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The Modification of Essential Carboxylic Acid Side Chains of Trypsin*

Gad Feinstein,† Peter Bodlaender, and Elliott Shaw

ABSTRACT: In attempts to chemically modify carboxyl groups of importance for the function of trypsin, particularly one(s) presumably responsible for its specificity, conditions of reaction with isoxazolium salts were studied. Both the structure of the reagent and the pH of the modification reaction were important in achieving a selective modification. *N*-Methyl-5-phenylisoxazolium fluoroborate and *N*-ethyl-5-phenylisoxazolium at pH 3.8 resulted in the most specific reaction, producing nearly complete inactivation by the modification of two to three carboxyl groups. This effect was largely prevented when the reaction was carried out in the presence of a competitive inhibitor, benzamidine. The enol esters produced by activation with isoxazolium salts were treated with glycine ethyl ester and *O*-methylhydroxylamine, forming the corresponding amides in high yields. This second change did not affect the degree of inactivation. *N*-Methylhydroxylamine, on the other hand, although fully displacing the activating reagent, was not incorporated and restored tryptic activity. Hydroxylamine also quantitatively displaced the reagent with some reactivation, the extent of which varied with reaction time and degree of initial modification. Possible explanations for these phenomena are discussed. Lossen degradation of the trypsin hydroxamic acid derivative indicated modification of both aspartic and glutamic acid residues had taken place. The observation that diisopropyl phosphorofluoridate reacted to a greater extent than 1-chloro-3-tosylamido-7-amino-2-heptanone with several modified trypsin derivatives indicated the existence of enzyme species with altered enzymatic activity in the product mixture.

At least two stages of functional change were produced by carboxyl modification affecting specificity and serine reactivity in that order.

In its hydrolytic action, trypsin shows a strong preference for positively charged substrates either in simple derivatives (Neurath and Schwert, 1950) or in proteins (Canfield and Anfinsen, 1963). This specificity suggests the participation of a carboxylic acid side chain in the binding site. Studies have shown that the binding of various competitive inhibitors is dependent upon a group with a pK of 4.6–4.7 in agreement with this idea (D'Albis and Bechet, 1967; East and Trowbridge, 1968). Specific chemical modification offers the possibility of identifying this charged group and confirming its functional role. Thus, removal of the charge should be reflected in altered enzymic properties.

In the hope of converting the carboxyl group (or groups) of trypsin responsible for specificity into an amide, the use of an isoxazolium salt for activation followed by treatment with amines was undertaken. In the preceding paper (Bodlaender *et al.*, 1969) the essential chemical properties governing the behavior of this class of reagents with proteins in aqueous solution were defined. Trypsin was used as a model protein in that study, without regard to functional change, to establish conditions of selective modification of carboxylic acid side chains. It was shown that in the pH range of carboxyl ionization (pH 3–5), side reactions involving other amino acid residues could be avoided. In a limited modification under these conditions the chemistry of the reaction of isoxazolium salts with trypsin could readily be interpreted on the basis of the formation of a reactive enol ester which could be subsequently derivatized with nucleophiles as originally outlined in the use of these reagents in peptide synthesis (Woodward *et al.*, 1961).

Results are now described of attempts to modify carboxyl group(s) in trypsin essential for its specificity. Carboxyl groups may also be of critical importance to this enzyme for other reasons (Scrimger and Hofmann, 1967) as discussed later. In the primary sequence studies on trypsin, it has been reported that the enzyme has seven (Mikes *et al.*, 1966) or possibly eleven (Walsh and Neurath, 1964) carboxylic acid side chains. Because the isoxazolium salts are positively charged, the possibility was considered that they might be active site directed and therefore preferentially labeled the specificity site. In addition, since the bulky enol ester could be displaced by several nucleophiles, the effect of geometry and charge of these substituents could ultimately be tested.

* From the Biology Department, Brookhaven National Laboratory, Upton, New York 11973. *Received May 7, 1969.* Send reprint requests to this address. This research was performed at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

† Present address: Department of Biochemistry, Tel-Aviv University, Tel-Aviv, Israel.

