EVIDENCE FOR N ⟶ O TRANSFER IN THE DEACYLATION OF AN ACYL-α-CHYMOTRYPSIN

Keith Brocklehurst

Department of Biochemistry and Chemistry,
St. Bartholomew's Hospital Medical College,
Charterhouse Square, London, E.C.1., England.

Kenneth Williamson

Department of Chemistry, John Dalton College of Technology,
Chester Street, Manchester, 1, England.

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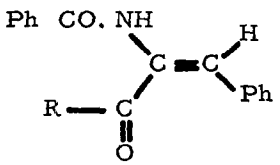
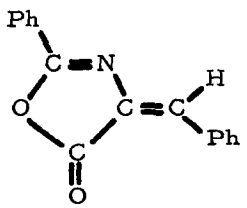
We recently reported the α-chymotryptic hydrolysis of methyl α-benzamido-cis-cinnamate (I) in which the enzyme probably binds the N-acylamino-side-chain of (I) but not the β-phenyl group (Brocklehurst and Williamson, 1966).

We now report that 4-cis-benzylidene-2-phenyl-2-oxazolin-5-one (II) an acylating agent which possesses the β-phenyl group of (I) but not the N-acylamino-side-chain, acylates α-chymotrypsin rapidly in the pH-range 5 - 11 to provide an acyl-enzyme (III) which subsequently undergoes deacylation to provide α-benzamido-cis-cinnamic acid (IV).





(I) R = OMe (II)

(III) R = enzyme

(IV) R = OH

When a solution of (II) in dioxan is added to a solution of α-chymotrypsin (in molecular excess) in acetate buffer pH 5, the absorption band of (II) [λ_{max} = 364 mμ] is rapidly replaced by the absorption band of III [λ_{max} = 305 mμ] which changes only slowly into the absorption band of (IV) [λ_{max} = 275 mμ]. Similar results are obtained at higher pH's

except that in the pH range 6.5-7.5, where deacylation is fast, although the absorption band of (II) disappears within 15 seconds of the admixture of (II) with the enzyme, the spectrum observed initially is always a mixture of those of (III) and (IV). At pH 8, where deacylation to provide (IV) is again slow, the rapid disappearance of (II) is still observed but the absorption maximum of (III) is now 285 mμ. Although caution must be exercised in identifying acylated centres in enzymes by the positions of their ultraviolet absorption bands, the data in the Table suggest that the centre acylated at pH 5 could be the imidazole group of a histidine residue and that acylated at pH 8 could be the hydroxyl group of a serine residue. α-Benzamido-cis-cinnamate ion and (I) absorb at longer wavelengths, by 5.5 and 6.5 mμ respectively, than the corresponding unsubstituted trans-cinnamoyl compounds. In the agreement with the assignment of the centre acylated at pH 8 to a serine hydroxyl group, the acyl-enzyme (III) prepared at this pH absorbs at about the same wavelength as (I) and at longer wavelength by 4.5 mμ than O-trans-cinnamoyl-N-acetylserinamide. The acyl-enzyme (III) prepared at pH 5 absorbs at slightly shorter wavelength (by 2 mμ) than N-trans-cinnamoylimidazole (NTCI). Even in the absence of environmental effects of the enzyme, α-benzamido-cis-cinnamoylimidazole might be expected to absorb at shorter wavelength than the value predicted from a consideration of the other compounds in the Table, due to steric inhibition of conjugation. This possibility is being examined by a study of model compounds.

Table

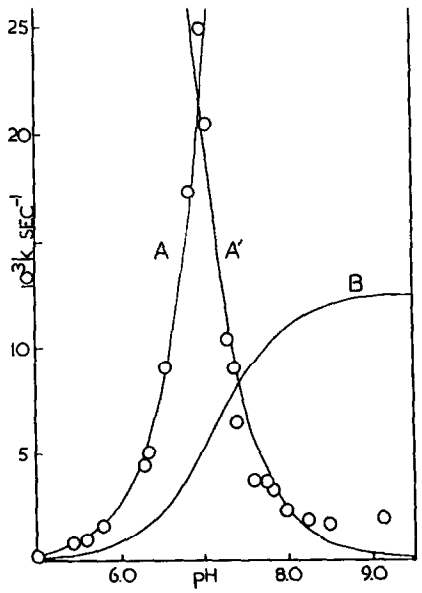
Absorption spectra of acyl-α-chymotrypsins and reference compounds

