Intramolecular Nucleophilic and General Acid Catalysis in the Hydrolysis of an Amide. Some Comments on the Mechanism of Catalysis by Serine Proteases

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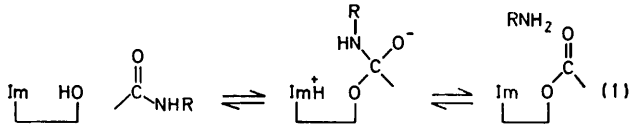
The lactonisation of *N*-(2-aminoethyl)-6-*endo*-hydroxybicyclo[2.2.1]heptane-2-*endo*-carboxamide shows a sigmoid pH–rate profile which is interpreted, kinetically, in terms of the hydroxide-ion-catalysed hydrolysis of the amide with the terminal amino-group unprotonated and protonated. Reaction of the latter species occurs with a rate enhancement of *ca.* 10^9 compared with an amide lacking the hydroxy- and protonated amino-groups. This is attributed to intramolecular nucleophilic and general acid-catalysis. The relative effectiveness of these two processes are compared and it is concluded that intramolecular general acid-catalysis makes a relatively minor contribution to the rate enhancement even though the breakdown of the tetrahedral intermediate is thought to be a concerted process. Some comments are made about the mechanisms proposed for the chymotrypsin-catalysed hydrolysis of amides and concerted breakdown of the tetrahedral intermediate is suggested as a possible mechanism.

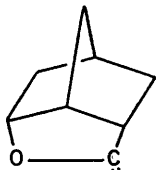
THE serine proteases catalyse the hydrolysis of amides with the intermediate formation of an acyl-enzyme by the transfer of the acyl group from the amide to a serine hydroxy-group in the protein.[1-3] The mechanism of this acyl transfer reaction is thought in turn to involve the formation of a relatively unstable tetrahedral intermediate by analogy with the corresponding simple reactions in solution.[4-7] It is generally accepted that the formation of the tetrahedral intermediate is assisted by general base catalysis by the imidazolyl group of histidine and that the imidazolium ion acts as a general acid to facilitate amine expulsion from the addition intermediate [1-3] [equation (1)]. However, there are several problems with this mechanism in its simplified form [8,9] and the exact nature of the rate-limiting step is not clear for the enzyme-catalysed hydrolysis of the 'natural' amide substrates.

It is often suggested that the transfer of a proton between electronegative atoms of substrates and those of acidic or basic groups in enzymes contributes to the large rate enhancement observed in enzyme-catalysed reactions.[10] Two aspects of the involvement of functional groups on the enzyme should be distinguished, (1) the rate enhancement brought about by 'chemical catalysis' relative to the 'uncatalysed' or 'solvent-catalysed' reaction, *i.e.*, the importance of catalysis by acidic or



basic species compared with that of proton transfer to or from water and (2) the rate enhancement brought about by 'chemical catalysis' occurring within the enzyme–substrate complex, and being of lower kinetic order than an analogous intermolecular reaction.[11]

In an effort to provide additional insight into these matters we have studied the hydrolysis of the amide (I). It is known that the neighbouring hydroxy-group greatly enhances, by *ca.* 10^7—10^8, the rate of hydrolysis of other hydroxy-amides analogous to (I) to give the lactone (II) as an intermediate. This rate enhancement is attributed to intramolecular nucleophilic catalysis similar to that proposed for the serine proteases.[12] We wished to determine the importance of intramolecular



general acid–base catalysis in the hydrolysis of (I). A preliminary report of this work has been published.[13]

EXPERIMENTAL

Materials.— N-(2-*Aminoethyl*)-6-endo-*hydroxybicyclo*-(2.2.1]*heptane*-2-*endo*-*carboxamide* (I). The lactone (II) [12,14] (0.5 g) was refluxed with redistilled 1,2-diaminoethane (35 cm^3) for 24 h. The excess of amine was removed under nitrogen and reduced pressure and the residue chromatographed on silica gel with chloroform–light petroleum (b.p. 40—60°). The product was obtained as a crystalline solid (100 mg) upon recrystallisation from ethyl acetate, m.p. 105—106 °C (Found: C, 60.4; H, 9.05; N, 14.1. Calc. for $C_{10}H_{18}NO_2$: C, 60.6; H, 9.1; N, 14.15%), $\nu_{max.}$ (Nujol) 3 410, 3 290, 3 150, 3 050, and 1 640 cm^{-1}, $\delta(CD_3OD)$ 4.22 (m, 6-*exo*-H), 3.30 (m, $NHCH_2$), and 2.73 (m, 2-*exo*-H and CH_2NH_2).