TABLE I: Reactions of ^{14}C-Labeled Nucleophiles with EPI-β-trypsin.[a]

Starting EPI-β-trypsin			Resulting β-Trypsin Derivative		
Ethylamine[b] (moles/mole of trypsin)	Act.[c] (%)	Nucleophile (2 M)	Ethylamine[b] (moles/mole of trypsin)	Act.[c] (%)	^{14}C Incorp[d] (moles/mole of trypsin)
2.24	20	$^{14}CH_3NHOH$[e]	Trace	81	0.39
2.19	17	$^{14}CH_3ONH_2$[f]	0.39	20	1.80
2.99	8	$^{14}CH_3ONH_2$[g]	0.31	9	2.90
1.76	22	$^{14}CH_3ONH_2$[g]	0.23	30	1.67
1.97	22	$^{14}CH_2(NH_2)CO_2CH_2CH_3$[h]	0.36	31	1.51

[a] EPI-activated β-trypsin (15 mg/ml) was incubated in 2.0 M nucleophile as described in Methods. Conditions for individual nucleophiles as cited in the table are given below. [b] Determined on the amino acid analyzer. [c] Determined by NPGB burst. [d] Radioactivity covalently bound to protein (determined by scintillation counting); trypsin molarity by amino acid analysis. [e] Incubated 6 hr at pH 5.5. Control with nonactivated β-trypsin lost less than 10% activity and incorporated 0.04 equiv of nucleophile. [f] Incubated 5 hr at pH 5.7. [g] Incubated 5 hr at pH 6.5. Control with nonactivated β-trypsin lost less than 10% activity and incorporated 0.35 equiv of nucleophile. [h] Incubated 3 hr at pH 7.0. Control with nonactivated β-trypsin lost less than 10% activity and incorporated 0.10 equiv of nucleophile.

Materials[1]

[^{14}C]DFP and [^{14}C]*O*-methylhydroxylamine were purchased from New England Nuclear and diluted with unlabeled reagents obtained from Aldrich and City Chemical Co., respectively. The hydroxylamine derivative was then triturated vigorously several times with methylene chloride and crystallized twice from anhydrous ethanol and ether before use. Diaminobutyric and diaminopropionic acids (Cyclo, Grade I) were used as standards for amino acid analysis. Benzamidine-HCl (Aldrich) and 1-ethyl-3-(dimethylaminopropyl)carbodiimide (Ott Chemical Co.) were used as supplied. [^{14}C]TLCK[2] was synthesized by the published procedure (Shaw *et al.*, 1965) using uniformly labeled L-lysine (New England Nuclear).

Methods[1]

Activation of Carboxylic Acid Side Chains of β-Trypsin with EPI. The procedure which resulted in greatest specificity is described. Solid EPI, gently ground just before use to break up the larger crystals, was added (20 mg/ml) to a solution of β-trypsin (5 mg/ml) in 0.1 M calcium chloride at pH 3.8 and 21–23°. The pH was maintained by automatic titration with 0.20 M sodium hydroxide. Rapid sodium hydroxide uptake began 1–3 min after addition of the reagent. Reaction times of 15–20 min (measured from the time of addition of EPI) usually resulted in a protein containing on the average three modified carboxyl groups per mole of protein. Reactions were terminated by adjusting the pH to 2.5 with formic acid and, following centrifugation, were gel filtered through Bio-Gel P-2 columns with 10^{-3} M HCl. If subsequent reaction with a nucleophile was desired, then centrifugation and gel filtration were carried out at 5° to minimize possible rearrangement of the enol ester to the imide form. Analytical data were obtained from the gel-filtered solution which was subsequently lyophilized. These samples were then stored at −15° and were used within 24 hr if reaction with nucleophiles was carried out.

Reaction of Activated Carboxyl Groups of β-Trypsin with Nucleophiles. EPI-β-trypsin (15 mg/ml) was added to a 2.0 M solution of nucleophile at the desired pH (see Table I for details). The solution was also 0.02 M each in calcium chloride and in benzamidine to retard autolysis, and reactions were carried out at 21–23°. At the indicated times (Table I) solution was clarified by centrifugation and then gel filtered through Bio-Gel P-2 columns with 10^{-3} M HCl at room temperature. The protein-containing fractions were pooled and dialyzed for 24 hr at 5° against 10^{-3} M HCl. Analytical data were obtained from these dialyzed samples.

Amino Acid Analyses.[3] Diaminobutyric acid was determined under conditions identical with those described for the determination of methyl- and ethylamine in protein hydrolysates, and was eluted at 57 min with a ninhydrin constant of 91.7. Protein hydrolysates containing diaminopropionic acid were analyzed by using a 17-cm column of PA-35 Beckman spherical resin with 0.038 N sodium citrate buffer at pH 3.8 and 30° and a buffer flow rate of 40 ml/hr. Under these conditions diaminopropionic acid was eluted at 168 min with a ninhydrin constant of 16.1. A 3–5-mV range card was used in the recorder.

