The Use of Isoxazolium Salts for Carboxyl Group Modification in Proteins. Trypsin*

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ABSTRACT: The limitations and uses of isoxazolium salts for the modification of carboxylic acid side chains of proteins have been investigated. *N*-Alkyl-5-phenylisoxazolium salts have been shown to be suitable reagents for the activation of carboxylic acid side chains of trypsin, in reactions which take place rapidly under mild conditions, and are specific to carboxyl groups when carried out below pH 4.75. The activated carboxylic acid side chains, in the form of enol esters, are sufficiently stable to allow isolation of the protein, and a subsequent reaction with nucleophiles is then possible.

Such displacement reactions with [^{14}C]nucleophiles resulted in the formation of new trypsin derivatives in high yield. Although studies were limited to the carboxyl groups of trypsin, presumably the procedures described are generally applicable.

A number of reagents have previously been introduced for the modification of carboxylic acid side chains of proteins (Hoare and Koshland, 1967, and references therein). Emphasis has often been placed on achieving an extensive reaction involving either all the carboxyl groups of a protein or all of the exposed ones. In a few instances a single critical carboxyl group has been labeled in an enzyme by virtue of either unique reactivity toward a reagent not generally very specific for carboxyl groups, as in the action of iodoacetate on ribonuclease T_1 (Takahashi *et al.*, 1967) or phenacyl halides on pepsin (Erlanger *et al.*, 1966; Gross and Morell, 1966), or by virtue of being substrate like, as in the labeling of pepsin by diazo derivatives (among others, *cf.* Rajagopalan *et al.*, 1966; Ong and Perlmann, 1967).

In an attempt to locate the carboxyl group in the active center of trypsin which is probably responsible for its preference for positively charged substrates (Neurath and Schwert, 1950), reagents capable of achieving a specific modification by active-site direction were sought. *N*-Alkyl-5-phenylisoxazolium salts (I) appeared promising for this purpose since, not only have they been demonstrated to be very reactive toward carboxylic acids, activating them for peptide synthesis (Woodward *et al.*, 1961, 1966), but also they are positively charged and an initial complex at the specificity site of trypsin could be reasonably hoped for in view of the known affinity of trypsin for geometrically similar molecules such as the benzamidine cation (Mares-Guia and Shaw, 1965).

A limited number of observations of the action of isoxazolium salts on proteins and polypeptides have been made (Marfey *et al.*, 1965; Haines and Zamecnik, 1967; Bláha and Rudinger, 1965); however, these have not defined the chemistry of the interaction nor the conditions suitable for a specific reaction at carboxyl groups. In the present paper a systematic study is described which provides conditions for the use of isoxazolium salts as a group-specific reagent for carboxyl modification of proteins in aqueous solution. The observations are limited to trypsin, but it can be expected that the findings will be generally applicable.

While this work has been in progress, carboxyl groups have been invoked for additional, important roles in chymotrypsin which, by analogy, must be taken into account for trypsin, namely, the maintenance of an active conformation (Matthews *et al.*, 1967) and the heightening of reactivity in the histidine–serine catalytic mechanism (Blow *et al.*, 1969). Because of these and recent postulated roles in other hydrolytic enzymes (Blake *et al.*, 1967; Reeke *et al.*, 1967), methods for the selective, gentle modification of carboxyl groups in proteins should be of considerable use in evaluating these numerous, diverse possible functions.

The synthesis, chemical properties, and mechanisms of action of various isoxazolium salts have been intensively studied (Woodward and Olofson, 1961, 1966; Kemp and Woodward, 1965). Reactions of these reagents with carboxylates proceed rapidly at room temperature to form an enol ester in high yield. The reaction of an *N*-alkyl-5-phenylisoxazolium ion (I) with a carboxylate is outlined in Scheme I. This reaction is initiated by proton abstraction from the 3 position of the isoxazole by water or carboxylate, causing an immediate rearrangement to the highly reactive ketoketenamine intermediate (II) which then rapidly reacts with one molecule of carboxylic acid to form a β-acyloxy-*N*-alkylcinnamamide (III), an enol ester. These enol esters were shown to be efficient acylating agents and could therefore be further reacted with amines resulting in the formation of an amide (V) and *N*-alkylbenzoylacetamide (VI) as the side product. A competing reaction was reported to be a base-catalyzed rearrangement to the relatively inert imide, *N*-acyl-*N*-alkylbenzoylacetamide (IV). This rearrangement could however be retarded by increasing the bulk of the *N*-alkyl substituent. For example, β-acetoxy-*N*-ethylcinnamamide was shown to undergo rearrangement much more slowly then the *N*-methyl analog.

