl-chymotrypsin is at higher frequency than that of methyl cinnamate in ^2H_2O, and is thus presumably less strongly hydrogen-bonded than in aqueous solution; this is reflected in poor kinetic specificity (see Fig. 7 and Table 3). By contrast, dihydrocinnamoyl-chymotrypsin shows a productive band at 1692 cm^{-1}, which is well below the frequency of the model ester in ^2H_2O; we can thus conclude that the hydrogen-bonding of the carbonyl group when inserted in the oxyanion hole is stronger than that which occurs in aqueous media.

Dielectric constants experienced by acyl-enzyme carbonyl groups

Although it may be argued that application of the concept of bulk dielectric to consideration of the atomic-scale environment of the ester carbonyl group of acyl-enzymes is contentious, it is clear that useful deductions concerning polarity can be made. This is especially so if comparison is made between the enzymic system and relevant small model compounds in a well-defined environment. Fig. 7 shows that there is a linear relationship between the ester carbonyl frequency and the dielectric constant of the medium for methyl cinnamate. It is notable that solvents of low dielectric constant that can hydrogen-bond (e.g. chloroform) do not fall on the line, being displaced to a lower frequency than expected. By reading off the frequencies of the productive and non-productive bands, the apparent dielectric constants experienced by the carbonyl groups in the acyl-enzymes can be estimated. For cinnamoyl-chymotrypsin the non-productive conformer carbonyl group experiences a dielectric constant of 50, whereas the productive conformer experiences a value of 70. The determination of the values for the dihydrocinnamoyl derivative is more problematical, since methyl dihydrocinnamate does not

Table 2. Carbonyl frequencies and bandwidths of methyl esters of acyl groups used in studies of acyl-chymotrypsins

Spectra were taken in cells with $CaCl_2$ windows and a 50 μm Telfon spacer.

Acyl group	Frequency (cm^{-1})	Bandwidth (cm^{-1})	Solvent
3-(Indol-3-yl)acryloyl*	1721	25	Hexane
	1708	27	Acetonitrile
	1685	35	^2H_2O
Cinnamoyl	1722 (1730 sh†)	–†	Hexane
	1704	29	Chloroform
	1711	15	Acetonitrile
	1690	40	^2H_2O
[*carbonyl*-^{13}C]Cinnamoyl	1673	11	Acetonitrile
3-(5-Methylthien-2-yl) acryloyl*	1722	22	Hexane
	1709	30	Acetonitrile
	1686	–	^2H_2O
Dihydrocinnamoyl	1745 (1715‡)	10 (15‡)	Hexane
	1725	29	Chloroform
	1732	20	Acetonitrile
	1700	45	^2H_2O

* From Tonge & Carey [13].
† The presence of the shoulder makes estimation of the bandwidth inaccurate.
‡ This additional peak is present in the carbonyl region.

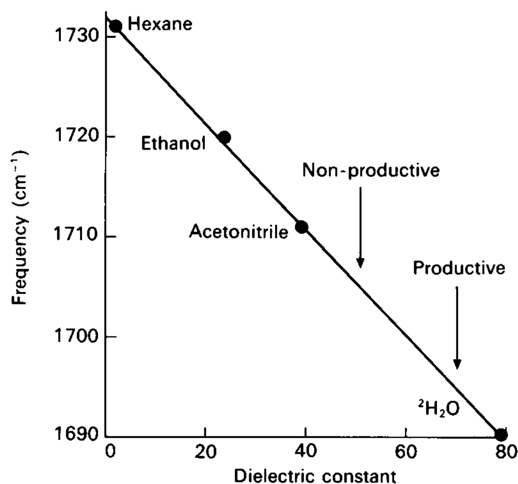

Fig. 7. I.r. carbonyl frequencies of methyl cinnamate as a function of solvent dielectric constant

Spectra were collected for 1 % (v/v) solutions of methyl cinnamate in the various solvents. For interpretation of the arrowed frequencies, which correspond to putative acyl-enzyme conformers, see the text.