Reaction of [^{14}C]DFP with β-Trypsinhydroxamic Acid and

[1] Methods and materials *not* described here appear in the accompanying paper (Bodlaender *et al.*, 1969).

[2] Abbreviations used are: MPI, *N*-methyl-5-phenylisoxazolium fluoroborate; EPI, *N*-ethyl-5-phenylisoxazolium fluoroborate; K, *N*-ethyl-5-phenylisoxazolium-3′-sulfonate; MBI, *N*-methylbenzisoxazolium fluoroborate; DL-BAPA, benzoyl-DL-arginine *p*-nitroanilide; NPGB, *p*-nitrophenyl *p*′-guanidinobenzoate-HCl; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone; EPI-β-trypsin, refers to β-trypsin derivatized with EPI; NH_2OH-EPI-β-trypsin refers to EPI-β-trypsin which has been subsequently treated with hydroxylamine; EDC, 1-ethyl-3-dimethylaminopropylcarbodiimide; DABA, diaminobutyric acid; DAPA, diaminopropionic acid.

[3] We are grateful to Dr. Standish Hartman for helpful suggestions with regard to the analysis of diaminopropionic and diaminobutyric acids.

O-Methylhydroxamic Acid Derivatives. The protein sample (5 mg) was dissolved in 1 ml of 0.25 M Tris-maleate buffer (pH 7.0), 0.02 M in calcium chloride, and treated with 0.10 ml of a 0.10 M solution of [^{14}C]DFP in isopropyl alcohol. The reaction was allowed to proceed for 2 hr at 21–23° following which the pH was dropped to 2.75 with a 1.25 M formate buffer of that pH, centrifuged, and dialyzed against 10^{-3} M HCl. Analytical data were obtained from the dialyzed samples. Control reactions with β-trypsin resulted in stoichiometric incorporation of radioactivity. Increased reaction times did not significantly affect the results.

Reaction of [^{14}C]TLCK with β-Trypsinhydroxamic Acid and O-Methylhydroxamic Acid Derivatives. The proteins (12 mg) were dissolved in 2.0 ml of 0.25 M Tris-maleate buffer (pH 7.0) which was also 0.02 M in calcium chloride; 3.0 mg of [^{14}C]TLCK was then added and the reaction was allowed to proceed for 2 hr. The pH was dropped to about 4 by the addition of 1.25 M formate buffer. Solutions were centrifuged if any cloudiness developed, and then dialyzed for 48 hr. Analytical data were obtained from the dialyzed solution. Control reactions with β-trypsin resulted in stoichiometric incorporation of [^{14}C]TLCK. Increased reaction times did not significantly affect the results.

Lossen Rearrangement of Trypsinhydroxamic Acid Derivatives. Method a: the procedure was essentially that described by Gallop *et al.* (1960). Method b: the β-trypsinhydroxamic acid (5 mg/ml) was added to a 0.1 M solution of EDC which was maintained at pH 4.75 by the pH-Stat addition of 0.5 N HCl for 3 hr; the pH was then adjusted to 2.5 with formic acid and the reaction mixture was filtered through a Bio-Gel P-2 column with 10^{-3} M HCl. The eluted protein was hydrolyzed for 24 hr in 6 N HCl at 110°, and the hydrolysate was analyzed for DABA and DAPA.

Determination of Trypsin Activity. The active-site titrant, NPGB, was used as previously described (Chase and Shaw, 1967). In the case of modified proteins which tended to exhibit relatively high postburst turnover rates, extrapolations back to zero time were made to obtain corrected burst heights.

Results

The approach was aimed at finding conditions for the reaction of isoxazolium salts, *i.e.*, MPI, EPI, K, and MBI, with β-trypsin which would cause maximum inactivation with minimal modification. Reactions were followed by analyzing aliquots from the reaction mixture, at various time intervals, for extent of modification and for enzymatic activity. The degree of modification was revealed by the moles of ethyl- or methylamine per mole of trypsin found in protein hydrolysate (Bodlaender *et al.*, 1969). Initially, enzymatic assays were performed with both DL-BAPA, a rate assay (Erlanger *et al.*, 1961), and with *p*-NPGB, an all-or-none active-site titrant (Chase and Shaw, 1967). Since both methods gave similar results, subsequent assays were performed only with the active-site titrant. As a test for specificity, the effect of benzamidinium ion, a highly effective trypsin inhibitor (Mares-Guia and Shaw, 1965), was studied.

