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Catalytic Activity of α-Chymotrypsin in which Histidine-57 has been Methylated

By R. HENDERSON*
Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K.

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The properties of a derivative of α-chymotrypsin in which histidine-57 has been methylated have been examined. Although the modified enzyme binds substrate with the same affinity as does native α-chymotrypsin, acylation and deacylation occur at much decreased rates. As for native α-chymotrypsin, a basic group of pK_a approx. 7 is involved in both acylation and deacylation. The significance of these results is considered in relation to the normal function of histidine-57.

During the investigation of the residual activity of a number of chemically treated α-chymotrypsins in which the side chain of histidine-57 had been specifically modified, it was found that the product of the reaction of α-chymotrypsin with methyl *p*-nitrobenzenesulphonate (Nakagawa & Bender, 1969, 1970) has a much higher specific activity than any of the others. It is the purpose of this paper to give a brief account of the kinetic behaviour of the enzyme thus modified. Several specific substrates were investigated and the results are considered in the context of the normal function of histidine-57 in native α-chymotrypsin.

It was found that the dissociation constants for the binding of *N*-acetyl-L-tyrosine ethyl ester and proflavine to the modified enzyme are identical within experimental error with those observed with native chymotrypsin. The rate constants for acylation and deacylation, however, are much smaller and may indicate that the hydrolysis reaction occurs via a different mechanism. Nakagawa & Bender (1969, 1970) have shown that the modified enzyme contains one additional methyl group, substituted at histidine-57 to form 3-methylhistidine-57. The demonstration below that this modified chymotrypsin can form a relatively stable enzyme–substrate complex and an acylated intermediate with specific substrates may prove to be of value in the crystallographic investigation of the enzyme's binding site (Steitz, Henderson & Blow, 1969; Henderson, 1970).

* Present address: Department of Chemistry, Yale University, 225 Prospect Street, New Haven, Conn. 06520, U.S.A.

EXPERIMENTAL

Preparation of methylated α-chymotrypsin. A sample (100mg) of bovine α-chymotrypsin (Worthington Biochemical Corp., Freehold, N.J., U.S.A.; lot CDI8LK; 3× crystallized and freeze-dried) was dissolved in 7ml of 0.1M-sodium phosphate buffer, pH7.8, and 0.7ml of acetonitrile containing 6mg of methyl *p*-nitrobenzenesulphonate (sixfold excess) was added. After 10min, 2mg of PMS† fluoride in 0.15ml of propan-2-ol was added and the solution left for a further 5min. The enzyme, now partially (approx. 50%) having reacted with methylating reagent and the remainder having been inactivated with PMS fluoride, was then quickly passed through a short column of Sephadex G-25F equilibrated with 0.1M-sodium citrate buffer, pH5.61, and the eluent applied to a chromatographic column (0.9cm×90cm) of Bio-Rex 70 (200–400 mesh), previously equilibrated with 0.1M-sodium citrate, pH5.61. Fig. 1 shows the elution profile. Peak I, present in different batches of enzyme in various quantities, is an impurity. Peak II is the methylated α-chymotrypsin and peak III is PMS-chymotrypsin.

The unmodified enzyme was treated with PMS fluoride before chromatography, to avoid the problem of autolysis normally present during chromatography of proteolytic enzymes, and because PMS-chymotrypsin was found to be easier to separate from chymotrypsins in which histidine-57 had been alkylated. A more complete reaction of chymotrypsin with the methylating reagent was avoided because of the possibility of reaction at other sites (Nakagawa & Bender, 1970). The modified enzyme used in the following experiments was obtained by combining the two or three main fractions of peak II. The material was thus composed almost entirely of a single molecular species of α-chymotrypsin containing 3-methylhistidine-57. If ^{14}C-labelled methylating reagent was used,

†Abbreviation: PMS, phenylmethanesulphonyl.