Compound	λ_{max} mμ	Solvent	Reference†
trans-cinnamate ion	269.5	water	a
α-benzamido-cis-cinnamate ion	275	4.8% dioxan	b
methyl-trans-cinnamate	279.5	3% acetonitrile	a
(I)	286	4.8% dioxan	b
O-cinnamoyl-N-acetyl-serinamide	281.5	10% acetonitrile	a
(III) prepared at pH 8*	285	4.8% dioxan	b
(III) prepared at pH 5*	305	4.8% dioxan	b
N-trans-cinnamoylimidazole	307	1.6% acetonitrile	a
trans-cinnamoyl-α-chymotrypsin*	292	1.6% acetonitrile	a

*Difference spectra vs. α-chymotrypsin

†(a) Bender, Schonbaum and Zerner, 1962; (b) this work.

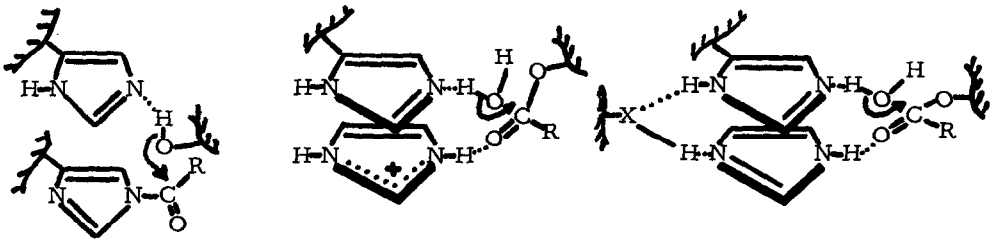
The tentative assignment of the centre acylated at pH 5 to an imidazole group and that acylated at pH 8 to a serine hydroxyl group finds support in the unusual rate-pH profile for the deacylation of (III). Whereas the deacylation of trans-cinnamoylchymotrypsin (TCC) is dependent upon a single ionizing group of pK_{app} 7.15 required in the base form (Bender, Schonbaum and Zerner, 1962) the pH-dependence of the first order rate constant for the deacylation of (III) is complex (see Figure). In the pH range 5 - 7 it is dependent upon the base form of a single ionizing group of pK_{app} ca. 7.7 [i.e. $k = \bar{k}/(1 + [H^+]/K_b)$] whereas above pH 7 it is dependent upon the acid form of a single ionizing group of pK_{app} ca. 6.4 [i.e. $k = \bar{k}/(1 + K_a/[H^+])$]. The data in the pH range 5 - 8 do not fit a single rate equation for acid-base catalysis, i.e. $k = \bar{k}/(1 + K_a/[H^+] + [H^+]/K_b)$. At alkaline pH the deacylation appears to be subject to catalysis by hydroxide ion. This is being investigated further and will form the subject of a future publication. The deacylation of TCC is susceptible to hydroxide ion catalysis only when the active site is exposed by treatment with urea (Bender, Schonbaum and Zerner, 1962). A possible interpretation of these spectrophotometric and kinetic data is stated briefly below.



Figure

Deacylation of (A, A') α-benzamido-cis-cinnamoyl-α-chymotrypsin and (B) trans-cinnamoyl-α-chymotrypsin. (A, A'): Buffers : acetate, phosphate and tris; 4.8% dioxan; $\underline{I} = 0.1$; 25.0°; the rate constants were determined from the first order decay of the absorbance at 310 mμ; the curves are theoretical for (A) $\bar{k} = 130 \times 10^{-3}$ sec^{-1}; $pK_{app} = 7.68$ and (A') $\bar{k} = 100 \times 10^{-3}$ sec^{-1}; $pK_{app} = 6.40$ (B) is taken from Bender, Schonbaum and Zerner (1962); the curve is theoretical for $\bar{k} = 12.5 \times 10^{-3}$ sec^{-1}; $pK_{app} = 7.15$.