Plots of residual activity *vs.* degree of modification, obtained with reagents EPI and K for reactions carried out at pH 4.75 at room temperature, were linear and extrapolated to zero activity corresponding to the modification of six to seven carboxyl groups. In addition, the reaction was relatively insensitive to the presence of benzamidine (Figure 1). Similar results were obtained for the much more reactive reagent MBI, even when the reaction was carried out at pH 4.20 at 2°. However, decreasing the pH to 4.20 for reactions with EPI and MPI caused activity to drop faster with extent of modification, and benzamidine was now found to have a clearly protective effect (Figure 2). The greatest degree of specificity was obtained by carrying out the reaction at pH 3.8. Under these conditions, nearly complete inactivation occurred with the modification of two to three carboxyl groups (Figure 3), and in the presence of benzamidine the reaction proceeded with relatively little loss in activity (Figure 4). The results indicated that the degree of specificity obtained was in the descending order: MPI > EPI > K. Decreasing the pH further, that is to 2.95, did not increase the specificity of the reaction (Figure 4). It was of interest that α-trypsin which has one more carboxyl group than β-trypsin due to a

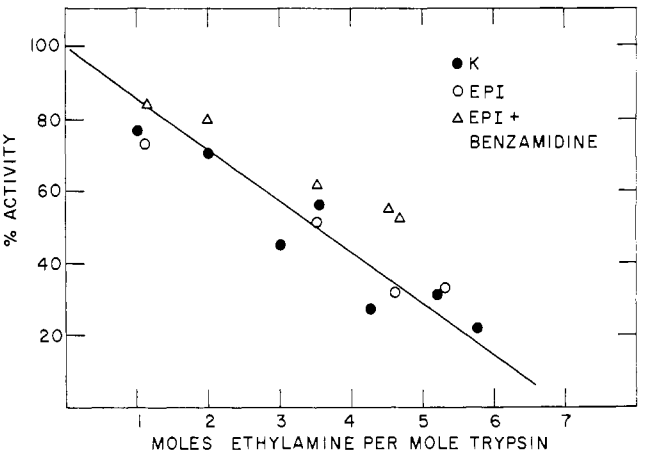

FIGURE 1: Modification of β-trypsin with EPI and K at pH 4.75 and 21–23°. The initial reagent and protein concentrations were 10 and 5 mg per ml, respectively, for the reaction with EPI; 5 and 1.5 mg per ml, respectively, for the reaction with K. The effect of benzamidine hydrochloride (14 mg/ml) was tested on reactions with EPI.

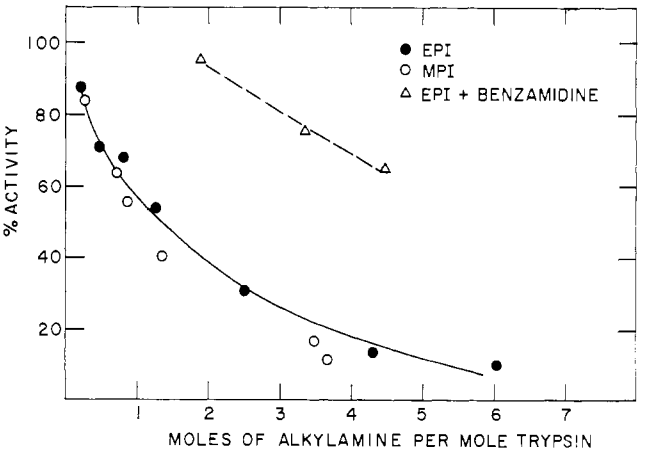

FIGURE 2: Modification of β-trypsin (5 mg/ml) with EPI (10 mg/ml) and with MPI (20 mg/ml) at pH 4.2 and 21–23°. The effect of benzamidine (14 mg/ml) is shown for the reaction with EPI.

TABLE II: Reactions of 2.0 M Hydroxylamine with EPI-β-trypsin at pH 6.0.[a]

Starting EPI-β-trypsin			Resulting β-Trypsin Derivative		
Ethylamine[b] (moles/mole of trypsin)	Act.[c] (%)	Reaction Time (hr)	Ethylamine (moles/mole of trypsin)	Act.[c] (%)	Δ Act.
2.80	17	4	Trace	27	10
2.95	10	5	0.0	23	13
2.50	12	5	0.0	28	15
1.76	22	5	0.0	47	25
3.60	8	2	Trace	40	32
3.23	7	2	Trace	50	43
2.50	12	2	0.0	38	27
2.26	19	2	Trace	51	32
1.76	22	2	0.0	55	33

[a] EPI-activated β-trypsin (15 mg/ml) was incubated in 2.0 M hydroxylamine, 10^{-2} M in benzamidine, at room temperature. [b] Determined on the amino acid analyzer. [c] Determined by NPGB burst.

split at Lys-131 (Schroeder and Shaw, 1968) gave results indistinguishable from β-trypsin in reactions with EPI.