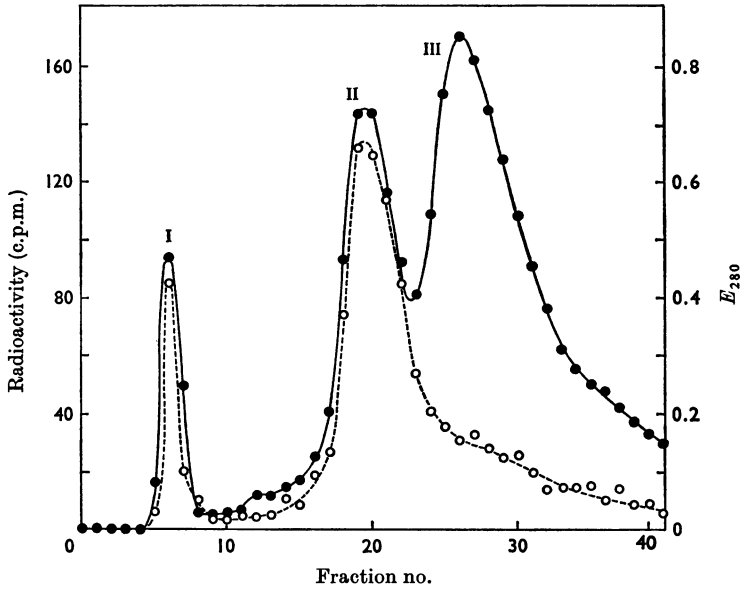

Fig. 1. Chromatography of methylated α-chymotrypsin from a column (0.9 cm × 90 cm) of Bio-Rex 70 (200–400 mesh) eluted at 20 ml/h with 0.1 M-sodium citrate buffer, pH 5.61, at 20°C. ●, E_{280}; ○, radioactivity (c.p.m.) from the methyl group. The fraction size was 10 ml. Peak I is an impurity, peak II is the methylated α-chymotrypsin and peak III is PMS-α-chymotrypsin.

0.9 ± 0.1 mol of ^{14}C label/mol of chymotrypsin, based on the extinction at 280 nm, was incorporated (Fig. 1).

Other derivatives of α-chymotrypsin, which had been treated with L-1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one ('tosylphenylalanyl chloromethyl ketone') (Schoellman & Shaw, 1963) or photo-oxidized in the presence of Methylene Blue (Koshland, Strumeyer & Ray, 1962) were similarly prepared. On chromatography of the photo-oxidation reaction product, two main peaks were obtained in addition to the PMS-chymotrypsin peak, presumably owing to the two most rapid oxidations of histidine-57 and methionine-192 (see Koshland *et al.* 1962). Only the larger of these two peaks was considered to be enzyme in which histidine-57 was photo-oxidized, since the other peak could be reactivated (at pH 2 and 40°C, as described by Gold & Fahrney, 1964) to give a partially active enzyme (approx. 20%), characteristic of modification of methionine-192 (Koshland *et al.* 1962; Knowles, 1965).

Assays of catalytic activity. Activity was assayed towards two specific ester substrates of chymotrypsin, *N*-benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester (Z-Phe-ONp) and *N*-acetyl-L-tyrosine ethyl ester (Ac-Tyr-OEt). Hydrolysis of amides was too slow to measure. With the *p*-nitrophenyl ester, hydrolysis above pH 7 was followed directly by the E_{400} due to *p*-nitrophenolate. With both substrates, below pH 9, reaction was followed indirectly by the proflavine-displacement technique (Bernhard, Lee & Tashjian, 1966). The dissociation constant for the binding of proflavine to modified chymotrypsin was found to be $2.3 \times (\pm 0.2 \times 10^{-5}) 10^{-5}$ M which is identical with the value found for native α-chymotrypsin under the same conditions. The difference spectrum was also identical with that found for native chymotrypsin with a peak at 470 nm and a trough at 430 nm. Monitoring of the reaction at 470 nm for displacement of proflavine thus showed almost no interference (<4%) due to *p*-nitrophenolate absorption centred at 400 nm. A typical assay was conducted by adding 50 μl of a solution of Z-Phe-ONp in acetonitrile to 3.0 ml of methylated chymotrypsin in 0.1 M-sodium phosphate buffer, pH 7.9, so that the final concentrations of enzyme, measured by the E_{280}, and substrate were 20 μM and 5 μM respectively. With Ac-Tyr-OEt, 50 μl of the substrate dissolved in dioxan was added. Before all assays, at 5 min before addition of substrate, 20 μl of a solution of PMS fluoride in propan-2-ol was added to the enzyme solution (to final concn. 0.1 mM-PMS fluoride) to eliminate the last traces of any fully active enzyme which might overwhelm the small residual activity of the modified enzyme. Treatment with PMS fluoride was not a necessary part of the procedure, however. Methylated α-chymotrypsin was also prepared by extensive reaction with the methylating reagent. The resultant material, without further purification, had essentially the same reactivity towards Ac-Tyr-OEt as the purified enzyme (peak II in Fig. 1). With Ac-Tyr-OEt such a large excess of substrate was used that even a small amount of fully active enzyme did not interfere with the initial acylation of the methylated enzyme. When present the proflavine concentration was between 40 μM and 0.1 mM. In all cases, the concentration of organic solvent was less than 3% (v/v). The temperature was 25°C.