In the pH range 5 - 7 the imidazole group of one of the two (Hartley, 1964) histidine residues of α-chymotrypsin is acylated by (II). The acyl moiety is then transferred, in the rate-limiting step, to a serine hydroxyl group, this transfer being dependent upon the base form of the other imidazole group in the enzyme [as in (V)]. The deacylation of the serine residue



(V) (VI) (VII)

occurs subsequently in a rapid step which involves general base catalysis by the base form of one imidazole group and general acid catalysis by the acid form of the other imidazole group [as in (VI)]. One version (Bender and Kézdy, 1964) of the currently accepted mechanism (see also Hartley, 1964) of α-chymotryptic hydrolyses involves the acid-base catalysis of the deacylation of an acylated serine residue by two imidazole groups required in the base form to achieve a concerted reaction via a proton transferring group XH [as in (VII)]. It is suggested that when α-chymotrypsin is acylated by (II) the active site is distorted, possibly as a result of "antiproductive" binding of the β-phenyl groups of (II) or (III) and/or the N-acylamino-side-chain which is generated when (III) is formed. It is suggested also that as a result of this distortion (a) the concerted reaction via XH is no longer possible (b) the active site may be exposed to hydroxide ions when the serine residue is acylated and (c) either the site of acylation in the pH range 5 - 7 is changed from serine to histidine or if a histidine residue is acylated first also in the hydrolysis of substrates, the transfer of the acyl moiety to serine becomes rate-limiting consequent upon the antiproductive binding.

As the pH is raised from 5 to 7, the rate of transfer of the acyl moiety becomes very fast and the rate of deacylation of the acylated serine residue

becomes slow, both rate changes being the result of the deprotonation of one imidazole group. It is suggested that the sharp break at pH ca. 7 in the rate-pH profile is the result of a change in the rate-limiting step of the deacylation from transfer of the acyl moiety to serine to the deacylation of the acylated serine residue. The latter step is dependent upon the acid form of a single imidazole group. The other imidazole group required in the base form is always in the base form in the pH range in question when the serine residue is acylated, since it is now perturbed by the close proximity of the protonated imidazole group [see (VI)]. Such a perturbation of the second imidazole group in the enzyme has been suggested previously (Bender and Kezdy, 1964) to account for the fact that whereas two imidazole groups are required in (VII) for mechanistic and stereochemical reasons, and although the participation of two such groups is suggested by their juxtaposition in the primary sequences of both chymotrypsin and trypsin (Smillie and Hartley, 1964; Hartley, 1964) only one such group has been detected kinetically (Kezdy, Clement and Bender, 1964).

A requirement of the mechanism involving (V) and (VI) is a change in the geometry of the active site on acylation of the serine residue. A change in conformation at this stage of α-chymotryptic catalysis is suggested by the fact that the N-terminal isoleucine-α-ammonium ion is probably required to maintain the geometry of the active site in the acylation by a substrate of the serine residue but is not required in its deacylation (see Bender and Kezdy, 1964).

That (II) acylates α-chymotrypsin in or near the active site is suggested by the protection which such acylation affords towards subsequent acylation at pH 5 by NTCI, a reagent which is known to acylate specifically the active site of the enzyme (Bender, Schonbaum and Zerner, 1962).

A preliminary study by spectrophotometry of the acid hydrolysis of the acyl-enzyme prepared at pH 5 shows that in the pH-range 1 - 2 it is converted rapidly into a product which has a spectrum closely similar to that of the acyl-enzyme prepared at pH 8. This is in agreement with the tentative assignment of the centre acylated at pH 5 to the imidazole group of a histidine residue. An acylated imidazole group would be protonated at low pH and its acyl moiety would therefore be transferred readily (Jencks and Carriuolo, 1959) to another nucleophilic centre, especially if this were

sterically aligned for reaction.

The configurations of (I), (II) and (IV) are those suggested by Buckles, Filler and Hilfman (1952). Currently, more direct evidence for these assignments is being sought. A detailed kinetic, spectrophotometric and spectropolarimetric study of the acylation of α-chymotrypsin by (II) and its trans-isomer is in progress.

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