give as good a linear relationship as that shown in Fig. 7 (results not shown). Extrapolation (to the equivalent of > 100 % ^2H_2O for the productive conformer) and interpolation of the regression line to the equivalent of Fig. 7 gives estimates of 40 for the non-bonded conformer, 70 for the non-productive conformer and 100 for the productive conformer. These values are very close to estimates obtained by an alternative method that is described below. A very good linear relationship between the fractional ^2H_2O composition of mixtures with acetonitrile and the carbonyl absorption frequency of methyl dihydrocinnamate (results not shown) allows the dielectric constants experienced by the carbonyl groups in the acyl-enzyme to be estimated as above by extrapolation and interpolation. Although the estimated dielectric constant for the productive conformer is well above that for aqueous media, the agreement seen between these two rather different methods lends confidence to the procedures employed. The 1710 cm^{-1} non-productive band of dihydrocinnamoyl-chymotrypsin gives a value of approx. 70, equal to that of the productive cinnamate conformer. The value of approx. 100 for the dihydrocinnamoyl-chymotrypsin productive conformer represents a very polar environment and is similar to that of formamide (109) (not unlike the species with which the carbonyl group interacts in the oxyanion hole), but much less than that of *N*-methylformamide (182). We here propose that the difference in apparent dielectric constants between the conformers is due to hydrogen-bonding and not to a change in other interactions, since there are no obvious candidate groups in the oxyanion hole region of the enzyme which might be responsible for a charge interaction. A notable feature is the high apparent polarity of the environment of all the conformers; a high apparent dielectric constant has been predicted in the interior of proteins from calculations and mutagenic studies of the pH-dependence of subtilisin [28,29].

Estimation of the catalytic power that may be provided by the oxyanion hole interaction

Concept of the catalytic building block. In this section we pose the question: To what extent does the oxyanion hole interaction provide catalytic rate enhancement? In order to aid our understanding of enzyme catalysis at the atomic level it will be useful if it proves possible to dissect enzyme catalytic mechanisms into a group of 'catalytic devices' that can be notionally assembled to form a complete system. Each catalytic device would be seen as contributing a certain fraction of the total rate enhancement, and it thus may prove possible to define a set of building blocks that may, in the future, be engineered into proteins or other materials with a view to the creation of 'tailor-made' catalysts. Although at the present time such a task seems difficult, the i.r.-spectroscopic studies reported here do provide an opportunity to address this question. It may be, of course, that a high degree of co-operativity occurs between catalytic devices and that enzyme

catalytic systems have evolved as complete entities; if this is so the building-block concept will not be easy to apply. Some experiments that have employed site-specific mutagenesis [8] and some computational studies [7,30,31] bear upon this question and are discussed below.

Estimation of catalytic rate enhancement from vibrational theory. If it is assumed that the ester carbonyl stretch vibration in the non-productive and productive conformations of acyl-enzymes can be approximated by the equation for simple harmonic motion:

$$V = (1/2\pi)\sqrt{(f/\mu)}$$

where V is vibrational frequency in cm^{-1}, f is the force constant and μ is the reduced mass, we can calculate the ratio of the force constants in the non-bonded and productive conformations of dihydrocinnamoyl-chymotrypsin as:

$$R_f = (V_{nb}/V_p)^2 = (1731)^2/(1692)^2 = 1.047$$

This represents a 4.7% change in the force constant.

Unfortunately there is no simple theoretical relationship between the force constant of a bond and its strength (ΔH) since the former relates to a small displacement and the latter to a large displacement (dissociation). If force constants for a wide range of bonds are plotted against the ΔH values, an empirical curved relationship is seen where the points for most bonds lie close to a single curve [32]. A tangent drawn to this curve at a ΔH value of 800 kJ/mol, which is the C=O bond strength, gives a slope of 1.4 ($\Delta f/\Delta\Delta H$). Thus a 4.7% change in force constant can be approximated to a 3.4% change in ΔH, namely a change in ΔH of 26.9 kJ/mol. This loss of bond enthalpy by hydrogen-bonding in the oxyanion hole represents ground-state electronic strain, and is directed towards transition-state formation and so can be subtracted from the overall activation free energy of the process. Direct application of absolute reaction rate theory allows calculation of a notional rate-enhancement factor that will result from this effect:

$$R_k \approx \exp(\Delta\Delta H/RT) \approx \exp[26900/(8.3 \times 298)] \approx 5.3 \times 10^4$$

We assume that any change in entropy will not be unfavourable relative to reaction from an unbonded state as far as the free energy of activation is concerned, since the ligands to which hydrogen-bonding occurs are fixed in space.

