Evidence for a pH-Dependent Irreversible Formation of a Stable Conformation of Phenacyl- α -Chymotrypsin

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(Received 19 August 1977)

A reinvestigation of the modification reactions of α -chymotrypsin with phenacyl bromide was carried out. Results conclusively demonstrate that the chemically and physically different modified enzymes prepared at pH4 and at pH7 both contain the phenacyl group at methionine-192 in the sulphonium salt form. Evidence to support this conclusion derives from ^{13}C nuclear-magnetic-resonance spectroscopic observations on [*methylene*- ^{13}C]phenacyl-enriched enzymes. More conclusively, the methionine-192-containing C-chain, derived by performic acid oxidative cleavage of radioactively-labelled enzyme prepared at pH7, was shown to contain the phenacyl moiety and to undergo dealkylation by 2-mercaptoethanol with loss of this moiety. In addition, thermolytic cleavage of the high-pH enzyme results in fragmentation of the polypeptide chain in a fashion analogous to model reactions of phenacylethionyl dipeptides and other methionine-192 sulphonium salts. A rationalization of the unusual nature of the high-pH phenacyl-modified enzyme based on the irreversible formation of stable conformation in which the phenacyl moiety is rigidly located in interior regions of the enzyme is presented and discussed.

We have previously shown that two characteristically different modified enzymes can be produced by the reaction of α -chymotrypsin with phenacyl bromide (Glover *et al.*, 1976). The low-pH enzyme, prepared by reaction at pH4, was shown to be the methionine-192-modified phenacyl sulphonium salt, as proposed originally by Schramm & Lawson (1963) and confirmed later by Naider & Bohak (1972) by using the 2-mercaptoethanol dealkylation technique. A noteworthy result of our initial studies was the finding that a high-pH enzyme, possessing unique chemical and physical properties, could be generated by reaction of α -chymotrypsin with phenacyl bromide at pH7 or by incubation of the low-pH enzyme at neutral pH. One of the characteristic features of the high-pH enzyme was its insensitivity to 2-mercaptoethanol dealkylation reaction conditions. On the basis of these limited observations, conclusions about the nature of the previously uncharacterized high-pH enzyme were tentative. Thus we postulated that the new modified enzyme was perhaps arising by chemical transformation of the low-pH enzyme involving transfer

of the *S*-phenacyl or *S*-methyl moiety at methionine-192 to nucleophilic centres in nearby amino acid residues (Scheme 1). Chemical changes of this nature could conveniently rationalize the pH-dependence of the process and the resistance of the high-pH enzyme to 2-mercaptoethanol dealkylation, which is specific for sulphonium salts.

Other explanations of these initial observations are of course possible. Therefore the object of the present investigation was to gain additional information about the nature of the new high-pH enzyme. Results from the present study, which used a combination of ^{13}C n.m.r. spectroscopy and chemical degradative techniques, strongly demonstrate that the phenacyl moiety of this modified enzyme is still located at methionine-192 as the sulphonium salt. Furthermore, evidence appears to suggest that the process occurring at pH7 to convert the initially formed low-pH enzyme into the high-pH enzyme corresponds to an irreversible conformational change that places the methionine-192 phenacyl group in an environment that blocks 2-mercaptoethanol dealkylation.

Scheme 1. Possible transformations involving *S*-phenacyl or *S*-methyl migration in phenacyl- α -chymotrypsin
 Abbreviations: Nu, nucleophile; CT, chymotrypsin.