RESULTS

The binding of proflavine to methylated α-chymotrypsin at pH 5, indicated by the difference in E_{470} between bound and free proflavine, is shown as a reciprocal plot in Fig. 2. Results obtained in the presence of dioxan and of dioxan plus Ac-Tyr-OEt are also shown. Both dioxan and Ac-Tyr-OEt bind to the methylated enzyme, and the binding is competitive with proflavine. The dissociation constants for the binding of proflavine and Ac-Tyr-OEt to methylated α-chymotrypsin obtained from similar results at pH 8 are 23 μM and 18 mM respectively. These are identical within experimental error with the values found for binding to unmodified α-chymotrypsin (Table 1).

Hydrolysis of Z-Phe-ONp by methylated α-chymotrypsin at pH 8, under conditions of excess of enzyme, occurred at a rate approx. one-ten-thousandth of that for unmodified α-chymotrypsin. Chromatographically purified tosylphenylalanyl chloromethyl ketone-treated chymotrypsin, PMS-chymotrypsin or photo-oxidized chymotrypsin had no measurable activity towards Z-Phe-ONp. An activity 1×10^{-7} times that of native α-chymotrypsin would have been detected.

The use of proflavine allowed acylation and deacylation to be followed separately (Fig. 3). With Z-Phe-ONp, acylation indicated by displacement of proflavine was found to follow closely the release of *p*-nitrophenolate. This experiment shows conclusively that all of the methylated chymotrypsin reacts with substrate since, if the hydrolysis was due to a small amount of highly active enzyme, resistant to PMS fluoride, significant displacement of proflavine would not be observed. Deacylation over a longer time-course (Fig. 3*a*) was observed as the proflavine recombined with the free enzyme.

In the reaction with Ac-Tyr-OEt, much higher concentrations of substrate (10 mM) were required. Some proflavine was therefore displaced immediately, owing to the reversible binding of the substrate as shown in Fig. 2. Subsequent acylation caused a further displacement of proflavine (Fig. 3*b*) until an equilibrium was reached, at which acylation and deacylation were equal. In the experiment shown in Fig. 3(*b*), 52% of the enzyme became acylated. Higher Ac-Tyr-OEt concentrations, up to 20 mM, resulted in the formation of larger amounts of acyl-enzyme. By analysing curves such as the one shown in Fig. 3(*b*), in terms of the kinetic scheme:

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} EA + P_1 \xrightarrow{k_3} ES + P_1 + P_2 \quad (1)$$

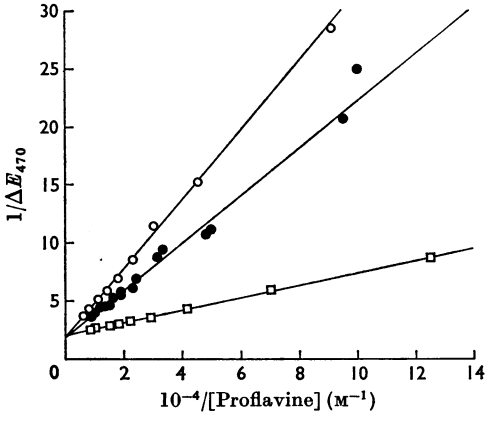

Fig. 2. Reciprocal plot of binding of proflavine to methylated α-chymotrypsin at pH 5.0, in 0.1 M-sodium phosphate buffer. □, Proflavine alone; ●, proflavine in the presence of 1.6% dioxan; ○, proflavine, in presence of 1.6% dioxan and 15 mM-Ac-Tyr-OEt. Similar results were obtained at pH 8.0 if Ac-Tyr-OEt was added immediately before the E_{470} was recorded.