Evidence was previously reported indicating that these derivatized carboxylic acid side chains are largely in the form of reactive enol esters. In order to examine the effect on enzymic activity, it was desirable to replace this relatively bulky enol derivative by smaller nucleophiles. EPI-activated β-trypsin was used exclusively in these studies since previous work had shown that its displacement proceeds with higher efficiency, since rearrangement to the relatively inert imide form is less rapid than for enol esters obtained with MPI (Bodlaender *et al.*, 1969). The extent of reaction was followed by using nucleophiles with carbon-14 labels (Table I). Reactions with *N*-[^{14}C]methylhydroxylamine resulted in nearly quantitative loss of ethylamine content of the protein, indicating nearly complete displacement of enol. However, the nucleophile was only incorporated in very low yields and resulted in a nearly complete restoration of tryptic activity. Reactions with [^{14}C]glycine ethyl ester and *O*-[^{14}C]methylhydroxylamine, on the other hand, proceeded with incorporation of nucleophile in high yield, and with only slight changes in the degree of inactivation. Reactions with hydroxylamine caused nearly quantitative displacement of enol along with a considerable increase in activity (Table II).

The reaction was more pronounced for reactions carried out for 2 hr than for 5 hr. However, this difference in degree of reactivation was diminished when the starting protein contained less than two modified carboxyl groups. Such reactions of EPI-activated β-trypsin should result in the conversion of the enol esters into hydroxamic acids. These new trypsin derivatives were therefore subsequently subjected to the conditions of the Lossen rearrangement. This is a reaction which converts hydroxamic acids into amines with loss of carbon dioxide. In proteins, the products from hydroxamic acid side chains of aspartic and glutamic acid residues can be quantitated in protein hydrolysates as DAPA and DABA, respectively, on the amino acid analyzer. In this work, two different procedures were followed to initiate the Lossen rearrangement. One method involved initial O

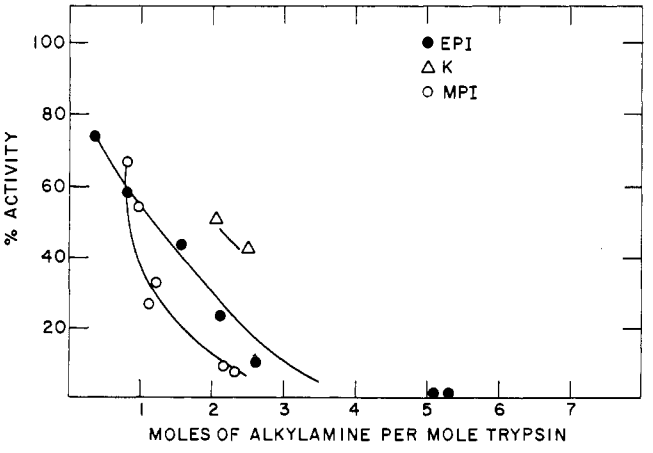

FIGURE 3: Modification of β-trypsin (5 mg/ml) with EPI (20 mg/ml), MPI (10 mg/ml), and K (20 mg/ml) at pH 3.8 and 21–23°.

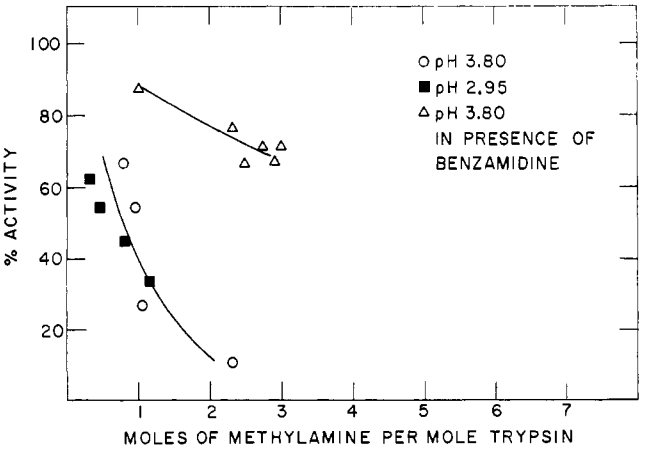

FIGURE 4: Modification of β-trypsin (5 mg/ml) with MPI (10 mg/ml) carried out at pH 3.80 and 2.95, at 21–23°. The effect of benzamidine (14 mg/ml) was tested on the reaction at pH 3.80.

arylation of the hydroxamic acid with fluorodinitrobenzene, and the rearrangement was then induced under basic conditions (Gallop *et al.*, 1960).

