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THE FORMATION AND ACTIVITIES OF SUBSTITUTED PHENACYLCHYMOTRYPSINS

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SUMMARY

1. The preparation of derivatives of α-chymotrypsin with *p*-nitrophenacyl bromide, phenacyl bromide and *p*-methoxyphenacyl bromide is described.

2. For the *p*-nitrophenacyl derivative, the new 350-nm absorption is pH independent from pH 2.88 to 11.25, mitigating against a major change in environment at the new chromophore over this range.

3. The enzyme derivatives (likely at Met-192) show different decreased but definite catalytic activities towards substrates of α-chymotrypsin. In each case, this activity is due to the modified enzyme itself.

4. Phenacyl- and *p*-methoxyphenacylchymotrypsin show generally increased dissociation constants and decreased catalytic constants. Below pH 8.5, *p*-nitrophenacylchymotrypsin towards ethyl-*N*-acetyl-L-tryptophanate has only decreased k_{cat} values compared to native enzyme, K_m being unaltered. The pH dependencies of k_{cat} below pH 8.5 and K_m over the entire range are identical for parent and nitrophenacyl enzymes. Above pH 8.5, k_{cat} for the enzyme derivative increases and is still rising at pH 10, having reached over twice its value at pH 8.5. The effect is reversible.

5. These results are taken to indicate that the conformational change occurring in α-chymotrypsin at pK 9 occurs for the nitrophenacyl enzyme as well, but, in the latter case, it reverses the original inhibition of k_{cat} by the nitrophenacyl group.

INTRODUCTION

The proteolytic enzyme α-chymotrypsin is one of the more well-defined enzymes in terms of molecular structure and chemical mechanism of catalysis. A rather detailed X-ray photograph is available[1], and the roles of several groups in the active site have been established by kinetic and modification studies. In general, it is agreed that the pH–rate parameter curves are caused by (a) the imidazole of His-57 which must act as

* Abbreviations: NPA-Br, *p*-nitrophenacyl bromide; L-ATrEE, ethyl-*N*-acetyl-L-tryptophanate; L-ATrME, methyl-*N*-acetyl-L-tryptophanate; D-ATrME, methyl-*N*-acetyl-D-tryptophanate; L-ATyEE, ethyl-*N*-acetyl-L-tyrosinate; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

a basic catalyst causing the dependence of the catalytic constant only on a base of $pK_a = 7$ (ref. 2) and (b) on the ionization of an N-terminus α-amino group of an isoleucine which keeps the enzyme in the active form when in the ammonium state and which has an apparent $pK_a = 9$ only manifested in the dissociation constant[3,4]. At Position 192, a methionine residue is three amino acids away from the active and obligatory serine but just outside the gourd shared active site and on the surface of the protein. Chemical modification studies have shown that it is quite accessible to chemical reagents and also that it can be reached by the γ-CH_2Br of a bromobutyryl-chymotrypsin[5,6]. When oxidized to the sulfoxide, little change in activity is apparent[7]. Other derivatives of this group show various reactivities, some somewhat more and some somewhat less active than the parent enzyme. We have studied a derivative of Met-192 reported to have little activity[8] in order to investigate further the role of small structural modifications fairly near to, but not in, the active site of the enzyme. We also had originally chosen this derivative because of the near-ultraviolet absorption it displayed that might be useful in studying possible conformational changes in the region involved in the formation of derivatives[9].

EXPERIMENTAL

Materials

Worthington 3 times crystallized, lyophilized α-chymotrypsin was used and, in some experiments, was filtered on Sephadex G-25 gel in 1 mM HCl prior to use.

Acetonitrile (Mallinckrodt Nanograde) was distilled from calcium hydride before use. Water was all double distilled.

Ethyl-*N*-acetyl-L-tryptophanate (L-ATrEE) was obtained from Mann Research Co., methyl-*N*-acetyl-D-tryptophanate (D-ATrME) from Cyclo Chemical Co., and ethyl-*N*-acetyl-L-tyrosinate (L-ATyEE) from Sigma Chemicals. Phenacyl bromide (Matheson) was recrystallized from methanol before use. *p*-Nitrophenacyl bromide (NPA-Br) was prepared by bromination of *p*-nitroacetophenone in acetic acid[10], and *p*-methoxyphenacyl bromide was synthesized by a similar route[11]; both were recrystallized several times from methanol. Spectra and melting points were in excellent agreement with literature values for these compounds.