Materials and Methods

General

All u.v.-visible spectrophotometric measurements were recorded by using a Beckman Acta III u.v.-visible spectrophotometer. ^1H n.m.r. spectra were determined with a Varian T-60 spectrometer with tetramethylsilane as an internal standard. The ^{13}C n.m.r. spectra were determined with a JEOL JNM-PS-100 spectrometer with $[\text{H}]$ chloroform or $^2\text{H}_2\text{O}$ as an internal lock and tetramethylsilane or acetonitrile as internal standards. Radioactivity was determined in Aquasol solutions (New England Nuclear Corp., North Haven, CT, U.S.A.) with a Beckman LS-100 liquid-scintillation system. All melting points were determined in capillary tubes with a Mel-temp melting-point apparatus and are reported uncorrected. Amino acid analyses were determined with a Beckman model 120C amino acid analyzer. All studies requiring accurately controlled pH were performed on a pH-stat (Radiometer, Copenhagen, Denmark).

Chemicals

[carbonyl- ^{14}C]Phenacyl bromide (sp. radioactivity 1.21×10^8 d.p.m./mmol), for the preparation of radioactively labelled enzymes, was prepared by the method described previously (Glover *et al.*, 1974). [methylene- ^{13}C]Phenacyl bromide (90% ^{13}C enrichment) was derived from [^{13}C]methyl iodide (Merck and Co., Croton, CT, U.S.A.) by using reported procedures for the preparation of acetophenone (Tegner, 1952) and phenacyl bromide (Cowper & Davidson, 1943). *N*-Acetyl-L-methionylglycine ethyl ester (Lawson *et al.*, 1962), *N*-(2,4-dinitrophenyl)alanine and *N*-(2,4-dinitrophenyl)glycine (Fraenkel-Conrat *et al.*, 1959), phenacyl acetate (Cavill & Solomon, 1955), ethyl phenacyl sulphide (Furuta & Ishimaru, 1969), α -(*NN*-diethyl)aminoacetophenone (Marvel & Du Vigneaud, 1924), α -ethoxyacetophenone (Yates, 1952), α -phenoxyacetophenone (Saburi *et al.*, 1967) and α -imidazol-1-ylacetophenone (Jones & Hysert, 1971) were all prepared by the reported procedures.

Protein chemistry

The high- and low-pH modified α -chymotrypsins were prepared by reactions of the native enzyme with phenacyl bromide and purified by procedures reported previously (Glover *et al.*, 1976). Thermolytic cleavage reactions of the modified phenacyl- α -chymotrypsins and the model compound, *N*-acetyl-S-phenacyl-L-methionylglycine ethyl ester, were carried out by the method of Lawson *et al.* (1962). Oxidative disulphide cleavage reactions with performic acid were carried out under the conditions described by Hirs (1956) and separations of the A-,

B-, and C-chains by gel filtration were by the method of Hoang *et al.* (1962). *N*-Terminal amino acid analyses with Sanger's reagent were carried out by the method suggested by Lawson & Schramm (1965). Amino acid analyses were by reported procedures (Liu, 1972). Determinations of enzyme esterase activities were made spectrophotometrically by using the procedure described previously (Glover *et al.*, 1974). The kinetic parameters k_{cat} and K_m were determined from Lineweaver-Burk plots (Gibian *et al.*, 1969) by using initial rates of hydrolysis of *N*-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester at pH 8.0.

S-Phenacylmethionine sulphonium bromide

To a solution of methionine (1 g, 7.4 mmol) in 20 ml of formic acid was added phenacyl bromide (5.9 g, 29.6 mmol). The reaction mixture was stirred for 24 h at 25°C. Water was added and the aqueous layer was extracted with diethyl ether, diluted to 360 ml with water and freeze-dried to give the solid *S*-phenacylmethionine sulphonium bromide. Spectral data for this compound are as follows: ^1H n.m.r. (trifluoroacetic acid) 2.60 (2 H, d, CH_2), 2.99 (3 H, s, CH_3), 3.59 (2 H, t, CH_2), 4.39 (1 H, m, CH), 5.15 (2 H, s, CH_2), 7.06–7.94 (5 H, m, Ar); ^{13}C n.m.r. [water, pH 3, δ (p.p.m.) relative to tetramethylsilane] 24.98 (q, CH_3), 26.41 (t, CH_2), 39.04 (t, CH_2), 52.73 (d, CH), 53.86 (t, CH_2), 130.25, 130.56 (d, *o*- and *m*-Ar), 134.31 (d, *p*-Ar), 137.06 (s, C-1 of Ar), 171.51 (s, carbonyl), 192.76 (s, ketone carbon); ^{13}C n.m.r. [water, pH 9, δ (p.p.m.) relative to tetramethylsilane] 27.74 (q, CH_3), 28.12 (t, CH_2), 42.43 (t, CH_2), 54.43 (d, CH), 66.37 (d, CH), 126.89, 127.76 (d, *o*- and *m*-Ar), 128.70 (d, *p*-Ar), 131.01 (s, C-1 of Ar), 178.15 (s, carbonyl), 181.84 (s, ketone carbon).