Although this method has been used successfully with other enzymes (Broomfield *et al.*, 1965; Gross and Morell, 1966), in the present work, combined yields of diaminobutyric and diaminopropionic acids usually present in equal amounts, were only 18–25% of the activated carboxyl groups per mole of enzyme in the starting protein.

An alternate method based on the observation that the product of the reaction between a hydroxamic acid and carbodiimide (water soluble) spontaneously undergoes the Lossen rearrangement (Hoare *et al.*, 1968) seemed more promising. An EPI-activated sample of β-trypsin which contained 2.95 activated carboxyl groups/mole of protein was subjected to the sequential treatment with hydroxylamine and a water-soluble carbodiimide (Hoare *et al.*, 1968). This resulted in the formation of 1.74 moles of diaminobutyric and 0.95 mole of diaminopropionic acid/mole of protein. However, a control reaction in which β-trypsin was subjected to the same treatment resulted in the formation of 0.61 mole of diaminobutyric and 0.13 mole of diaminopropionic acid. Nevertheless, after correction for these "blank" values, there remained a combined yield of 68%. Although the possibility has not yet been investigated, performing the reaction in 8.0 M urea may further improve the yields. The differences in yields obtained with these two methods may be a reflection of the ability of the reagents involved to penetrate into the region of the hydroxamic acid residues.

A question of considerable interest was whether the loss of enzymic activity caused by the modification of carboxylic acid side chains was limited to characteristic trypsin substrates. That is, had chemical modification resulted in a modified trypsin which was totally inactive as measured by both substrate-like reagents which are specific for trypsin, *i.e.*, NPGB (Chase and Shaw, 1967) and TLCK (Shaw *et al.*, 1965), as well as by active-site reagents which are general for serine proteases and esterases, *i.e.*, DFP (Hartley, 1960)? A difference in the extent to which these reagents react with modified trypsin may be indicative of a modification which has produced an active protein with altered specificity. Several EPI derivatives of β-trypsin which were further reacted with either hydroxylamine or *O*-methylhydroxylamine were subsequently treated with [^{14}C]TLCK or [^{14}C]DFP. The results (Table III) indicate a considerably greater extent of reaction with DFP than with TLCK and NPGB. This behavior was more pronounced for the β-trypsin-*O*-methylhydroxamic acid derivative than for β-trypsinhydroxamic acid. In addition, β-trypsin-*O*-methylhydroxamic acid samples, which had been treated with TLCK to destroy residual tryptic activity, still catalyzed the hydrolysis of *p*-nitrophenyl esters at a rate about ten times faster than the nonspecific catalysis observed for TLCK-inactivated β-trypsin. These differences in rates could be eliminated by a subsequent treatment of the modified trypsin with DFP.

TABLE III: Reactions of Modified β-Trypsin with Active-Site-Directed Reagents.[a]

Modified β-Trypsin[b]	NPGB (% Act)	[^{14}C]-TLCK[c]	[^{14}C]-DFP[c]
	14[d]	0.14	0.34
NH_2OCH_3-EPI-β-trypsin	28[e]		0.63
	44[f]	0.53	0.92
	40[d]	0.41	0.58
NH_2OH-EPI-β-trypsin	53[g]	0.47	0.61
	51[h]		0.66

[a] Use of the reagents is described under Methods. [b] The samples were prepared as described in Table I. [c] Moles bound per mole of trypsin. [d–g] Prior to nucleophile treatment, the initial degree of activation by EPI (as determined by molar equivalents of ethylamine per mole of trypsin) had been: [d] 3.6, [e] 1.5, [f] 3.2, [g] 2.3, and [h] 2.5.

Discussion

The modification of β-trypsin proceeded with greatest degree of specificity when reactions were carried out with 5-phenylisoxazolium salts, *i.e.*, MPI, EPI, and K at pH 3.8 (Figures 3). Results indicated that there may be, on the average, two to three carboxyl groups of similar reactivity under these conditions, and that one to two of these are important for tryptic activity. Since, in the presence of benzamidine, an efficient competitive inhibitor of trypsin (Mares-Guia and Shaw, 1965) modification proceeded with relatively little loss in activity (Figures 2 and 4), it appears that the protected carboxyl groups are in the active-site region.