Similarly the frequency difference between the non-productive (Henderson) and productive conformers can be used to calculate a putative rate-enhancement factor. In this case the value is only 100, but this represents only the difference between two hydrogen-bonded conformations, and not the whole of the realizable interaction as above. Similar calculations for cinnamoyl and dehydrocinnamoyl groups yield values of 16 and 32 respectively. That non-bonded conformations are not seen for the rigid unsaturated acyl groups may mean that they are unable to access this state for steric reasons.

Although no strong claim can be made for the exactitude of these estimates, it seems likely that a significant rate-enhancement dependent upon ground-state electronic strain induced by the oxyanion hole is seen in the deacylation of dihydrocinnamoyl-chymotrypsin. The significance of these values should be judged against the lack of specificity of the acyl groups (approx. 10^{-4}–10^{-3} of $k_{cat.}$ for acetyl-L-tyrosyl-chymotrypsin). Thus we might expect to see larger spectroscopic shifts and rate-enhancement factors when more specific substrates are studied.

While we propose, as above, that the contribution of ground-state electronic strain to rate enhancement can be estimated from the decrease in carbonyl bond strength, we propose also that the potential stabilization of the transition state is related to the enthalpy of the hydrogen bonds that form in the ground state. The degree to which these will be strengthened by lengthening of the C=O bond distance and accumulation of negative charge on the oxygen atom will depend upon how well aligned the interaction is in the ground state and hence on how strong these bonds are.

I.r.-spectroscopic estimation of hydrogen-bonding strength and associated catalytic rate enhancement

Lady & Whetsel [33] studied hydrogen-bonding in model systems using i.r. spectroscopy. Of interest to us here are the studies of hydrogen-bonding in aniline (and *N*-methylaniline)–ethyl acetate complexes in cyclohexane. The hydrogen bonds in these 1:1 complexes are expected to have a similar effect on the carbonyl vibration frequency compared with the bonds in the oxyanion hole. The N–H stretching vibration of the anilines was used to detect and measure hydrogen-bond formation. This frequency (3483 cm^{-1} for symmetric N–H stretch in free aniline) shifts to lower values on hydrogen-bonding and increases in intensity.

When the amount of ethyl acetate added to a fixed amount of aniline is varied, hydrogen-bond formation is 'titrated' and an equilibrium constant for bond formation can be extracted from the raw data. This experiment is repeated at several temperatures to allow extraction of ΔH and ΔS.

In order to allow correlation with our experimental observations, we have performed a related experiment in which spectra were measured in the ethyl acetate carbonyl stretching region as the amount of aniline in excess over a small fixed amount of ethyl acetate was varied, this is shown in Fig. 8. The purpose was to allow correlation between the measured spectroscopic shift in the carbonyl region with the thermodynamic parameters measured in the N–H stretching region. It is important to note that it is necessary to correct for the effect on the carbonyl frequency of the change in the dielectric constant of the medium as the amount of added aniline is increased. This is achieved by adding aniline to concentrations well beyond that required to saturate hydrogen-bonding (i.e. $> 20\%$, v/v). At concentrations of aniline above saturation there is a linear relationship between the carbonyl frequency and the concentration of added aniline, and this allows an extrapolation to zero aniline concentration. The frequency shift for hydrogen-bond formation is thus taken as the difference between the extrapolated value at zero aniline concentration and the measured value at zero aniline concentration. For both systems the frequency shift is -13 cm^{-1}. The associated ΔH values, taken from ref. [33], are -13 kJ/mol and -14.7 kJ/mol for aniline and *N*-methylaniline respectively. Thus the enthalpy for hydrogen-bond formation in the productive conformer relative to the non-productive conformer of the dehydrocinnamoyl acyl-enzyme will have approximately this strength, since the frequency shift between conformations is 13 cm^{-1}, whereas that for dihydrocinnamoyl-chymotrypsin (18 cm^{-1}) will be somewhat stronger and that for cinnamate (10 cm^{-1}) somewhat weaker. The enthalpy difference between non-bonded and productive conformers of dihydrocinnamoyl-chymotrypsin will be considerably larger and represent a very strong 'biological' hydrogen bond. These values compare with a range of approx. 5–40 kJ/mol for biological hydrogen bonds and with a value of approx. 21 kJ/mol for the N–H···O bond in amides [34]. It is important to appreciate that the enthalpy of formation of a hydrogen-bonding system represents the excess bonding energy allowing for weakening of the C=O and N–H bonds; the hydrogen bond itself is thus stronger than the sum of the losses in the N–H and C=O bonds.