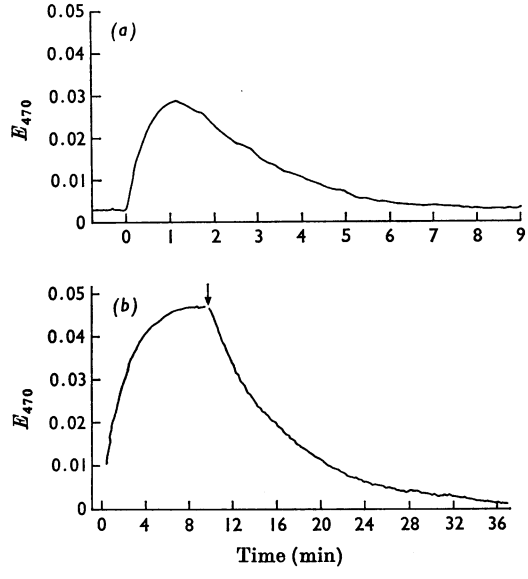

Fig. 3. Acylation and deacylation of methylated α-chymotrypsin by (*a*) Z-Phe-ONp and (*b*) Ac-Tyr-OEt. The trace shows the decrease in E_{470} owing to displacement of proflavine from the enzyme. In (*a*), $[E_0] = 15\,\mu M$, $[S_0] = 5\,\mu M$, [proflavine] approx. 70 μM, 1.6% acetonitrile, 0.1 M-sodium phosphate, pH 7.8 at 25°C. In (*b*), $[E_0] = 15\,\mu M$, $[S_0] = 10$ mM, [proflavine] = 40 μM, 1.6% dioxan, 0.1 M-sodium phosphate, pH 7.8 at 25°C. The arrow indicates addition of a small amount of α-chymotrypsin (0.5 μM) to hydrolyse rapidly all the Ac-Tyr-OEt. After the addition, the pH was 7.3.

where ES is the Michaelis complex, EA is the acyl-enzyme and P_1 and P_2 are the products resulting from hydrolysis of the substrate S; k_2 and k_3 were obtained separately. The value of K_s determined above was used in the calculation and account was taken of competitive inhibition by proflavine and dioxan. In addition, and as a further check on the validity of the calculation, the deacylation rate, k_3, was directly measured by rapidly hydrolysing all the Ac-Tyr-OEt once the acyl-enzyme had been formed. This was done by adding a small amount of fully active α-chymotrypsin to the mixture once it had equilibrated. The resultant trace (Fig. 3*b*) shows directly that the enzyme deacylates at a rate similar to that found with Z-Phe-ONp. Note that hydrolysis of the Ac-Tyr-OEt caused the pH to change to 7.3.

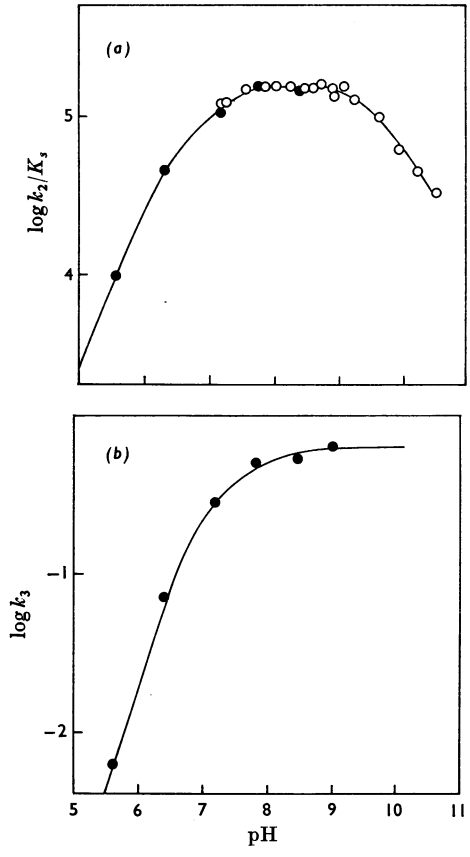

Fig. 4. pH–dependence of (*a*) acylation and (*b*) deacylation of methylated α-chymotrypsin by Z-Phe-ONp, followed ●, by the proflavine-displacement technique; or ○, by release of *p*-nitrophenolate: 25°C; buffers, 0.05M-sodium phosphate below pH 9, 0.05M-sodium borate above pH 9.