Reactions for preparation of derivatives

A typical preparative run was as follows. 112.5 mg of α-chymotrypsin were dissolved in 75 ml of 0.1 M (total) phosphate buffer (pH 6.86). 0.75 ml of 23 mM NPA-Br in acetonitrile was added to this solution. Concentrations are thus 48 μM enzyme and 0.23 mM NPA-Br. The reaction was kept at 4° for 24 h which was sufficient time for the assay to show constant activity.

At this time, an additional 0.5 ml of NPA-Br was added, and the mixture was left standing another 36 h. Activity was monitored throughout this period. The pH was then brought to 4.0 by gradual addition of 0.1 M HCl, and the solution was divided and put onto three separate 30-cm G-25 Sephadex columns with 1 mM HCl as eluent. The protein fractions were collected, combined and lyophilized. Spectra agreed well with those in the literature[8]. The lyophilized material is stable at 0° for at least 2 months. Other derivatives were prepared by analogous procedures.

Kinetic measurements

All assays were carried out spectrophotometrically at 25.0 ± 0.2° in a Cary-14 spectrophotometer. These methods are described in detail both in the literature[12] and in the following section.

The k_{cat} and K_m determinations as a function of pH were carried out by the spectrophotometric method as well, and all results are the average of 2–5 determinations. Using computer analysis, Lineweaver–Burk ($1/v$ *vs.* $1/[S]$ plots were constructed from spectrophotometric data, each from one run.

RESULTS

Preparation of derivatives of α-chymotrypsin using NPA-Br

The reaction for preparation of derivatives was carried out at pH 3–8, and loss of activity was monitored by performing rate assays *vs.* L-ATrEE with aliquots from the incubation mixture withdrawn at successive time intervals. NPA-Br was always in 3–20-fold excess over α-chymotrypsin. Gel filtration of the aliquots led to no change in results. The data for one such series of experiments at pH 4 are given in Table I.

TABLE I

A REPRESENTATIVE PREPARATION OF A DERIVATIVE OF CHYMOTRYPSIN WITH NPA-Br

Maximum velocities of assays of the incubation mixtures of a typical experiment. α-Chymotrypsin (96 μM) and NPA-Br (0.3 mM) in acetate buffer (pH 4.0). Assays were performed with 2.89 mM L-ATrEE in 0.1 M phosphate buffer (pH 7.8) (1.6% acetonitrile) with aliquots from the reaction mixture.

Time	$v_{max} \times 10^5$ *(M·sec⁻¹)*	$E_0 \times 10^7$ *(cell) (M)*
0	4.1 ± 0.1	7.75
10 min	3.4 ± 0.1	7.75
30 min	2.4 ± 0.1	7.75
1 h	2.0 ± 0.1	7.75
1.5 h	1.5 ± 0.1	7.75
2.5 h	1.2 ± 0.1	15.5
5.5 h	1.1 ± 0.1	15.5
8.5 h	0.52 ± 0.05	15.5
21 h	0.64 ± 0.06	15.5
48 h*	0.57	12.6

* At 21 h, a second aliquot of NPA-Br was added.

The reaction is first-order in each component, α-chymotrypsin and NPA-Br. The order of reactivity was pH 3 < pH 5 < pH 7 ~ pH 8. An attempt was made to demonstrate binding prior to reaction, but the solubility of NPA-Br precluded saturation of the enzyme*.