Although there is no theoretical correlation, there has been a long-standing contention enshrined in the Badger–Bauer re-

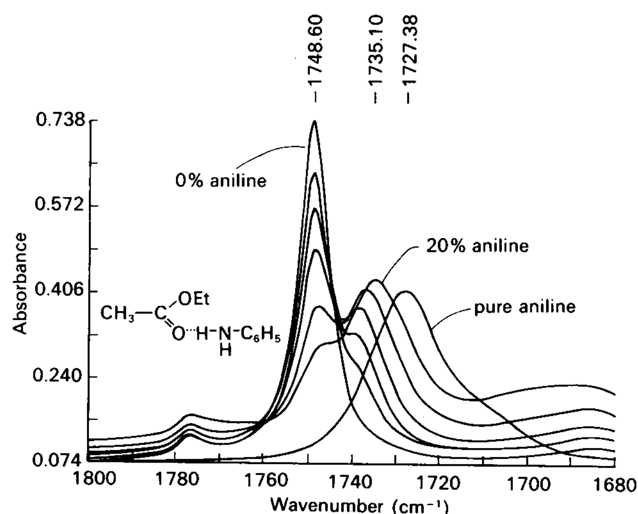

Fig. 8. Aniline-induced hydrogen-bonding shift in the frequency of the ethyl acetate carbonyl stretch vibration

Aniline (0–20%, v/v) was added to 10 μl of ethyl acetate in a total volume of 1 ml.

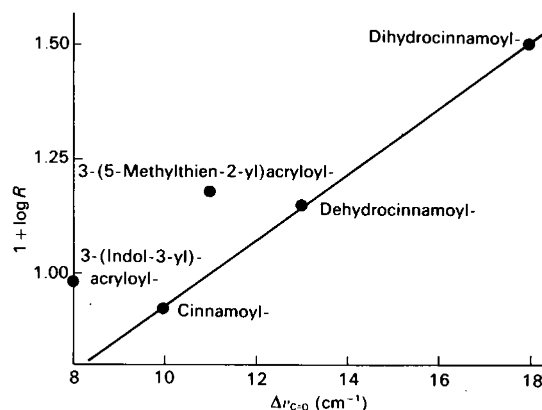

Fig. 9. Correlation between the carbonyl frequency shift induced by hydrogen-bonding and the logarithm of the corrected rate ratio for acyl-chymotrypsin hydrolyses

The carbonyl frequency shift was measured between the non-productive (Henderson [24]) conformer and the productive conformer in each case. The rate ratio R is the value of the first-order rate constant for hydrolysis of the acyl-chymotrypsin at high pH (i.e. His-57 fully deprotonated) divided by the apparent first-order rate constant for the hydrolysis of the equivalent acylimidazole measured at pH 10.5 [13]. Values of the rate constants are given in Table 3.