Although reactions with MPI were more specific, that is, produced a greater inhibitory effect, differences among these three reagents were not very great. In fact, EPI was judged the most useful, primarily because the resulting activated carboxyl groups are more readily modified by successive reaction with nucleophiles than are the activated carboxyls derived from MPI as previously described (Bodlaender *et al.*, 1969). Although reagent K was not studied as intensively, the limited results showed it to be somewhat less specific than EPI, presumably due to the presence of the sulfonate group. In preliminary studies MBI had been found to be least useful due to its diminished selectivity for carboxyl groups, and was also found to be less specific in the present study, probably a result of its extreme reactivity.

A rationale for the specificity observed with MPI and EPI may be twofold. Either the positively charged reagents are active site directed and reaction with a carboxylate group in this region of the enzyme is therefore favored, or the pK_a of the carboxyl group(s) important for enzymatic activity is unusually low and reaction with isoxazolium salts is more rapid as a consequence. These possibilities need not be mutually exclusive. The instability of isoxazolium salts preclude kinetic measurements of the usual kind that might detect selective reversible binding prior to modification. The existence of abnormally acidic carboxylic acid side chains in trypsin is indicated by physical studies (Lazdunski, 1964).

In interpreting the pH dependence of the reaction of isoxazolium salts with proteins it is important to keep in mind that the initial step may be reaction of a carboxylate group with the isoxazolium cation to remove a proton from the 3 position of the latter generating the highly reactive keto-

ketenamine (Woodward and Olofson, 1966) which then may combine with a carboxylic acid (protonated) to form the enol ester. Conceivably, these carboxyl groups need not be the same. Furthermore, ketoketenamines are also formed during the hydrolysis of isoxazolium salts which may then react with any carboxylic acid group. This rate of hydrolysis increases with pH and may account in part for the more rapid modification of protein as the pH of reaction increases (Bodlaender *et al.*, 1969). Such conditions may therefore be conducive to nonspecific modification of carboxylic acid side chains, as was encountered for reactions carried out at pH 4.75 with EPI and K (or at pH 4.2 with the more reactive reagent, MBI). If it is assumed that modification of β-trypsin under these conditions is limited exclusively to the seven carboxylic acid side chains, then the results of Figure 1 suggest that random modification has taken place. The specificity obtained at lower pH values (Figures 2 and 3) may be due to diminished reagent hydrolysis which then permits kinetic control by the more selective processes, such as prior binding or abnormally low carboxyl group ionization, to become important.

Samples of β-trypsin having carboxyl groups activated by EPI at pH 3.8 were treated with various nucleophiles to determine what effect such displacements would have on enzymatic activity (Table I). Treatment with *O*-methylhydroxylamine or with glycine ethyl ester resulted in new trypsin derivatives and very little change in residual, low tryptic activity. The failure of substitution of the rather bulky enol portion of the esters by smaller molecules to result in an increase in enzymic activity suggests that loss of an ionic charge rather than a steric effect was the primary cause for loss of activity. Reactions with hydroxylamine, however, consistently caused partial reactivation of enzymic activity. The extent of reactivation depended both upon reaction time with hydroxylamine and upon the degree of initial modification by EPI (Table II). The initial reaction of hydroxylamine with reactive esters is known to result in the rapid formation of an unstable *O*-acylhydroxylamine, which then may react again with another molecule of hydroxylamine to yield the stable hydroxamic acids (Jencks, 1958). Although the displacement of enol ester side chains was complete within 2 hr, the subsequent reaction resulting in the formation of hydroxamic acid was probably not. Hydrolysis of that portion of product existing as *O*-acylhydroxylamine, leading to regeneration of the carboxyl groups, could therefore be responsible for the observed dependency of reactivation upon shorter reaction time with hydroxylamine. However, the dependency of extent of reactivation upon the degree of modification of starting EPI-β-trypsin raises the possibility that the effectiveness of at least one carboxyl group important to trypsin activity is not noticeably impaired when it is converted into a hydroxamic acid. That is, there may be two "important" carboxyl groups, and conversion of either one into the enol ester results in loss of activity. However, if only one of these is detrimental to enzymatic activity in the form of a hydroxamic acid, then reactivation is understandable, and is expected to be a function of the extent of initial modification with EPI.