Other materials used for kinetics were of AnalaR grade. Freshly boiled, deionised water was used throughout and the ionic strength maintained at 0.20M with potassium chloride unless otherwise stated.

Product Isolation and Kinetics.—These were carried out as described previously.[12] For the pH–rate profile of an ionisable substrate the rate constants for the two forms of the substrate and the dissociation constant were treated as disposable parameters.[15]

RESULTS

As described for analogous hydroxy-amides,[12] the alkaline hydrolysis of amide (I) proceeds rapidly to give initially

Observed and calculated pseudo-first-order rate constants for the lactonisation of hydroxy-amide (I) in water at 30.0° and ionic strength 0.20M (KCl)

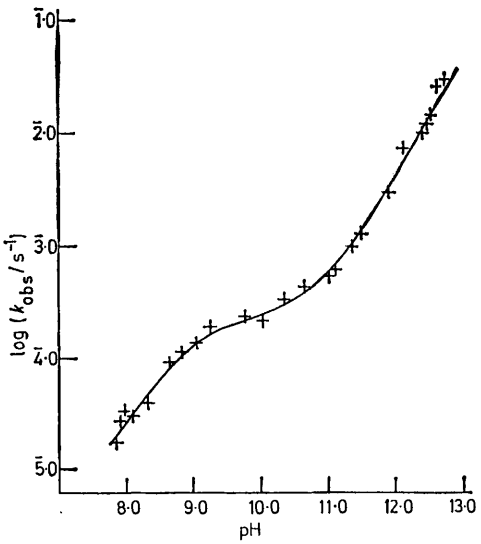
pH	$10^4 k_{obs}/s^{-1}$	$10^4 k_{calc}$ [a]/s^{-1}
12.81	271	215
12.72	240	175
12.62	135	140
12.55	115	119
12.46	96.5	97.3
12.18	69.0	52.1
11.94	27.7	30.9
11.53	12.4	13.3
11.39	9.65	10.3
11.14	5.98	6.70
11.05	5.16	5.85
10.68	3.91	3.70
10.40	3.20	2.92
10.08	2.14	2.41
9.80	2.34	2.11
9.74	2.43	2.05
9.30	1.94	1.58
9.10	1.32	1.33
8.86	1.11	1.01
8.69	0.898	0.803
8.34	0.387	0.447
8.11	0.289	0.287
8.03	0.316	0.244
7.95	0.267	0.207
7.88	0.175	0.179

[a] Calculated from equation (3) with k_1 0.223 l mol^{-1} s^{-1}, k_2 17.3 l mol^{-1} s^{-1}, K_a 1.17 × 10^{-9} mol l^{-1}, and K_w 1.48 × 10^{-14}.

the lactone (II) followed by the hydroxide-ion catalysed hydrolysis of this intermediate to the corresponding hydroxy-acid. The rate of hydrolysis of the hydroxy-amide (I) increases linearly with sodium hydroxide concentration. (Table). No buffer catalysis was observed (up to 0.15M-buffer) with amine, diamine, or phosphate buffers. Observed pseudo-first-order rate constants as a function of pH were determined (Table) and shown graphically in the

$$\text{Rate} = k_1[RNH_2][OH^-] + k_2[\overset{+}{R}NH_3][OH^-] \quad (2)$$

Figure. The pH–rate profile is sigmoid and indicative of two terms in the rate law (2); hydroxide-ion-catalysed hydrolysis of the hydroxy-amide with the terminal amino-group non-protonated (RNH_2) (I) and protonated ($\overset{+}{R}NH_3$) (III). The solid line of the Figure is calculated from equation (3) using K_w 1.48 × 10^{-14}, k_1 0.223 l mol^{-1} s^{-1} (s.d. 5.8%), k_2 17.3 l mol^{-1} s^{-1} (s.d. 4.5%), and K_a 1.48 × 10^{-9} mol l^{-1} (s.d. 6.8%). K_a is the acid dissociation constant of the terminal amino-group and corresponds to a pK_a of 8.93, which may be compared with a value of 9.05 for 1-acetamido-2-aminoethane.[16]