Results and Discussion

^{13}C n.m.r. spectroscopic investigations

^{13}C n.m.r. spectroscopy, which has shown wide application as a technique in exploring the nature of modified proteins (Gurd & Keim, 1973; Nigen *et al.*, 1972), appeared ideally suited for our initial investigations of the novel enzyme produced by phenacyl bromide modification of α -chymotrypsin at high pH. With this technique the phenacyl α -methylene carbon could be used as a probe to determine the location of the phenacyl moiety on the polypeptide backbone. Accordingly, if chemical processes were responsible for production of the high-pH enzyme, the phenacyl grouping could be covalently linked to a number of different nucleophilic centres, including the original methionine sulphur in the sulphonium ylide form or derived homocysteine, the hydroxyl oxygen atoms of serine, threonine and tyrosine, the ϵ -amino group

of lysine, the imidazole nitrogen atoms of histidine or the carboxylate oxygen atom of aspartic acid. The actual site functionalized might be determined by comparing the chemical shift of the phenacyl α -methylene carbon atom in the high-pH enzyme, prepared from enriched [methylene- ^{13}C]phenacyl bromide, with those of model systems.

A number of model compounds, designed to mimic portions of potentially modified enzymes resulting from migration of the *S*-methyl or *S*-phenacyl fragment of the methionine-192 sulphonium salt to nearby nucleophilic centres, were prepared (Table 1). Analyses of the proton noise-decoupled and coupled ^{13}C n.m.r. spectra of each of these compounds allowed unambiguous assignments to be made to resonances associated with the phenacyl α -methylene carbon atoms. The spectroscopic data, summarized in Table 1, suggested that the α -carbon resonances are sufficiently sensitive to the type of functional group attached to the phenacyl moiety to be potentially useful in probing the site of modification in the high-pH enzyme.

The preparation of the low- and high-pH enzymes with ^{13}C -labelled phenacyl α -carbon atoms was also studied. Although we expected the background signals due to the natural abundance of ^{13}C in the large number of carbon atoms found in these enzymes to be significant, an approximate calculation indicated that distinguishable resonances for the phenacyl α -methylene carbon atoms would be detectable if 90% isotopic enrichment at this position could be accomplished. As a result, [methylene- ^{13}C]phenacyl bromide was prepared by the reaction of methyl-lithium (from ^{13}C]methyl iodide, 90%-enriched) with lithium benzoate followed by bromination of the intermediate acetophenone (Tegner, 1952; Cowper &

Davidson, 1943). A final check on the enrichment obtained by this procedure was performed by using integrated areas on ^1H n.m.r. resonances for [^{12}C]- and [^{13}C]-phenacyl bromides, and shown to be the anticipated 90%. Modification reactions of α -chymotrypsin were carried out with this reagent under the conditions reported previously (Glover *et al.*, 1976). ^{13}C n.m.r. spectra of the low- and high-pH enzymes (Figs. 1b and 1c) contained broad resonances for the enriched phenacyl α -methylene

Fig. 1. ^{13}C n.m.r. spectra of (a) α -chymotrypsin (12mm) in water, (b) the low-pH enzyme (14mm) in water, prepared by reaction of α -chymotrypsin with [methylene- ^{13}C]phenacyl bromide at pH4, and (c) the high-pH enzyme (15mm) in water, prepared by incubation of ^{13}C -enriched low-pH phenacyl enzyme at pH7.

