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DEUTERIUM ISOTOPE EFFECTS ON ACYLATION OF SUBTILISIN

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SUMMARY

^2H_2O effects on the hydrolyses of nonactivated alkyl and the corresponding activated alkyl thiol and *p*-nitrophenyl esters by subtilisin, Type Carlsberg, were investigated. Similar effects were obtained with the activated and nonactivated esters of acetic and cinnamic acids. This rules out the formation of a transient acyl-imidazole intermediate in the catalysis by serine proteases proposed by Hubbard, C. D. and Kirsch, J. F. (1972) *Biochemistry* 11, 2483–2493, for nonspecific-activated ester substrates. Stereochemical considerations based on model building of the active site of subtilisin offer further evidence against nucleophilic catalysis by the histidine residue.

It has been shown previously that in the catalysis by serine proteases, like chymotrypsin, the histidine residue at the active site is a general base rather than a nucleophile[1]. By contrast, it has recently been proposed that in the hydrolysis of alcohol-activated substrates by chymotrypsin, nucleophilic catalysis by the imidazole group is operative[2]. This notion appears to be accepted in the literature[3]. The evidence presented in this paper rules out the possibility of nucleophilic catalysis by the histidine residue of serine proteases, even in the case of the afore-mentioned special substrates.

It is known that proton transfers proceed approximately 2–3 times as fast in water as in ^2H_2O, which is evidence for general base or general acid catalysis, whereas the rate of a nucleophilic reaction is not affected by ^2H_2O (*cf.* ref. 1). Deuterium isotope effects of about 2, found with typical non-specific activated esters, like *p*-nitrophenyl trimethyl acetate in the acylation of chymotrypsin[4] and *p*-nitrophenyl acetate in the acylation of subtilisin, Type Novo[5], were interpreted in terms of general base catalysis. Therefore, the recent finding of Hubbard and Kirsch[2], that the ratios of the second-order rate constants of acylation of chymotrypsin measured in water and in ^2H_2O, $k_{H_2O}/k^2_{H_2O}$, are 1.07 and 1.64 with *p*-nitrophenyl-*p*-trifluoromethyl benzoate and 2,4-dinitrophenyl benzoate, respectively, is of some note. They inter-

Abbreviation: Z = *N*-benzyloxycarbonyl.

preted these low values as compelling evidence for nucleophilic catalysis by the histidine residue. They claimed that in the case of non-specific activated ester substrates, such as the two benzoates mentioned above, acylation of chymotrypsin proceeds through a pathway involving the transient formation of acyl-imidazole intermediate en route to the acyl-serine enzyme[2].

In order to distinguish between nucleophilic and general base catalysis by the histidine residue, in this work the study of ^2H_2O effects on acylation by non-specific activated esters was extended to the non-activated, alkyl esters containing the corresponding acyl moiety. If deuterium isotope effects are similar with the two types of esters, nucleophilic catalysis by the imidazole group can be ruled out since simple alkyl esters are hydrolysed by imidazole as a general base[6]. Only activated esters are hydrolysed by imidazole as a nucleophile[6].

As a comparison, alkyl thiolesters, were also examined. This type of ester can be regarded as another example of activated esters, since the alkyl thiolate ion, in contrast to the corresponding alcoholate ion, is a good leaving group[7]. Hence acylation of serine proteases with alkyl thiolesters and *p*-nitrophenyl esters proceeds *via* a similar mechanism which does not involve general acid catalysis[8].

For the present investigations the serine protease subtilisin was preferred to chymotrypsin, since the pH-independent maximal rate constant of acylation, k_{limit}, can be calculated more precisely from the sigmoid pH–rate profile of acylation of subtilisin, than from the bell-shaped pH–rate profile of acylation of chymotrypsin.

In Table I, k_{limit} values of acylation of subtilisin with three sets of substrates are shown. The second-order acylation constants (k_2/K_S) are complex constants involving the first-order acylation and the binding constants. They were calculated from pseudo first-order reactions measured at different pH values, as described previously[8]. In the case of *p*-nitrophenyl acetate, the second-order rate constants were calculated from Lineweaver-Burk plots, also ($k_2/K_S = k_{cat}/K_m$, *cf.* ref. 1). The

TABLE I

^2H_2O EFFECT ON THE KINETIC PARAMETERS OF REACTIONS CATALYSED BY CARLSBERG SUBTILISIN

Conditions: 25 °C, 1.7% acetonitrile, k stands for the pH-independent maximal second-order rate constant of acylation ($M^{-1}s^{-1}$).