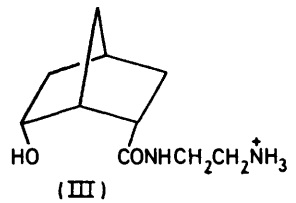


pH–Rate profile for the lactonisation of hydroxy-amide (I) in water at 30.0° and ionic strength 0.2M (KCl). The line is calculated from equation (3) using the constants described in the text and in the Table

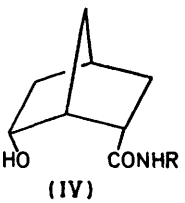
$$k_{obs} = \frac{k_1 K_w K_a / 10^{-pH} + k_2 K_w}{10^{-pH} + K_a} \quad (3)$$

DISCUSSION

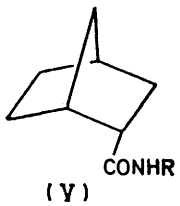
The rates of the hydroxide-ion-catalysed hydrolysis of the hydroxy-amide (IV) is *ca.* 10^6—10^7 fold faster than those of the analogous amides lacking the neighbouring hydroxy-group (V), which is attributed to neighbouring-group participation by the hydroxy-group in (IV) to give the intermediate lactone (II).[12] The rate constant k_1 for



(III)



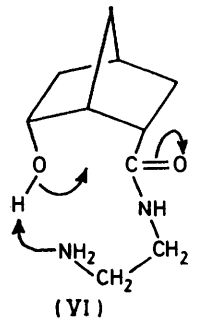
(IV)



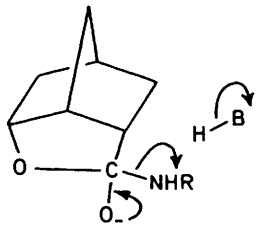
(V)

the hydroxide-ion-catalysed hydrolysis of the *N*-2-aminoethyl-hydroxy-amide (I) agrees well with that predicted from a Brønsted plot of the rate constants as a function of the basicity of the leaving group.[12] However, the rate constant k_2 for the hydroxide-ion-catalysed hydrolysis of the hydroxy-amide with the terminal amino-group protonated (III) is *ca.* 150 times greater than that predicted for the hydrolysis of an amide (IV) where the pK_a of the conjugate acid of the departing amine is 7.5.[12] This rate enhancement is indicative of some form of direct interaction between the terminal protonated amino-group and the reaction centre. The term k_2 is, of course, kinetically equivalent to a spontaneous hydrolysis of the unprotonated hydroxy-amide (I). A spontaneous or uncatalysed reaction is not observed for the other hydroxy amides that have been studied ($k_{H_2O} < 10^{-6}$ s^{-1} [12]). The rate enhancement could therefore be attributed to intramolecular general base catalysis (VI) but this would involve the formation of a ten-membered cyclic transition state, which seems unlikely. Furthermore, the observation of intermolecular general base catalysis in the hydrolysis of the hydroxy-amides (IV) is ascribed to the kinetically equivalent mechanism of general acid-catalysed break-

down of the tetrahedral intermediate (VII).[17] The simplest interpretation, therefore, of the rate enhancement is that the protonated terminal amino-group acts as an intramolecular general acid catalyst by donating a proton to the departing amino-group in the tetrahedral



(VI)



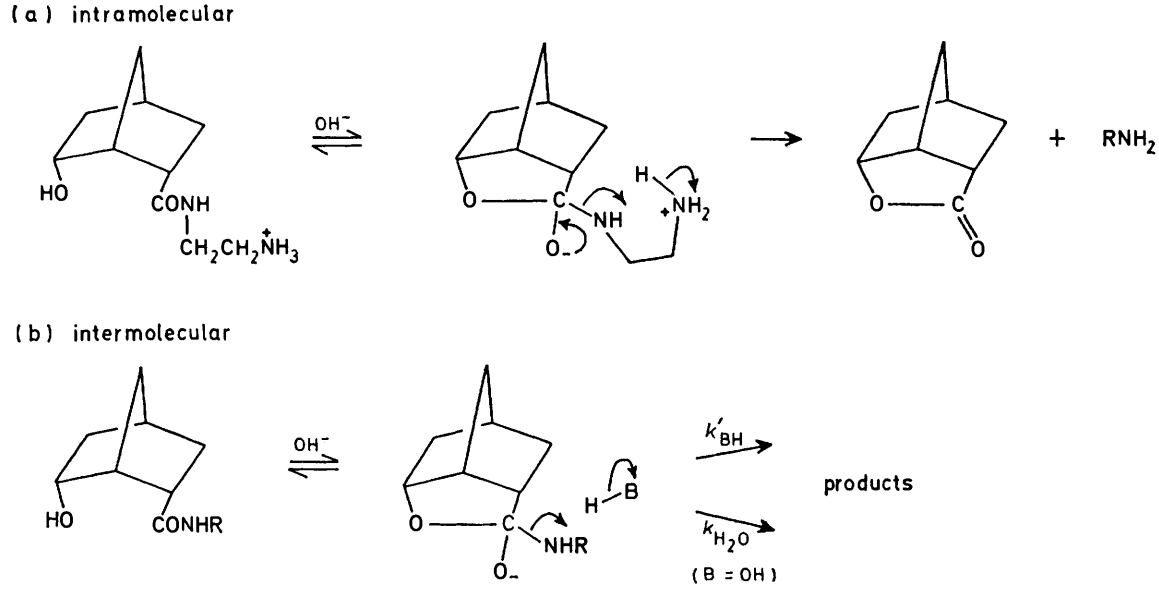
(VII)