The following instrument parameters were used in each case: sweep width, 6400 Hz; filter, 7000 Hz; tilt angle, $18\mu\text{s}$ when 90° pulse equals $54\mu\text{s}$; repetition rate, 2.2 s; broad-band proton decoupling with ^1H -irradiation frequency, 99.53925 MHz. The phenacyl α -methylene carbon resonances in spectra (b) and (c) are indicated by arrows and the chemical shifts given are relative to acetonitrile as an internal standard.

Table 1. Phenacyl α -methylene carbon chemical shifts for a series of model compounds ($R = \text{PhCO}$)

Chemical shifts are recorded relative to tetramethylsilane as an internal standard.

Structure	Phenacyl α -carbon chemical shift (p.p.m.)
$\text{R}-\text{CH}_2-\text{S}(\text{CH}_3)-\text{CH}_2-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{CO}_2\text{H}^*$	53.86
$\text{R}-\text{CH}=\text{S}(\text{CH}_3)-\text{CH}_2-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{CO}_2\text{H}^\dagger$	66.37
$\text{R}-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}_3$	36.63
$\text{R}-\text{CH}_2\text{O}_2\text{C}-\text{CH}_3$	66.01
$\text{R}-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_3$	73.45
$\text{R}-\text{CH}_2-\text{O}-\text{C}_6\text{H}_5$	70.45
$\text{R}-\text{CH}_2-\text{N}(\text{CH}_2-\text{CH}_3)_2$	59.59
$\text{R}-\text{CH}_2-\text{N} \begin{array}{c} \diagup \text{N} \diagdown \\ \diagdown \text{C} \diagup \end{array}$	52.45

* Run in aqueous solution ($^2\text{H}_2\text{O}$) at pH3.

† Run in aqueous solution ($^2\text{H}_2\text{O}$) at pH9.0.

carbon atom at 52.64 p.p.m. (W_H 20 Hz) and 52.50 p.p.m. (W_H 35 Hz) respectively. Comparison of these values with those recorded in Table 1 shows that the observed chemical shifts for the enriched carbon atoms in both enzymes are near those expected for a phenacyl group covalently linked to a histidine or methionine residue as *N*-amino or *S*-sulphonium salt functions. However, an unambiguous assignment of the exact location of the phenacyl moiety in the high-pH enzyme is precluded by the fact that the signals for the α -methylene carbon atom of this group are considerably broadened. In addition, our studies have demonstrated that the chemical-shift differences for this resonance in imidazole and methionine models would be small. Thus, although the ^{13}C n.m.r. spectroscopic data obtained for the low- and high-pH enzymes suggest that the phenacyl groups in both are bound as sulphonium salts at methionine residues, additional chemical evidence is required.

Dealkylation of performic acid-cleaved enzymes

In our previous studies (Glover *et al.*, 1976) several alternative explanations for the pH-dependent nature of the phenacyl- α -chymotrypsins had been considered. In one of these, the inertness of the high-pH enzyme under 2-mercaptoethanol dealkylation conditions was temporarily rationalized on the basis of irreversible formation of the sulphonium ylide at methionine-192. It was thought that conformational features present in the high-pH modified enzyme might retard the rate of proton transfer to re-form the sulphonium salt. The failure to detect dealkylation after the high-pH enzyme is reversibly heat-denatured under acidic conditions (Glover *et al.*, 1976) appeared to rule out this explanation, as well as others that invoked unusual conformations in the methionine-192 region to rationalize the resistance of the high-pH enzyme to 2-mercaptoethanol. This comprised part of the evidence used to tentatively conclude that the phenacyl moiety in this modified enzyme was not on the methionine residue. On the other hand, one interpretation of the almost identical phenacyl α -methylene carbon chemical shifts in spectra of the

high- and low-pH enzymes, appears to contradict the initial findings. Clearly, further chemical investigations were necessary.