Titration of methylated α-chymotrypsin with the reagent indolylacryloylimidazole (Bernhard & Tashjian, 1965) also proved possible although the reaction proceeded rather slowly. In the presence of a sixfold excess of indolylacryloylimidazole, a 10μM solution of methylated α-chymotrypsin at pH 7.2 was 90% converted in 3 days. Desalting of the reaction mixture on Sephadex G-25F gave a derivative with a u.v.-absorption spectrum close to that of normal indolylacryloyl-α-chymotrypsin, with a peak at approx. 350nm. The E_{350}/E_{280} ratio was 0.21, which is close to the value for indolylacryloyl-α-chymotrypsin (0.24) at the same pH value. In native α-chymotrypsin, indolylacryloylimidazole reacts to acylate serine-195 (Charney & Bernhard, 1967; Henderson, 1970). The fact that the spectra of the modified and native enzymes, when treated with indolylacryloylimidazole, are very similar suggests that the acyl linkage also occurs at serine-195 in methylated α-chymotrypsin. The resultant indolylacryloyl derivative appears to be stable for several weeks at pH 7.

The pH profile for acylation and deacylation by Z-Phe-ONp is shown in Fig. 4 and a comparison of all the catalytic rate constants and dissociation constants with those of native α-chymotrypsin is given in Table 1. Acylation by Ac-Tyr-OEt had a similar pH-dependence.

Table 1. *Comparison of some dissociation and rate constants between α-chymotrypsin and the methylated derivative*

All measurements were made in 0.1M-sodium phosphate buffer, pH 7.9 at 25°C.

	α-Chymotrypsin	Methylated derivative	Ratio
Proflavine K_i (M)	2.2×10^{-5} ($\pm0.2\times10^{-5}$)	2.3×10^{-5} ($\pm0.2\times10^{-5}$)	1.0
Ac-Tyr-OEt K_s (mM)	18*	18 (±6)	1.0
Ac-Tyr-OEt k_2 (min^{-1})	3.2×10^{5}*	1.6 (±0.5)	2×10^{5}
Ac-Tyr-OEt k_3 (min^{-1})	1.2×10^{4}*	0.23 (±0.04)	5×10^{4}
Z-Phe-ONp k_2/K_s ($min^{-1}\cdot M^{-1}$)	$\sim3\times10^{9}$†	1.5×10^{5} ($\pm0.2\times10^{5}$)	2×10^{4}
Z-Phe-ONp k_3 (min^{-1})	3.1×10^{3}†	0.6 (±0.1)	5×10^{3}

* Values taken from McConn, Ku, Himoe, Brandt & Hess (1971).

† Estimated by assuming $k_{cat.}/K_m=k_2/K_s$, $k_{cat.}=k_3$, and using measured values of $k_{cat.}=3.1\times10^{3}\,min^{-1}$, K_m, approx. 10^{-6}M (cf. Zerner & Bender, 1963).

DISCUSSION

It is surprising that methylated α-chymotrypsin should retain any catalytic activity at all in view of the chemical modification of one of the residues previously thought to be essential. However, there is an enormous difference between the catalytic rates of the modified enzyme and native chymotrypsin, such that a different mechanism could well apply. Indeed, the results shown in Table 1 reinforce the importance of histidine-57 for the normal activity of α-chymotrypsin and may provide some indication of which factors contribute most to the rate enhancement observed in reactions catalysed by chymotrypsin.

An answer to the question of how methylated α-chymotrypsin hydrolyses the above specific ester substrates would help to resolve further the roles of serine-195 and histidine-57 in catalysis by the normal enzyme. The fact that substrate is bound to the modified enzyme with the same affinity as to native α-chymotrypsin means that the modification is a relatively minor one and that no gross reorganization of the polypeptide chain is involved. That an acyl-enzyme can still be formed is further evidence of the integrity of the region of the enzyme near to serine-195. There may be small local changes, however, especially in the precise positions and orientations of the side chains of histidine-57 and serine-195. In view of the hydrogen bond normally found between N-3 of histidine-57 and the hydroxyl group of serine-195 (Sigler, Blow, Matthews & Henderson, 1968), some repositioning must occur on methylation of the histidine residue.