* Our very approximate overall rate at pH 4 is $1 \cdot 10^{-6}$ M·min^{-1}. Using the n values of PEARSON *et al.*[13] for various sulfur nucleophiles, the approximate value of 50 M^{-1}·min^{-1} as a second-order rate constant for the reaction of *p*-bromophenacyl bromide and thiourea in methanol[14], (assuming an s value of about unity for the phenacyl halides) gives an overall rate for our concentrations of about $1 \cdot 10^{-7}$ M·min^{-1} in the organic system. We have not and cannot correct for solvent, since the reaction is on the protein. The *p*-nitro compound ought to be faster

Well after catalysis of L-ATrEE hydrolysis by aliquots of the reaction mixture became constant with time (> 10 half-lives), a second aliquot of NPA-Br (again 3–20-fold excess over initial α-chymotrypsin) was added to the already reacted solution, and another several half-lives produced no further decrease in activity. Gel filtration of the reaction mixture produced essentially no (< 5%) change in behavior of the enzyme derivative. Blanks were performed using NPA-Br (no α-chymotrypsin) which showed neither catalysis nor inhibition of L-ATrEE hydrolysis.

For kinetic and spectral studies, the reaction mixture of *p*-nitrophenacylchymotrypsin was filtered on Sephadex G-25 gel and then was lyophilized.

Properties of p-nitrophenacylchymotrypsin

Amino acid analysis (on a Beckmann 120C) of the enzyme derivative showed no loss of any residue other than methionine. That this methionine is at Position 192 has been shown by SIGMAN AND BLOUT[8] and is not unexpected on the basis of the known behavior of α-chymotrypsin towards a large number of reagents and substrates. The absorption at 350 nm is in agreement with the literature.

While it was reported that the enzyme derivative had no activity[8], we found that we could not destroy the residual activity (about 10%, *vide infra*) by forcing further reaction with NPA-Br as described above. This decreased but significant activity is manifest towards methyl-*N*-acetyl-L-tryptophanate (L-ATrME), L-ATrEE and L-ATyEE but not at all towards D-ATrME. No catalysis of ester hydrolysis occurs in 8 M urea. Reaction of *p*-nitrophenacylchymotrypsin with the chymotrypsin-specific inhibitor *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) destroyed the activity as shown in Table II*.

TABLE II

INHIBITION OF *p*-NITROPHENACYLCHYMOTRYPSIN BY TPCK AND UREA

p-Nitrophenacylchymotrypsin (24.9 μM) and 0.4 mM TPCK in 0.1 M phosphate buffer (pH 6.98). Aliquots assayed as usual with L-ATrEE in Tris buffer (pH 8.09).

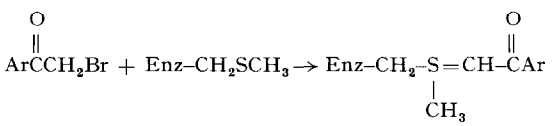
Time of incubation (h)	$v_1 \times 10^6$ $(M \cdot sec^{-1})$
0	1.75
0.5	1.43
7	1.11
22	0.81
46	0.80
46 + 2nd aliquot TPCK (approx. 1 min)	0.65
46 + 24	0.44
46 + 48	0.31
46 + 48 (control, no TPCK)	1.63
0	2.01
8 M urea (approx. 1 min)	approx. 0

than the *p*-bromo derivative, and we do not really know the *s* value for this α-halo ketone series. The calculation is thus extremely approximate but does at least show about the same order of magnitude for the two reactions.

* TPCK reacts with chymotrypsin much as does a substrate, which is its great usefulness as a site-specific reagent for this protein[15,16]. We could expect that the decrease in rate of reaction with *p*-nitrophenacylchymotrypsin might be about the same as that of the L-ATrEE reaction. It has been shown with native chymotrypsin that extraneous reactions use up TPCK after a certain

The hydrolysis of L-ATrEE catalyzed by trypsin has been studied kinetically[17], and, in order for our observed activity to be due to trypsin, it would have had to constitute 10% of our original enzyme samples. Rate assays with our preparation and a trypsin substrate showed very little catalysis, and we can thus eliminate trypsin as the source of activity. All our results were reproducible with material of two different Worthington lot numbers. It is our conclusion from this evidence that (a) the residual activity is due to a protein and (b) this protein is Met-192 *p*-nitrophenacylchymotrypsin. Even if there was alkylation at another residue, our great excess and length of time of reaction with the derivatizing agent should have produced further reaction at the methionine*.