In view of these well-established chemical properties,

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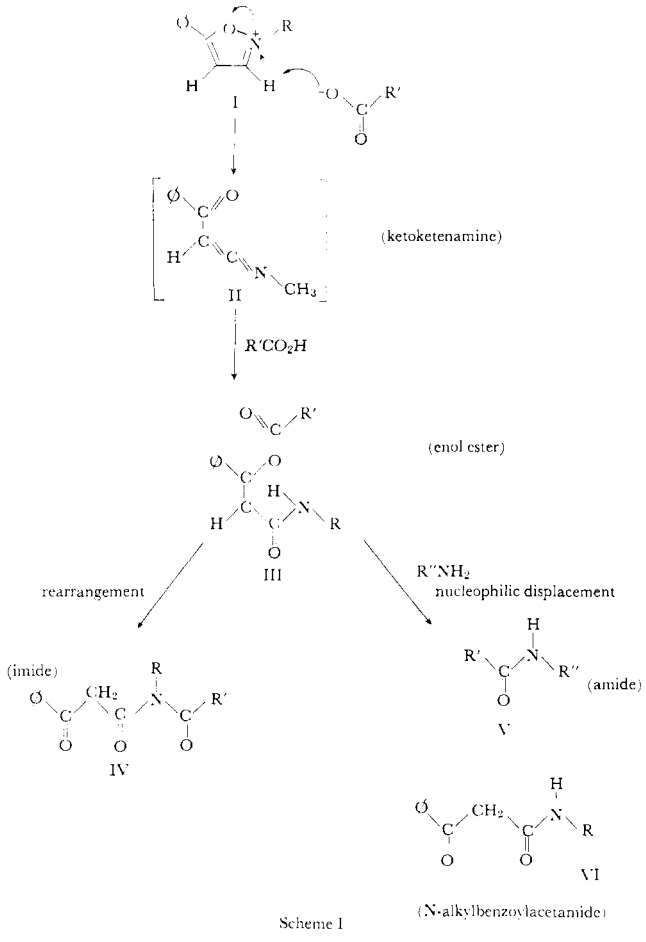
SCHEME I

Reaction of an Isoxazolium Salt
With Sodium Acetate at Room Temperature



Scheme I

several potential applications of isoxazolium salts in the modification of carboxyl group side chains of proteins become evident. (1) Carboxylic acid side chains of proteins could be reacted under mild conditions in aqueous solutions. This would be especially valuable if reactions involving other amino acid residues were negligible. Furthermore, resulting enol esters or imides, if any rearrangement has taken place, should liberate a molecule of alkylamine for each carboxyl group modified during acid hydrolysis of the protein. A preliminary investigation had established that both ethylamine and methylamine can be quantitated on the amino acid analyzer, thus making it possible to determine the extent of protein modification. Alternatively, radioactive labels could be introduced into the reagent. (2) The initially formed enol esters might be further reacted with suitable nucleophiles, providing a method for the incorporation of molecules of various dimensions and chemical properties, and therefore also a route to more stable protein derivatives. (3) By varying the structure of isoxazolium salts and reaction conditions, selective modification of certain carboxylic acid side chains of special interest may be possible. (4) Under suitable conditions, intramolecular cross linking might be initiated, *i.e.*, by the nucleophilic attack on the enol ester by a correctly oriented ϵ-amino group of a lysine residue.

This paper deals with establishing the conditions whereby isoxazolium salts can be used to modify carboxylic acid side chains in proteins, as exemplified by trypsin, leading to their conversion into substituted amides without effect on other amino acid side chains. Results of attempts to modify a carboxyl group essential for the specificity of trypsin form the basis of a subsequent paper.