Although attempts to quantitate hydroxamic acid side chains by techniques involving the Lossen rearrangement were only partly successful, results did suggest that both glutamic acid and aspartic acid residues had been modified, a result of interest since trypsin is said to contain five aspartic acid side chains but only two of glutamic acid (Mikes *et al.*, 1966).

In contrast to other nucleophiles, reactions of EPI-activated β-trypsin with *N*-[^{14}C]methylhydroxylamine proceeded with very little incorporation of nucleophile and nearly full restoration of enzymatic activity. Apparently *N*-methylhydroxylamine catalyzes the hydrolysis of enol esters, regenerating the carboxylic acid side chains. It had been postulated that nucleophile displacement resulting in *N*-methylhydroxamic acid formation was effectively retarded by steric interference of the *N*-methyl group (Bodlaender *et al.*, 1969). These results were found to be quite reassuring as a demonstration that trypsin is not irreversibly denatured during these successive modification steps and support the conclusion that losses in activity with other nucleophiles are a direct result of the chemical modification of important carboxylic acid side chains.

Early in this work the decision was made to avoid rate assays as a guide to the modification studies. It was realized that ultimately a change in specificity of trypsin might be achieved by modification that would lead to a loss of esterase activity toward arginine or lysine esters without a comparable loss toward a neutral or chymotrypsin-specific substrate. However, initially it was essential to establish conditions for achieving selective modification with the highly reactive class of reagents under study, and since the products were clearly mixtures, it did not appear that rate assays would be the best guide. Enzyme titrants avoid the difficulties inherent in the interpretation of rate assays accompanying chemical modification (Ray and Koshland, 1963) and consequently the use of NPGB for measurement of trypsin active centers (Chase and Shaw, 1967) was routinely used. Guided by this reagent, modified trypsins were prepared which still retained some tryptic activity but a considerably greater proportion of intact hydrolytic centers as measured by DFP incorporation (Table III). The differential use of these titrants permits one to establish the extent to which a form of trypsin with modified specificity has been produced. In this work the yield of such a form has ranged from 20 to 50%.

The fractionation of this mixture is now in progress to characterize the components and to determine the sites of modification and their effect on the structure and function of trypsin. It is evident that there are at least two stages of effect in the limited modification obtainable at pH 3.8. Trypsin-like character, as measured by susceptibility to NPGB and TLCK, disappears more rapidly than the reactivity of the active-center serine. However, eventually this property is also lost on carboxyl modification.

Crystallographic studies on chymotrypsin have shown the close proximity of two buried carboxyl groups to the active center region. The function of one of these, Asp-194, is said to be invoked on activation for the maintenance of a functional conformation by ion pairing with the free amino group of Ile-16 (Matthews *et al.*, 1967; Sigler *et al.*, 1968). A second, Asp-102, is surmised to play an indirect role heightening the reactivity of the functional serine residue, Ser-195 (Blow *et al.*, 1969). It is not known whether similar arrangements exist in the structure of trypsin. The homology of these enzymes leads one to expect so (Walsh and Neurath, 1964; Sigler *et al.*, 1968). A specificity-site carboxyl group in trypsin would then make a total of three in this region. The

disruption of the ion pair in trypsin analogous to that involving Asp-194 in chymotrypsin has presumably been achieved by nitrous acid treatment and was said to result in a simultaneous loss of ability to incorporate DFP (Scrimger and Hoffmann, 1967). In the present work, effects on specificity were seen with carboxyl modification of trypsin without loss of ability to incorporate DFP and presumably the carboxyl groups modified in such products do not include the one participating in zymogen activation. It has been reported possible to modulate the specificity of trypsin in the sense of inhibiting proteolytic activity relative to esterase activity by a treatment presumably modifying only tryptophan residues (Coletti-Previero, *et al.*, 1969) and resulting in a conformational change. Such a treatment may have indirectly influenced the effectiveness of a carboxyl group whose function is to increase the nucleophilicity of the active-center serine as proposed for chymotrypsin (Blow *et al.*, 1969). In any case, the probable roles of carboxyl participation in the proteolytic action of trypsin have increased in variety and number. It appears that the methods we have described are relatively gentle and lead to limited modifications of trypsin which may involve more than one of these important carboxyl groups, particularly since two levels of effect were seen, namely loss of trypsin-like specificity followed by loss of serine reactivity. The present approach offers great promise for the identification of the responsible carboxyl groups and of more extensive study of their individual roles.

Acknowledgment

We are indebted to Mr. G. Latham for his assistance in the analytical procedures.

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