lationship [35] that there is an empirical linear correlation between the frequency shifts of hydrogen-bonding species (e.g. O–H, N–H and C=O stretch vibrations) and the enthalpy of formation of the hydrogen bond. This has been both supported (see, e.g., refs. [36] and [37]) and questioned (see, e.g., ref. [38]) in a large number of studies. Three studies that support the existence of the Badger–Bauer relation can be used to estimate hydrogen-bond strengths from spectroscopic shifts. A computational study of gas-phase hydrogen-bonding of a range of donors to formaldehyde [37] gives $-\Delta H$ values of 30.6, 22 and 17.2 kJ/mol respectively for dihydrocinnamoyl-, dehydrocinnamoyl- and cinnamoyl-chymotrypsin (non-productive relative to productive conformations) respectively; clearly these values are larger than those estimated from the ethyl acetate studies. The very large value of 66 kJ/mol is predicted for the enthalpy difference for non-bonded and productive conformers for dihydrocinnamoyl-chymotrypsin. A study of ethyl acetate hydrogen-bonding in carbon tetrachloride [36], albeit using inorganic Lewis acids, predicts $-\Delta H$ values of 9.6, 6.9 and 5.3 kJ/mol respectively for the differences for non-productive and productive conformers and 21 kJ/mol for the difference for the non-bonded and productive conformers as above.

A recent very extensive study [39] using trichloroethylene as solvent and *N*-methylpyrrolidinone as carbonyl acceptor predicts free energies of −8.4, −4.1, −1.5 and −26.5 kJ/mol respectively. Thus the predicted values for hydrogen-bond strength cover a considerable range; although these correlations provide useful estimates that may be compared with the enthalpy of activation of the deacylation process (61 ± 4 kJ/mol for cinnamoyl-chymotrypsin; S. Ingram & C. W. Wharton, unpublished work), it will be necessary to refine these methods before more accurate and consistent estimates will become available.

We propose that the strength of the ground-state hydrogen-bonding will be dependent upon the kinetic specificity of the acyl group, and so, in view of the poor specificity of the acyl groups studied here, very strong hydrogen bonds may be seen in highly specific substrates (Tonge & Carey [13] have seen lower frequencies than are reported here for productive modes of acyl-enzymes using resonance Raman spectroscopy). In this connection Kollman and co-workers [7] have calculated that oxyanion-hole hydrogen-bonding stabilizes the acylation tetrahedral intermediate in trypsin catalysis by 48 kJ/mol, and thus this interaction when transition-state stabilization is also taken into account is predicted to play a major role in rate enhancement.

Correlation of spectroscopic frequency shifts with kinetic parameters

As stated above, we propose that the strength of the ground-state hydrogen-bonding interaction in the oxyanion hole represents a measure of the potential transition-state stabilization that may be achieved when this state is reached. If this is so and the Badger–Bauer relation is valid in the environment of the enzyme active centre, it is predicted that there should be a linear relationship between the logarithm of the kinetic-rate ratio of an acyl-enzyme (a measure of the activation energy) and the frequency shift seen in the ester carbonyl group between the non-productive and productive conformers. It is necessary to correct for the intrinsic reactivity of the acyl groups and to use the kinetic-rate ratio so that only the enzyme-induced kinetic effect is taken into account, as described in ref. [13]. The kinetic parameters used to construct Fig. 9 are given in Table 3. That there is such a linear relationship, at least among groups that possess some structural similarity, is shown in Fig. 9. Notably the acetylenic compound has high intrinsic reactivity, which means that the observed deacylation rate is also high. The correlation may be interpreted in terms of what we shall call 'steric demand'. Thus dihydrocinnamate is very flexible and shows a highly disperse non-productive conformation, and this will be most easily deformable to the productive conformer. Dehydrocinnamate, which is essentially a rigid rod and therefore not deformable, may be able to access the productive conformation by a simple rotation about the serine β-C–O bond or the O–C=O axis. The cinnamate group is relatively well defined in both conformers and is proposed to flip between these two states; the rigid planar structure may require complex motions in order to negotiate the pathway between the conformations and will then be less stable in the acquired state. We would expect a similar relation to occur between non-bonded and productive conformers, but, since we do not see these for any acyl-enzyme except dihydrocinnamoyl-chymotrypsin, this cannot be tested.