At the beginning of this work, we were under the impression that (as originally reported) the 350-nm band which appears upon formation of this derivative was due to a charge transfer interaction between the *p*-nitrophenacyl group as an acceptor and some donor moiety on the protein. It has now been shown that the absorption arises because the reaction has produced not a sulfonium salt but a stable sulfur ylide that shows essentially the same absorption in the non-protein system[18].



In either case, this absorption, since it occurs at a wavelength remote from any others of the protein, can be used to test for environmental changes in its vicinity. Table III

TABLE III

pH DEPENDENCE OF THE SPECTRUM OF GEL FILTERED *p*-NITROPHENACYLCHYMOTRYPSIN
Each line represents a separate experiment.

pH	$A_{282\ nm}$	$A_{350\ nm}$	$\frac{A_{282\ nm}}{A_{350\ nm}}$
2.88	1.60	0.18	8.9
3.00	1.71	0.18	9.5
3.80	1.54	0.18	8.6
5.04	1.49	0.18	8.3
6.50	1.46	0.18	8.1
7.60	1.66	0.20	8.3
7.63	1.72	0.22	7.8
7.64	1.44	0.17	8.5
8.86	1.39	0.16	8.6
9.89	1.33	0.15	8.7
10.7	1.20	0.14	8.6
11.25	1.72	0.20	8.6
Av. for all pH values			8.6 ± 0.3

incubation time so that the reaction does not go to completion until a second aliquot is added. This is due to a competing reaction and is pronounced in our study because of the decreased activity of *p*-nitrophenacylchymotrypsin. The inhibition still occurs too rapidly to be accounted for by trypsin activity or by reaction at a site other than the active site of chymotrypsin.

* Further evidence for this activity being that of *p*-nitrophenacylchymotrypsin itself is that with unsubstituted and *p*-methoxyphenacyl bromide a lower and different activity is obtained.

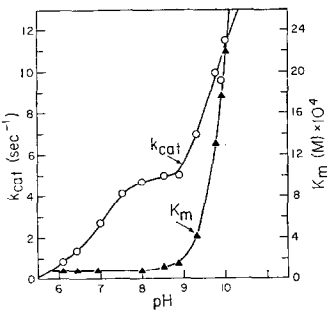


Fig. 1. Kinetic parameters of the *p*-nitrophenacylchymotrypsin catalyzed hydrolysis of L-ATrEE as a function of pH.

shows that pH from 2.9 to 11.2 had no effect on the band, the ratio of molar absorptivity at 282 nm to that at 350 nm being 8.6 ± 0.3.

This is in accord with the ylide assignment; it is known that at pH 9, a conformational change occurs in this region of the protein that would likely have affected a charge transfer interaction[3,4], but would not necessarily be expected to affect the ylide absorption.

Of primary interest, then, was the determination of the nature of the residual activity, or more specifically, the reasons for the remaining activity being so small since Met-192 is not involved in catalysis and the activity of chymotrypsins with derivatives at this position is usually only somewhat altered.

Fig. 1 and Table IV show the results of a study of the pH dependence of the kinetic parameters in the hydrolysis of L-ATrEE by *p*-nitrophenacylchymotrypsin. All data are at least duplicate runs plotted independently in the Lineweaver–Burk manner. Significant features are that (a) K_m has the same values and the same pH dependence as that of α-chymotrypsin[12]; (b) below pH 8.5, k_{cat} is close to 10% of the value for α-chymotrypsin and behaves identically as a function of pH (dependence on a base of pK 7, presumably the imidazole group of His-57); and (c) above pH 8.5, k_{cat} increases, reaching a value of 11 sec^{-1} at pH 10. The latter behavior is different