It was anticipated that the hydrolysis of substrate by methylated α-chymotrypsin might be due to proximity and orientation effects alone, involving the uncatalysed reaction of serine-195 with the bound substrate. However, the pH profile (Fig. 4) shows the normal pK_a values of 7 and 9.5 in acylation and 7 in deacylation, thus implying that, as in native α-chymotrypsin, a basic group with pK_a approx. 7 and an acidic group of pK_a 9.5 are involved in the reaction. No uncatalysed reaction of the serine-195 anion was detected even at pH 10.5. The pK_a of 9.5 is slightly higher than usually found presumably because of the presence of a small amount of organic solvent (Kaplan & Laidler, 1967) and may be attributed to the deprotonation of the α-amino group of isoleucine-16 (Oppenheimer, Labouesse & Hess, 1966) and its effect on substrate binding. The pK_a of 7 obtained for both acylation and deacylation is more puzzling. In native α-chymotrypsin, a similar decrease in activity at low pH values with pK_a 7 is observed, and this has been widely interpreted as being due to the protonation of the imidazole moiety of histidine-57 and more recently to protonation of an active-site hydrogen-bond system found between the three residues aspartate-102, histidine-57 and serine-195 in the intact enzyme (Blow, Birktoft & Hartley, 1969), the exact positions of the protons and distribution of charge being uncertain. If the hydrogen bond between histidine-57 and aspartate-102 is still formed in the methylated enzyme, it is difficult to imagine how the new pK_a of 7 could arise. Moreover, the pK_a of 1-methylimidazole is 7.25, so that it seems likely that the hydrolysis reaction catalysed by methylated α-chymotrypsin involves 3-methylhistidine-57 in its unprotonated form and with a conformation different from that in native α-chymotrypsin. Possibly there is an equilibrium with most of the enzyme in an inactive conformation similar to that of native α-chymotrypsin, but, for a fraction of the time, other conformations may exist from which catalysis can occur. Preliminary X-ray results suggest that this may be so (C. Wright, personal communication).

It seems likely that in methylated α-chymotrypsin, the modified histidine-57 acts as a general base on the hydroxyl group of serine-195, directly or through a water molecule, to give an acyl-enzyme, which is subsequently hydrolysed by a similar mechanism. However, the allowed positions of N-1 of 3-methylhistidine-57 in a model of α-chymotrypsin (Matthews, Sigler, Henderson & Blow, 1967) permit only a poor hydrogen bond to be formed with the hydroxyl group of serine-195 and this may be the main cause of the low rates of hydrolysis relative to the native enzyme (see Fig. 5).

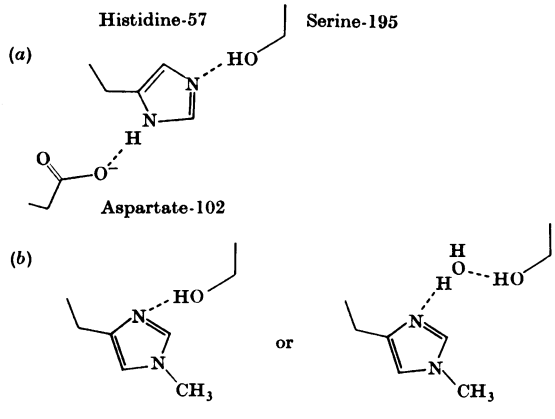

Fig. 5. (*a*) Diagrammatic representation of the active-site hydrogen-bond network in α-chymotrypsin. (*b*) Possible hydrogen-bonding in the methylated enzyme which might explain its activity and pH-dependence. Only a poor hydrogen bond can be formed directly between N-1 of histidine-57 and the serine hydroxyl group.

If the above arguments are correct, then (*a*) since a hydrogen bond cannot be made between aspartate-102 and the methylated imidazole group at the same time as catalysis occurs, it is clear that the contribution of aspartate-102 to the rate enhancement in native chymotrypsin catalysed reactions can be at most 5×10^3, possibly much less, and (*b*) although the modified chymotrypsin is a considerably poorer enzyme than the native, it is still quite a good catalyst. It therefore appears that those factors responsible for catalysis in methylated chymotrypsin may also be of most importance for the rate enhancement observed for the hydrolysis reaction catalysed by normal chymotrypsin. The methylated enzyme may prove to be a useful tool in gaining a deeper understanding of catalysis by chymotrypsin.

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