A study of the oxidative cleavage products of the modified enzymes was then carried out. Radioactively labelled high-pH enzyme (sp. radioactivity 1.03×10^8 d.p.m./mmol) was prepared from [^{14}C]phenacyl bromide by the procedure described previously (Glover *et al.*, 1974, 1976). Performic acid oxidative cleavage (Hirs, 1956) of the disulphide linkages of this enzyme generated the A, B and C polypeptide chains, which were separated by gel filtration (Hoang *et al.*, 1962). Most of the radioactivity associated with the original enzyme was found in the separated C-chain (sp. radioactivity 0.88 d.p.m./mmol), which contains the original methionine-192 residue. Importantly, treatment of the radioactively labelled C-chain with 2-mercaptoethanol led to removal of 87% of the radioactivity present initially. This observation appears to confirm conclusions from the ^{13}C n.m.r. experiments that the phenacyl group in the high-pH enzyme is still covalently linked as a sulphonium salt at methionine-192.

Thermal cleavage of the modified enzymes

An alternative chemical approach has been used to gather further evidence on this point. Witkop and co-workers (Lawson *et al.*, 1962) have shown that sulphonium salts of methionyl dipeptides, e.g. *N*-acetyl-*S*-alkylmethionylglycine ethyl ester (1; $\text{R} = \text{alkyl}$), undergo degradative cyclization when heated in aqueous solution (see Scheme 2). The heterolytic fragmentation pathway followed in this process is analogous to that occurring in the CNBr cleavage of methionine peptides and leads to generation of homoserine lactone, a methyl thioether and glycine ethyl ester. Moreover, this technique has been used to identify methionine-192 as the site of alkylation in the affinity-controlled bifunctional modification of α -chymotrypsin by *p*-nitrophenyl *N*-bromoacetyl- α -amino-isobutyrate (Lawson & Schramm, 1965). Thus this method appears to be directly applicable

Scheme 2. Degradative cyclization of *N*-acetyl-*S*-alkylmethionylglycine ethyl ester

to location of the phenacyl moiety in the high-pH enzyme, since the identities of the *N*-terminal amino acid residues in the C-chain fragments derived by oxidative and sequential thermal and oxidative cleavage of this modified enzyme should vary if the sulphonium salt function is present at methionine-192.

Preliminary experiments were carried out with *N*-acetyl-*S*-phenacilmethionylglycine ethyl ester (1; R = PhCOCH₂) to demonstrate that the phenacyl grouping would not change the nature of this reaction. Indeed, amino acid analysis of the mixture obtained after heating this modified dipeptide at 110°C for 70min showed the presence of glycine ethyl ester corresponding to 53% cleavage of the methionylglycine peptide bond.

This technique was then used to gain information about the nature of the modified enzyme. Solutions containing α -chymotrypsin, and the low- and high-pH phenacyl-enzymes were subjected to thermal-cleavage conditions (110°C for 70min) and then to performic acid oxidation. The C-chains and C-chain fragments were separated from larger polypeptides

by gel filtration. Under the conditions used, the normal α -chymotrypsin-derived C-chain and both C-chain fragments generated by thermal cleavage co-chromatograph and therefore are obtained as mixtures. Mixtures containing the C-chain fragments were then treated with Sanger's reagent, 1-fluoro-2,4-dinitrobenzene (Lawson & Schramm, 1965), and hydrolysed. Chromatographic separation of the diethyl ether-soluble *N*-dinitrophenylated glycine and alanine gave pure materials that could be quantitatively analysed by visible-spectrophotometric techniques. Additional control-reaction sequences that omitted the thermal cleavage steps were carried out with the native and modified enzymes.