TABLE IV

KINETIC PARAMETERS FOR THE CATALYSIS OF L-ATrEE HYDROLYSIS BY *p*-NITROPHENACYLCHYMOTRYPSIN; pH DEPENDENCE

pH	k'_{cat} (*sec*$^{-1}$)	$K_m' \times 10^4$ (*M*)	$k'_{cat}/K_m' \times 10^{-3}$ ($M^{-1} \cdot sec^{-1}$)
6.10	0.81	0.88	9.2
6.43	1.40	0.82	17.1
7.00	2.70	0.82	32.9
7.54	4.04	0.85	47.4
7.96	4.61	0.92	50.1
8.55	5.05	2.23	22.6
8.90	4.97	3.38	14.7
9.28	7.45	4.82	15.5
9.73	10.1	13.2	7.6
9.88	9.6	17.6	5.4
10.00	11.1	21.6	5.1

from that of α-chymotrypsin for which k_{cat} is pH independent from pH 8 to at least pH 11.5 (ref. 3). The rates above pH 9 have been carefully corrected for OH^- and buffer-catalyzed hydrolysis but would not be within several orders of magnitude rapid enough to account for the observations in any case. There was no loss of nitrophenacyl group or other irreversible change since the protein could be incubated at pH 10 for the time of several experiments and then be used in a kinetic experiment at pH 7.5 to give the same results as an enzyme originally dissolved at pH 5 or 7 and assayed at pH 7.5.

With L-ATyEE as a substrate at pH 7.00 (0.1 M phosphate buffer), two separate experiments with *p*-nitrophenacylchymotrypsin showed $k_{cat} = 13.7 \pm 1.3$ sec^{-1} and $K_m = 13.8 \pm 1.8 \cdot 10^{-4}$ M. For α-chymotrypsin, $k_{cat} = 147$ sec^{-1} and $K_m = 9.75 \cdot 10^{-4}$ M at this pH and our conditions. Thus here the overall "velocity" is decreased by something greater than 10-fold, and the effect almost entirely again is in k_{cat}. One cannot investigate this substrate at a high pH because of ionization of the phenolic hydroxyl group.

We have also, by analogous procedures, prepared the unsubstituted phenacylchymotrypsin and *p*-methoxyphenacylchymotrypsin. It is significant that for these two derivatives the overall activity is less than that of the *p*-nitrophenacyl compound (about 20-fold decreased from native enzyme) and also that this decreased activity is manifest in both k_{cat} and K_m. There is the same pK 7 dependence but then no increase in k_{cat} at higher pH values.

DISCUSSION

The previous section has described results which are in accord with the conclusion that NPA-Br reacts with α-chymotrypsin to form an alkylated *p*-nitrophenacylchymotrypsin which has a well-defined catalytic activity. This result is in itself not very surprising, since a number of derivatives of chymotrypsin with reacted Met-192 have either unchanged or even increased activity. That the entire alteration is manifest in k_{cat} and not at all in K_m when this non-catalytic group is alkylated is, however, a somewhat unexpected situation. The methionine can be oxidized to the sulfoxide[7], alkylated with small aliphatic groups or with reagents containing carboxylate[5,6] (or presumably other hydrophilic groups) somewhere on a short chain with little change in catalytic activity.

In contrast, alkylation with the various phenacyl groups and also with the $ArNHCOCH_2$–[19] group resulted in significant loss of activity. Our data might suggest that in these latter cases the aromatic ring is at least partly binding to or disrupting some catalytic group near the active site, while the former non-hydrophobic derivatives are probably largely projecting into the aqueous solution.

The pH dependence of *p*-nitrophenacylchymotrypsin is in reasonable agreement with a theoretical sigmoid dependence on a base of p$K_a = 7$ for k_{cat}. The Michaelis constant, K_m, is pH independent from pH 5 to about 8.5. Both of these are properties of native α-chymotrypsin, with a k_{cat} decreased only in absolute magnitude. A rather small shift in the relative positions of the catalytic functions at the active site of the protein might reasonably be expected to produce significant alterations in the magnitude of the catalysis.