An empirical derivation (not shown) predicts a linear relation

Table 3. Rate constants for the hydrolysis of acyl-chymotrypsins and acylimidazoles at 25 °C

$k_{obs.}$ is the first-order rate constant for the hydrolysis of the acyl-enzymes at high pH (in water), where His-57 is fully deprotonated. For k_{OH^-}, hydrolysis of acylimidazoles was measured in 0.1 M-carbonate buffer, pH 10.5, containing 0.1 M-NaCl.

Acyl group	$10^2 \times k_{obs.}$ (s^{-1})	$10^2 \times k_{OH^-}$ (s^{-1})	Ratio
3-(Indol-3-yl)acryloyl*	0.21	0.22	0.96
Cinnamoyl	1.86†	2.25	0.83
3-(5-Methylthien-2-yl) acryloyl*	2.0	1.30	1.53
Dehydrocinnamoyl	23.3‡	16.3	1.43
Dihydrocinnamoyl	28§	8.8	3.18

* From Tonge & Carey [13].
† Measured at 310 nm in 0.2 M-carbonate buffer, pH 10.5.
‡ Determined by measurements in the range pH 4–6.5 in 0.1 M-acetate and phosphate buffers and fitting of the rate constants to a sigmoid pH-dependence curve to obtain the limiting value at high pH.
§ From Kogan *et al.* [40].

between the difference in the squares of the frequencies of the non-productive and productive conformers (assumed to be simple harmonic oscillators) and the logarithm of the corrected rate ratio; this plot (not shown) is also linear for related acyl groups, as in Fig. 9. The reason for this is that the difference of two squared terms can be factorized to give the sum and difference. The sum term, the sum of two large and similar numbers, will not alter much, but the difference will show considerable change. For this reason plots of the frequency shift will, in practice, have a similar form to plots of the differences of the squares of the frequencies. Thus a deviation from simple harmonic motion for the carbonyl vibrations is not implied; this may also explain why the Badger–Bauer relation works in many cases.

Distribution and energy differences between conformers

The enthalpy of hydrogen-bond formation estimated from frequency shifts (e.g. -13 kJ/mol for a 13 cm^{-1} shift in the ethyl acetate/aniline system) would suggest that the productive conformer should be very strongly favoured (an approx. 200-fold predominance for an enthalpy difference of -13 kJ/mol) if hydrogen-bond formation were the only factor controlling the distribution of conformers. For cinnamoyl-chymotrypsin (10 cm^{-1} shift), whose spectrum has been analysed by deconvolution, the ratio of intensities of non-productive to productive peaks is approximately unity at pH 4, but the productive bandwidth is half that of the non-productive; there is thus a 2-fold difference in population if the absorption coefficients of the conformers are assumed to be equal. Plainly some other factor has an important role in defining the distribution of conformers. Whereas the non-productive conformers are somewhat restrained, the non-bonded conformer of dihydrocinnamoyl-chymotrypsin represents a state of low steric energy in which the acyl group finds an unstrained energy minimum in the relatively hydrophobic environment of the specificity binding pocket. When the productive hydrogen-bonding conformer is formed most of the enthalpy of the hydrogen bonds is used to overcome steric strain. This behaviour is not unexpected for non-specific acyl groups, but will have important consequences regarding the role of steric strain in enzymic catalysis if it persists in more specific acyl-enzymes. We have not yet fully established the exchange status of the conformers; for cinnamoyl-chymotrypsin in reacting conditions at pH* 6 both conformations decay to zero with the same first-order rate constant. This finding suggests that the conformations are in equilibrium, but further characterization is necessary.