Substrate	$pK_a(H_2O)$	$pK_a(^2H_2O)$	k_{H_2O}***	$k^2_{H_2O}$	$k_{H_2O}/k^2_{H_2O}$
Ethyl acetate			0.065	0.034	1.91
Ethyl thiolacetate	7.27	7.82	3.03	1.55	1.95
p-Nitrophenyl acetate	7.10	7.65	2 250†	1 280	1.76
p-Nitrophenyl acetate*	7.18	7.80	2 400	1 180	2.03
Ethyl cinnamate	7.40	7.85	2.10	1.18	1.78
Ethyl thiolcinnamate	7.50	7.77	48.5	30.5	1.59
p-Nitrophenyl cinnamate	7.45	7.95	44 000	20 500	2.14
Ethyl Z-glycinate	7.20	7.78	308	150	2.05
Ethyl Z-thiolglycinate	7.18	7.67	25 600	17 000	1.51
p-Nitrophenyl Z-glycinate	7.20	7.65	1 730 000	1 400 000	1.23
p-Nitrophenyl acetate**	7.13	7.48	0.96	0.315	3.05

* Calculated from Lineweaver-Burk plots.
** Deacetylation, where k stands for k_{cat} (s^{-1}).
*** Data of ref. 8.
† Corrected.

rate constants measured with the two different methods are similar within experimental error (Table I). From the Lineweaver-Burk plots k_{cat} values, which are equal to the first-order deacylation rate constants, were also determined.

It is seen from Table I that in the case of acetic and cinnamic acid derivatives, ^2H_2O has similar effects ($k_{H_2O}/k^2_{H_2O} \approx 2$) on the acylation by non-activated and activated esters, and thus nucleophilic catalysis by the imidazole group can be ruled out in the acylation of serine proteases. However, in a few instances, such as the acylation of subtilisin with *p*-nitrophenyl Z-glycinate (Table I) or the acylation of chymotrypsin with *p*-nitrophenyl-*p*-trifluoromethyl benzoate[2], $k_{H_2O}/k^2_{H_2O}$ is extremely low. As expected, in deacylation (Table I and ref. 9) deuterium isotope effects reflect typical general base catalyses.

The reason for the low $k_{H_2O}/^2_{H_2O}$ ratios is not known. Two possibilities may be mentioned here. First, K_S may decrease in ^2H_2O thereby lowering $k_{H_2O}/k^2_{H_2O}$. This is possible since ^2H_2O promotes binding by increasing hydrophobic interactions and hydrogen bond formation[10]. Since there are considerable differences in the binding modes of even similar substrates with subtilisin[11], this effect should depend to a large extent on the structure of the substrate and it is difficult to interpret it with a given substrate. The second possibility is that the formation of the tetrahedral intermediate and the proton transfer are not absolutely concerted processes, and in the rate-limiting transition state some of the zero point energy of the proton is maintained (*cf.* ref. 12). In other words, the attachment of the proton to the donor or acceptor atom may have some covalent bond character in the transition state.

Finally, I should like to point out that nucleophilic catalysis by the imidazole group seems very unlikely in the light of the steric structure of the active site of serine proteases. Such a mechanism implies a fast nucleophilic attack by the serine hydroxyl group on the carbonyl carbon atom of the transient acyl-imidazole. However, the hydroxyl group is not a good nucleophile. Its high reactivity in serine proteases is due to the catalysis by the imidazole base, which is sacrificed if the imidazole group has been acylated. The three-dimensional model of the active site of chymotrypsin[13] or subtilisin[14] shows that there is no other amino acid side chain in the neighbourhood of the hydroxyl group that could accept the proton. One can also build the model of the acyl-imidazole intermediate on the basis of the atomic coordinates given for *N*-acetyl-L-alanyl-glycyl-L-phenylalanylmethylene subtilisin[15], a derivative at His-64 of subtilisin BPN alkylated by the corresponding chloromethyl ketone. Inspection of this model shows that the hydroxyl group of Ser-221 is effectively shielded from the solvent by the substrate and the side chain of Met-222. Thus, the serine hydroxyl group cannot transfer its proton even to a water molecule.

EXPERIMENTAL

Subtilisin type Carlsberg and substrates, as well as the method of determination of the rate constants, were the same as described in a previous paper[8].

$p^2H = pH + 0.4$ (ref. 16)

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