The results of these reaction sequences (Scheme 3) are summarized in Table 2, and show that yields of dinitrophenylated alanine obtained from the C-chain and C-chain fragments of each of the enzymes were in general high (approx. 85%). In addition, *N*-terminal glycine production, determined by the ratio of dinitrophenylated glycine and alanine obtained, occurred to a much greater extent in those degradative sequences in which modified enzymes were used and

Scheme 3. Thermal degradation of the C-chain of modified α -chymotrypsin
Abbreviation: Dnp, 2,4-dinitrophenyl.

Table 2. Analysis of *N*-terminal amino acids of C-chain and C-chain fragments derived by thermal-oxidative cleavage sequences

N-Terminal glycine/alanine ratios were determined by visible-spectrophotometric analysis of separated dinitrophenylated amino acids. Concentrations of C-chain and C-chain fragments were estimated by amino acid analysis.

Enzyme	<i>N</i> -Terminal glycine/alanine ratio	<i>N</i> -Terminal alanine production as a fraction of total C-chain and C-chain fragments
α -Chymotrypsin*	0.07	
Low-pH enzyme*	0.05	0.85
High-pH enzyme*	0.06	0.86
α -Chymotrypsin†	0.1	—
Low-pH enzyme†	0.40	0.87
High-pH enzyme†	0.41	0.88

* Enzyme subjected to performic acid oxidation, only, before dinitrophenylation and hydrolysis.

† Enzyme heated at 110°C for 70 min before performic acid oxidation, dinitrophenylation and hydrolysis.

initial thermal cleavage steps were included. The ratios of *N*-terminal glycine to alanine in these cases reflect efficiencies for the thermal cleavage reactions of about 32%. These values are close to that obtained by Lawson & Schramm (1965) with methionine-192-modified α -chymotrypsin derived by reaction with *p*-nitrophenyl *N*-bromoacetyl- α -aminoisobutyrate and in the range expected on the basis of yields for analogous cleavage reactions of the model phenacyl sulphonium salts (1). Lastly, thermal-oxidative cleavage sequences were carried out with [*methylene*-¹⁴C]phenacyl-labelled high- and low-pH enzymes. The amounts of radioactivity lost as a result of thermolytic cleavage of these enzymes (50 and 52% from the low- and high-pH enzymes respectively) correlate well with the overall yields of dinitrophenylated glycine produced.

Conclusions

Results from the spectroscopic and chemical investigations described above provide convincing evidence in support of the conclusion that the phenacyl moieties in both the high- and low-pH enzymes are covalently linked to methionine-192 in the sulphonium salt form. From these observations we are able to provide a more precise explanation of the differences between these modified enzymes. We have previously discussed a tentative chemical rationale for the significant finding that the high-pH enzyme, formed by alkylation of α -chymotrypsin at pH 7 or by incubation of the low-pH enzyme at

pH 7, is inert under the normal 2-mercaptoethanol dealkylation conditions. Accordingly, it was thought that intra-enzyme transfer of the potentially labile phenacyl group from methionine-192 to nearby nucleophilic centres would conveniently explain the pH effect and chemical properties observed. Obviously, the present findings invalidate this rationalization as well as others that are based on changes in the methionine-192 sulphonium salt function.

Another and now more plausible explanation of these features involves the irreversible formation of an unusually stable conformation for the modified α -chymotrypsin in which the methionine-192 phenacyl group resides in an interior region of the enzyme and is thus not susceptible to attack by the nucleophilic reagent, 2-mercaptoethanol. The chemical and physical data strongly suggest that the buried group in the high-pH enzyme is in the sulphonium salt rather than ylide form. It might have been expected that deprotonation of the sulphonium salt would have generated the uncharged ylide at methionine-192 capable of associating with hydrophobic interior regions of the enzyme. However, it is possible for a conformation having the buried sulphonium salt residue to be highly stabilized by ionic interactions with a carboxylate group.