General discussion, conclusions and overview

As rehearsed by Knowles [2], there has been a dichotomy in our thinking concerning the source of enzyme catalytic power. These conventional wisdoms have on the one hand focused upon the sequence of chemical catalytic steps that occur upon the reaction co-ordinate, and have on the other hand focused upon the ability of enzymes to bind and stabilize transition states. In reality, of course, these approaches are closely complimentary and lead to both dynamic and static visions (albeit still somewhat primitive) of how enzymes function. The role, if any, of ground-state strain has received little attention, although its potential importance has been noted [41]. There is little evidence for steric strain in ground states of enzyme-catalysed reactions, although this does not rule out its involvement. By contrast, there is now considerable evidence for polarization of carbonyl groups in enzyme–substrate complexes [15–17]. A notable feature of all these studies has been a lack of detailed quantitative analysis of the consequences of carbonyl polarization in terms of energetics and kinetics. Examination of the chemical literature readily reveals the reason for this in that analysis can be attempted at two levels, both of which prove difficult to drive to a successful and secure conclusion. Firstly, an analysis may be based upon the concepts and mathematical analysis of chemical physics. Virtually all of this work is conduced in the gas phase, the reacting systems are di- or tri-atomic, and the equations require accurate knowledge of molecular parameters. It has not proved possible to adapt these equations in any simple way such that they may describe the relative 'black box' of the enzyme active site, although computational work has made progress in this direction [7,8]. It should be possible to build an effective interface with computational studies such as those by Kollman and co-workers [7], since vibrational frequencies could be computed from estimates of bond energies. A promising semi-empirical approach, as yet in its infancy, which relates bond strengths, lengths and energies has been developed by Burghi & Dunitz [42]. The alternative approach is based on the concepts and methods of physical-organic chemistry and describes work primarily done in the solution phase. Although advances are being made in this area (see, e.g., ref. [39] and references cited therein), the correlations that have been established between such as the carbonyl frequency shift and hydrogen-bonding strength are highly empirical. Clearly solvation effects are important, and so this restricts the effective use of these correlations in that they are probably only relevant to the particular solvents in which the studies have been done and to the particular molecules that were chosen as donors and acceptors.

The rate-enhancement factor of approx. 5.0×10^4 calculated from the differences in the enthalpies of the non-bonded and productive conformations of dihydrocinnamoyl-chymotrypsin represents (on a logarithmic scale) perhaps half the total rate enhancement of the enzyme-catalysed reaction. The rate enhancement predicted to result if the enthalpy difference between the non-productive and productive conformations is all that can be utilized to reduce the activation energy is only about 100. However, in the Henderson structures there will be some hydrogen-bonding via the water bridge to His-57, and so a true non-bonding reference will not have been achieved. We speculate that both of these factors will be considerably larger when more specific substrates are measured (this is suggested by the generally somewhat larger frequency shifts seen for specific enzyme–substrate complexes [15–17]). Although it may be the case that transition-state stabilization is the predominant binding factor in

rate enhancement, we propose that ground-state electronic strain also has a highly significant role to play. Further, we propose that it will prove beneficial to pay careful attention to ground-state interaction when planning strategies for the mutagenic alteration of existing catalysts or in the design of catalysts *de novo*. This is particularly so in view of the way in which it is envisaged that these interactions guide the ground state to the transition state. This is seen as being analogous to organizing the system so that it is aligned on the correct reaction co-ordinate and progressed along it by ground-state electronic strain such that the transition state will subsequently be achieved along the minimum-energy path.

Mutation of the subtilisin oxyanion hole: an apparent paradox

As outlined in the Introduction, the oxyanion hole of subtilisin can be mutated so as to remove one half of the interaction [8,43]. When this was done by replacing Asn-155 with Ala-155 or Leu-155 the K_m value changed by only 2–4-fold while $k_{cat.}$ was decreased by two to three orders of magnitude. These findings were interpreted to mean that the role of the oxyanion hole is to provide transition-state stabilization, and this view has been supported by computational studies that emphasize the electrostatic nature of the interaction in the oxyanion hole [30,31]. It is well established that in enzymic catalysis potential binding energy can be sacrificed to provide rate enhancement; this is demonstrated clearly in elastase-catalysed reactions [44]. If binding energy is used to strain a substrate whether electronically or sterically and this strain is directed towards transition-state formation, then the effect will always show up in $k_{cat.}$ and not in K_m. Thus the observation of a change in $k_{cat.}$ can have the attributes of a mirage in that it can equally well be ascribed to a change in a ground-state effect as to a change in transition-state stabilization. It is our contention that in the absence of a direct physical observation of the presence or absence of strain it is not possible to assign such effects in a meaningful way.

This argument strengthens our belief that ground-state electronic strain is a significant factor in enzyme catalytic rate enhancement.

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