intermediate, thus facilitating carbon–nitrogen bond fission (Scheme 1).

Effective Concentration of the Intramolecular General Acid Catalyst.—Although the absolute rate increase caused by intramolecular general acid catalysis [Scheme 1(a)] relative to the 'water' reaction [k_{H_2O}, Scheme 1(b)] is considerable, this is attributable mainly to the difference in acidities of the proton donors. The contribution of intramolecularity itself is small, in agreement with other intramolecular general acid–base catalysed reactions.[18] The second-order rate constant k_2 for the hydroxide-ion catalysed hydrolysis of (III) may be divided by the third-order rate constant.* k_{BH}, for the intermolecular general acid-catalysed reaction of hydroxide-ion with (IV; R = $CH_2CH_2CH_3$), *i.e.*, for the pathway proceeding *via* k_{BH}' [Scheme 1(b)]. This ratio of rate constants gives the effective molarity[18] of the protonated amino-group in the intramolecular reaction [Scheme 1(a)] compared with a protonated amine of similar acidity in the intermolecular reaction [k_{BH}', Scheme 1(b)]. The value obtained is *ca.* 1M and is little affected if the comparison is made with an intermolecular reaction in which the leaving group is trifluoroethylamine [(IV; R = CH_2CF_3)].

The effective molarity is small, in agreement with other intramolecular general acid–base-catalysed reactions,[18] and this is attributed to the 'loose' transition state involved in these proton-transfer reactions. The entropy associated with the low-frequency vibrations in the 'loose' transition state involved offsets the large loss of translational and rotational entropy that normally occurs in intermolecular reactions.[18]

The observation of a small effective concentration for intramolecular general acid catalysis in the present system is particularly interesting. Intermolecular general acid catalysis in the hydroxide-ion-catalysed hydrolysis of the hydroxy-amides (VII) is thought to be a *concerted* reaction in which there is a certain amount of coupling between proton-transfer and carbon–nitrogen bond fission.[17] Most effective concentrations that have been reported for intramolecular general acid–base-catalysed reactions involve a stepwise mechanism in which a transport process is usually rate limiting and the proton itself is in a potential energy well in the transition state.[19] The stepwise mechanism for acid–base catalysis represents the limit of a 'loose' transition state in which the reactant molecules are diffusing together and it is not surprising, therefore, that the entropy change associated with such bimolecular steps is small and that there is little rate advantage to be gained by covalently linking the reactant molecules



SCHEME 1

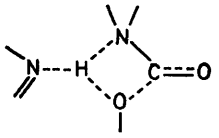
* k_{BH} is kinetically equivalent to the observed rate constant for general base catalysis $k_{BH} = k_B K_a / K_W$.[17]

together in an intramolecular reaction.[18,20] It appears that the same is also true even if the proton is ' in flight ' in the transition state of the intermolecular reaction, *i.e.*, the reaction mechanism is one of concerted proton transfer. This is expected in view of the relatively small entropy changes associated with hydrogen-bonding equilibria.[18]

The observations of low effective concentrations in intramolecular general acid–base-catalysed reactions may be extrapolated to determine the catalytic advantage of having a general acid or base catalyst as part of the protein structure in enzyme-catalysed reactions.[20,21] The catalytic advantage appears to be minimal. Although it may be necessary for the proton acceptor or donor to be at the active site, as the equivalent intermolecular catalyst may be sterically prevented from reaching the enzyme-bound substrate, the fact that the general acid or base is *part* of the enzyme apparently makes little contribution to the enormous rate enhancement brought about by the enzyme.[20,21]