Unfortunately, little direct precedent can be found to support explanations based on the irreversible conformational change postulated. Significant effects of pH on the conformation of the dimer of α -chymotrypsin have been observed by using X-ray crystallography (Tulinsky *et al.*, 1973; Vandlen & Tulinsky, 1973; Mavridis *et al.*, 1974). A change in pH from 3.6 to 8.3 causes dramatic relocations of the aspartate-194 and methionine-192 residues, which are located in the same general region of the enzyme (Blow & Steitz, 1970). The repositioning of the aspartate grouping appears to be strongly coupled to deprotonation of the isoleucine amino group, which disrupts the structure-stabilizing ionic bond between aspartate-194 and isoleucine-16. The flexibility of the polypeptide backbone around methionine-192 is indicated by the large reorientation that occurs with this residue.

It is noteworthy that methionine-192 remains on the surface of the enzyme throughout the pH-dependent conformational reorganization. On the other hand, it is known that the normal benzyl bromide alkylation of methionine-192 (Schramm & Lawson, 1963) fails to occur at pH values where the ionic bond between aspartate-194 and isoleucine-16 is disrupted (Wedler & Bender, 1969). Thus it is quite possible that sufficient movement of the positively charged methionine-192 sulphonium residue into the interior of the phenacyl-modified enzyme takes place as a result of conformational changes of this type to allow ionic bonding to the carboxylate anion of aspartate-194.

Also, this interaction could lead to formation of stable conformations in the locale of methionine-192 that have the phenacyl α -methylene carbon atom rigidly held in a position inaccessible to nucleophilic reagents.

The less-flexible positioning of the phenacyl group in the high-pH enzyme is indicated by the increased band-width of the phenacyl α -methylene carbon resonance in the high-pH enzyme (35 Hz) compared with the low-pH enzyme (20 Hz). This parameter has been correlated with rotational freedom in a number of biopolymer systems (Govil & Smith, 1973). Lastly, a comparison of $k_{\text{cat.}}$, K_m and calculated $k_{\text{cat.}}/K_m$ values for hydrolysis by α -chymotrypsin and the modified enzymes of *N*-benzyloxycarbonyl-tyrosine *p*-nitrophenyl ester were determined (Table 3). If the reasonable assumption is made that the rates of acylation are large for these systems and invariant with the nature of the modifying group, the observed variations in the $k_{\text{cat.}}/K_m$ ratios reflect substrate-binding constants, K_s , for these enzymes (Zerner & Bender, 1964). Thus it is possible that the greatly decreased value of $k_{\text{cat.}}$ for the high-pH enzyme as compared with that of both the low-pH and native enzymes again reflects the formation of stable conformations in which catalytic functions in the active-site region are reoriented by a reorganization of electrostatic interactions involving the aspartate-194 residue.

In summary, since its inception our study of the deceptively simple modification reactions of α -chymotrypsin by phenacyl bromide have continued to reveal the unusual nature and behaviour of the phenacyl bromide have continued to reveal the unusual nature and behaviour of the phenacyl- α -chymotrypsins. We are now able to conclude with reasonable certainty that both modified enzymes have the phenacyl group at methionine-192. The abnormal physical and chemical nature of the high-pH enzyme appears fully consistent with an irreversible conversion into a stable conformation that places the methionine-192 phenacyl sulphonium salt

grouping in a buried environment. As a result, these modified enzymes present unusual opportunities for crystallographic studies that seek insight into factors controlling the stability of protein conformations.

This research was supported by grants from the Robert A. Welch Foundation and the National Science Foundation. Partial support of this research by the donors of the Petroleum Research Fund administered by the American Chemical Society is gratefully acknowledged. P. S. M. acknowledges support from the Camille and Henry Dreyfus Foundation for the Teacher-Scholar grant, 1975-1980.