The potentially most significant behavior of *p*-nitrophenacylchymotrypsin is its

kinetic behavior above pH 8. As can be seen in Fig. 1, the conformational change occurring in native α-chymotrypsin that is dependent on an acid of $pK_a \simeq 9$ and manifest only in K_m occurs here as well. In fact, K_m for L-ATrEE is identical to that of native enzyme at all pH values. Since this substrate is a "specific" one, we conclude that rather little conformational change has occurred upon preparation of the derivative. Actually K_m and k_{cat} are complex constants, as defined below, where ES is the adsorptive enzyme–substrate complex, ES' is a covalent acyl-enzyme, P_1 is alcohol and P_2 acid from the original ester, S.

$$E + S \overset{K_s}{\rightleftarrows} ES \overset{k_2}{\rightarrow} ES' \overset{k_3}{\rightarrow} E + P_2$$
$$\qquad\qquad\qquad\quad + \\ \qquad\qquad\qquad\quad P_1$$

$$K_m = K_s \cdot \frac{k_3}{k_2 + k_3} \qquad k_{cat} = \frac{k_2 k_3}{k_2 + k_3}$$

Assuming identical changes for k_2 and k_3 *vs.* L-ATrEE in *p*-nitrophenacylchymotrypsin*, and representing this change by a factor x and the new parameters by K_m' and k'_{cat} the equations are now:

$$K_m' = K_s' \frac{x\, k_3}{x\,(k_2 + k_3)} \qquad k'_{cat} = \frac{x^2 k_2 k_3}{x\,(k_2 + k_3)}$$

$$K_m' = K_s' \frac{k_3}{k_2 + k_3} \qquad k'_{cat} = x \frac{k_2 k_3}{k_2 + k_3}$$

Since $K_m' = K_m$ at all pH values:

$$K_s = K_s' \quad \text{and} \quad k'_{cat} = x k_{cat}$$

In terms of the kinetic parameters of α-chymotrypsin, K_s would be unchanged for this situation.

Returning now to the high pH dependence, K_s', since it behaves just as does K_s for α-chymotrypsin, is very likely showing essentially the same conformational change. However, for α-chymotrypsin, this change is an all-or-none situation; *i.e.*, binding would seem to occur with one given efficiency if the $pK_a = 9$ group is protonated and not at all if unprotonated. The ES complex for α-chymotrypsin at high pH should be the same as that at lower pH because k_{cat} and k_3 are completely unaffected by the $pK = 9$ group. In *p*-nitrophenacylchymotrypsin there is a definite dependence of k'_{cat} on pH in the higher pH range, this dependence being an increase. Significant effort was expended in trying to obtain data at pH > 10, but we were unable to obtain reliable data for the same reasons that native enzyme cannot be rigorously studied at pH > 11 (see ref. 3). In Fig. 1 we have drawn k'_{cat} as passing through all the points in the absence of any compelling reason to do otherwise**. The only conclusion about the pH dependence of k'_{cat} that would seem to be justified by our data is that there is an inflection point at pH 7 followed by a narrow flat region

* Since $K_m' = K_m$, this is a likely situation. Inhibition by *N*-acetyl-L-tryptophanamide is about equally effective for *p*-nitrophenacylchymotrypsin and native enzyme ($K'_i \simeq K_i$).

** However, its fit to a theoretical curve of $pK =$ approx. 9 is certainly possible. The problem is that one must assume a "flat" at the high pH end (>10.5), and it is possible to fit almost any smoothly rising data to either a given pK or a given "flat" if a value for one of the variables is assumed.

and then a subsequent rather sharp increase noticeable at about pH 8.5–9 and still rising at pH 10. All this behavior is due to a reversible effect on the protein but is not due to an acid–base reaction at the methionine ylide since its ultraviolet absorption is unchanged.

We would like to tentatively propose that the increase in k'_{cat} with pH is not only concomitant with the conformational change taking place with a $pK = 9$ but is a consequence of it. The fact that k'_{cat} is decreased for *p*-nitrophenacylchymotrypsin at the lower pH values while K_m' is undisturbed indicates one of two possibilities; (a) a direct steric interaction between a catalytic group and a segment of the protein that has changed chemically or conformationally because of the derivatization or (b) a movement of one catalytic group relative to another that normally acts in concert with the first. The increasing k'_{cat} at higher pH values could represent a reversal of the effect that caused the decrease in the first place. Significantly, the phenacyl and *p*-methoxyphenacyl derivatives, which show less favorable binding and catalysis, do not show a $pK = 9$ dependence.

Circular dichroism studies of *p*-nitrophenacylchymotrypsin at high pH would be very useful in elucidating the structural changes occurring.

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