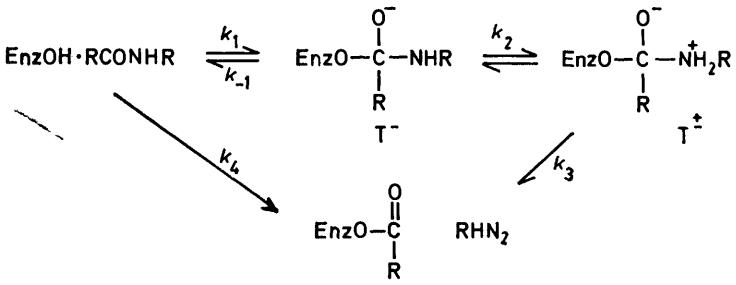
There is an interesting contrast between the large contribution (a factor of *ca.* 10^8) to the rate enhancement of intramolecular and enzyme-catalysed reactions by nucleophilic catalysis and the much smaller contribution (*ca.* 1—10) of general acid–base catalysis.[20-22] This is exemplified in the hydrolysis of the hydroxy-amide (III) in which the intramolecular nucleophilic hydroxy-group shows an effective concentration of *ca.* 10^8M (attributable almost entirely to the entropy effect) [12] whereas the intramolecular protonated amino-group shows an effective concentration of *ca.* 1M (attributable to the relatively small entropy change in the *intermolecular* reaction and possibly unfavourable strain effects and unfavourable entropy changes associated with loss of internal rotation in the *intramolecular* reaction).

Chymotrypsin.—The basic features that are generally accepted for the mechanism of the chymotrypsin-catalysed hydrolysis of amides were outlined in the introduction. Recently, it has been suggested that the reaction does not proceed through a stepwise pathway but rather by a concerted S_N2-type displacement in which the tetrahedral intermediate T^- (Scheme 2) does not have a discrete existence,[9] presumably $<10^{-13}$ s.[23] Although a fully concerted mechanism is not impossible, it seems unlikely. It has been proposed that the imidazole group ' simultaneously ' removes a proton from the serine hydroxy-group and donates it to the nitrogen of the departing amino-group;[9] presumably this is equivalent to a ' bifurcated ' hydrogen bond (VIII).



(VIII)

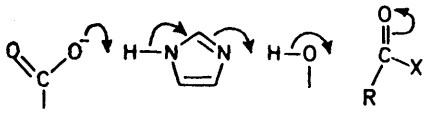
Proton donation to the departing amine nitrogen is thought to occur *before* carbonyl carbon–serine oxygen bond formation is complete.[9] The arguments presented to substantiate the completely concerted mechanism [9] are questionable. For natural substrates, the pK_a of the amine nitrogen would change from *ca.* −10 in the amide to *ca.* 7—10 in the tetrahedral intermediate[8] (Scheme 2). Proton transfer from the imidazolium cation of pK_a *ca.* 7 to the departing amine nitrogen *only* becomes thermodynamically favourable when serine oxygen–carbonyl carbon bond formation is complete. Also ' hydrogen bonding ' between the histidine and serine [as in (VIII)] will decrease the rate of proton transfer.[24] Despite these problems it is suggested that proton transfer *must* take place before serine oxygen–carbonyl carbon formation is complete.[9] It is stated that the rate of protonation of the departing amino-nitrogen in the tetrahedral intermediate, k_2 (Scheme 2) is 2.7×10^2 s^{-1} and as this is 3—4 orders of magnitude greater than the observed rate constants for cleavage of amides catalysed by chymotrypsin it is indicative of a concerted pathway, k_4 (Scheme 2). This is not necessarily true. Jencks has many times pointed out that a ' fast step ' can be rate-limiting if it is preceded by an unfavourable equilibrium.[25] In the present case, it simply requires $k_{-1} < k_2$ and it is well known that rates of breakdown of tetrahedral intermediates are often greater than 10^2 s^{-1}, and yet the mechanism may occur by a stepwise pathway.[8,9,23] It is argued [9] that the tetrahedral intermediate ' should be immediately protonated ' and although this is conceivable there is no ' should ' involved; it is well known that even thermodynamically favourable processes often require ' time ' to occur. It is suggested that if ' the imidazolyl cation of histidine and the tetrahedral intermediate (T^-, Scheme 2) were