References

- Blow, D. M. & Steitz, T. A. (1970) *Annu. Rev. Biochem.* **39**, 63-100
- Cavill, G. W. K. & Solomon, D. H. (1955) *J. Chem. Soc.* 4426-4429
- Cowper, R. M. & Davidson, L. H. (1943) *Org. Synth.*, Coll. Vol. 2, 480-481
- Fraenkel-Conrat, H., Harris, J. I. & Levy, A. L. (1959) *Methods Biochem. Anal.* **2**, 359-425
- Furuta, T. & Ishimaru, Y. (1969) *Nippon Kagaku Zasshi* **90**, 936-939
- Gibian, M. J., Rubenstein, P. A. & Ficklin, W. H. (1969) *Biochim. Biophys. Acta* **191**, 677-685
- Glover, G. I., Mariano, P. S., Wilkinson, T. J., Hildreth, R. A. & Lowe, T. W. (1974) *Arch. Biochem. Biophys.* **162**, 73-82
- Glover, G. I., Mariano, P. S. & Petersen, J. R. (1976) *Biochemistry* **15**, 3754
- Govil, G. & Smith, J. C. P. (1973) *Biopolymers* **12**, 2589-2597
- Gurd, F. R. N. & Keim, P. (1973) *Methods Enzymol.* **27**, 836-911
- Hirs, C. H. W. (1956) *J. Biol. Chem.* **219**, 611-621
- Hoang, D. V., Rovey, M. & Desnuelle, P. (1962) *Biochim. Biophys. Acta* **58**, 613-616
- Jones, J. B. & Hysert, D. W. (1971) *Can. J. Chem.* **49**, 325-332
- Lawson, W. B. & Schramm, H.-J. (1965) *Biochemistry* **4**, 377-386
- Lawson, W. B., Gross, E., Foltz, C. M. & Witkop, B. (1962) *J. Am. Chem. Soc.* **84**, 1715-1718
- Liu, T.-Y. (1972) *Methods Enzymol.* **25**, 44-55
- Marvel, C. S. & Du Vigneaud, V. (1924) *J. Am. Chem. Soc.* **46**, 2093-2099
- Mavridis, A., Tulinsky, A. & Liebman, M. N. (1974) *Biochemistry* **13**, 3661-3666
- Naider, F. & Bohak, Z. (1972) *Biochemistry* **11**, 3208-3211
- Nigen, A. M., Keim, P., Marshall, R. C., Morrow, J. S. & Gurd, F. R. N. (1972) *J. Biol. Chem.* **247**, 4100-4102
- Saburi, Y., Minami, K. & Yoshimoto, T. (1967) *Nippon Kagaku Zasshi* **88**, 557-560
- Schramm, H.-J. & Lawson, W. B. (1963) *Hoppe-Seyler's Z. Physiol. Chem.* **332**, 97-100

Table 3. Kinetic constants for hydrolysis of *N*-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester by α -chymotrypsin and the low- and high-pH enzymes at pH 8.0

Enzyme	K_m (app.) (μM)	$k_{\text{cat.}}$ [$\Delta A \cdot \text{ml} \cdot$ (mg of protein) $^{-1} \cdot$ s^{-1}]	$10^{-5} \times k_{\text{cat.}}/K_m$
α -Chymo- trypsin	0.109	72	6.6
Low-pH enzyme	0.364	76	3.45
High-pH enzyme	0.222	30	1.35

Tegner, C. (1952) *Acta Chem. Scand.* **6**, 782-790
Tulinsky, A., Vandlen, R. L., Morimoto, C. N., Mani,
N. V. & Wright, L. H. (1973) *Biochemistry* **12**, 4185-4192
Vandlen, R. L. & Tulinsky, A. (1973) *Biochemistry* **12**,
4193-4200

Wedler, F. C. & Bender, M. L. (1969) *J. Am. Chem. Soc.*
91, 3894-3899
Yates, P. (1952) *J. Am. Chem. Soc.* **74**, 5376-5381
Zerner, B. & Bender, M. L. (1964) *J. Am. Chem. Soc.* **86**,
3669-3674