SCHEME 2

formed and the tetrahedral intermediate had a lifetime long enough to be called an intermediate, the imidazoyl cation should easily lose its proton to the surrounding water . . . and no effective acid catalysis by imidazole could be expected '.[9] There are thermodynamic and kinetic arguments against this claim. *If* the imidazole residue is at equilibrium with respect to the bulk solvent then at the operational pH of the enzyme there will be a significant concentration of the imidazolyl cation. More important the authors themselves point out that proton transfer from imidazoyl cation to the more basic nitrogen of T^- (Scheme 2) can occur rapidly, faster in fact than the unfavourable proton transfer to water. Finally it is stated that the imidazoyl ring has considerable rigidity [9] and that the rate-limiting conformational change suggested previously [8] cannot occur. There is little evidence to support this claim. Internal rotations of groups inside proteins is well established.[26]

It has been suggested that the ' charge-relay system ', where proton relay from the hydroxy-group to the

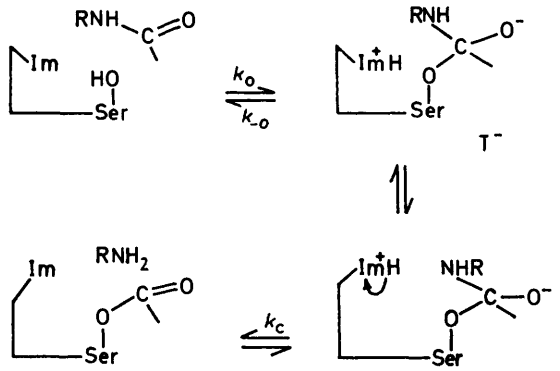


(4)

carboxy-group, *via* the imidazoyl group [equation (4)] contributes to the catalytic ability of the enzyme.[1,27] It is difficult to assess the contribution of this factor, if it occurs,[28] to the rate enhancement brought about by the enzyme. Intramolecular general acid–base catalysis is not particularly effective [18,20–22] and does not appear to depend precisely upon the orientation of the reacting groups.[29] There is no obvious chemical advantage if the carboxylate group is also involved. An important criticism of this mechanism is that it is usually written as shown in equation (4), depicting *formation* of the tetrahedral intermediate, T^-. It seems likely that the rate-limiting step for the chymotrypsin-catalysed hydrolysis of the ' natural ' amide substrates involves *breakdown* of the addition intermediate or a rate-limiting conformational change of the imidazole residue.[8] Increasing the rate of formation of this intermediate *cannot* therefore facilitate the reaction unless, which seems unlikely, it stabilises the intermediate and thus increases its concentration. It is conceivable that the ' charge-relay system ' could increase the already fast rate of proton transfer to the nitrogen of the departing amine but the actual contribution of this to the rate enhancement would be minimal.[11]

Based on observations of the mechanism of the aminolysis of esters several pathways for the chymotrypsin-catalysed hydrolysis of amides have been proposed.[8] Stepwise mechanisms for proton transfer and breakdown of the tetrahedral intermediate were favoured by analogy with the non-enzymic reaction.[9] However, it was pointed out that if the tetrahedral intermediate, $T^{\pm}$, was too unstable to exist, a concerted pathway involving simultaneous proton transfer and carbon–nitrogen bond fission was possible. It has now been shown that such a mechanism can occur in the intramolecular alcoholysis of amides.[17] Concerted general acid catalysed expulsion of amine from the tetrahedral intermediate T^- (VII) is possible when proton transfer is thermodynamically unfavourable.[19] However, it appears that the ' concerted ' mechanism can still occur when proton transfer is thermodynamically favourable by 2—3 pK units.[17] Based on these observations and those reported herein we would like to propose that the mechanism for the enzyme-catalysed pathway may involve a concerted breakdown of the tetrahedral intermediate.

Scheme 3 shows the initial formation of the tetrahedral intermediate T^- which may not be rate limiting for ' natural ' amide substrates. Carbon–oxygen bond fission, k_{-0}, could be faster than proton transfer and carbon–nitrogen bond fission ($Im\overset{+}{H}$ increases rate of breakdown, k_{-0}, as well as increasing the rate of formation, k_0, of the intermediate). The rate-limiting step could be k_c, the concerted breakdown of T^-, *i.e.*, proton transfer from the imidazoyl cation is coupled to carbon–nitrogen bond fission. This is analogous to the scheme proposed for the model system [Scheme 1(a)]. A concerted pathway for breakdown of the intermediate is compatible with the experimental observations.



SCHEME 3

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