Materials

β-Trypsin was prepared from twice crystallized, lyophilized, salt-free trypsin (Worthington Biochemical Corp.) according to the procedure of Schroeder and Shaw (1968).

Woodward's reagent K and 5-phenylisoxazole were obtained from Pilot Chemicals Inc., Watertown, Mass. EPI[1] and MPI were synthesized by the procedure of Woodward and Olofson (1966), through alkylation of 5-phenylisoxazole with triethyloxonium fluoroborate and trimethyloxonium fluoroborate, respectively. Oxonium salts were prepared according to the method of Meerwein (1966). MBI was prepared according to a procedure described for the synthesis of the *N*-ethyl analog (Kemp and Woodward, 1965). Thus, benzisoxazole was alkylated with trimethyl rather than triethyloxonium fluoroborate. An alternative synthetic approach is described below.

The following isoxazolium salts were previously unreported: MPI, mp 148–153. *Anal.* Calcd for $C_{10}H_{10}B_1F_4N_1$: C, 48.62; H, 4.08; N, 5.71. Found: C, 48.20; H, 3.93; N, 5.60. MBI, mp 123–129. *Anal.* Calcd for $C_8H_8B_1F_4N_1$: C, 43.48; H, 3.65; N, 6.34. Found: C, 44.09; H, 3.76; N, 6.24.

Miscellaneous reagents and their sources were as follows: hydroxylamine hydrochloride (Baker); *N*-methylhydroxylamine hydrochloride (Aldrich); *O*-methylhydroxylamine hydrochloride and silver tetrafluoroborate (City Chemical); glycine ethyl ester hydrochloride (Eastman); ethyl bromide (Fisher).

All radioactive reagents were purchased from New England Nuclear.

Methods

N-[^{14}C]*Methylhydroxylamine Hydrochloride.* The precursor, *N*-[^{14}C]methyl-α-phenylnitrone, was prepared according to a procedure of Buehler (1967) by alkylation of *anti*-benzaldoxime with [^{14}C]methyl iodide (1 mCi). The nitrone (5.8 g; 43 mmoles) was hydrolyzed in 80 ml of concentrated HCl at 105° for 12 min (Buehler and Brown, 1967). After cooling to room temperature, the reaction mixture was evaporated to dryness on a rotary evaporator. The residual solid was taken up in absolute ethanol and then precipitated with anhydrous ethyl ether. If the product separated as an oil, crystallization could be induced by cooling to −15° for several hours. The white, crystalline solid was collected by suction filtration and then vigorously triturated with methylene chloride. The product was dissolved in a minimum of absolute

[1] Abbreviations used are: EPI, *N*-ethyl-5-phenylisoxazolium fluoroborate; MPI, *N*-methyl-5-phenylisoxazolium fluoborate; MBI, *N*-methylbenzisoxazolium fluoroborate.

ethanol, five volumes of methylene chloride were added, and then just enough anhydrous ethyl ether to cause a permanent turbidity. Crystallization commenced immediately and the product was isolated by suction filtration. This recrystallization procedure was repeated three more times, yielding 1.6 g (19 mmoles, 44%) of *N*-[^{14}C]methylhydroxylamine hydrochloride (3.89×10^4 dpm/mmole): mp 86–88, lit. (Beilstein; Syst. No. 380) mp 88–89.

The product obtained under the same conditions, in a preliminary investigation of the synthetic steps, using unlabeled reagents, exhibited the same melting point and infrared spectrum as the ^{14}C-labeled product. Strong bands were observed at 3.45, 6.9, 7.25, 9.7, and 11.1 μ. Elemental analysis was obtained for the unlabeled material. *Anal.* Calcd for $C_1H_6Cl_1N_1O_1$: C, 14.38; H, 7.24; N, 16.77. Found: C, 14.83; H, 7.24; N, 16.17.

Infrared spectra were obtained of the Nujol mull on a Perkin-Elmer grating infrared spectrophotometer, Model 137B; melting points were determined on a microscope hot stage.

N-Ethyl-5-phenylisoxazolium Fluoroborate (an Alternative Procedure). With the aid of 25 ml of anhydrous ethyl ether, 9.75 g (50 mmoles) of anhydrous silver tetrafluoroborate was slowly washed into a vigorously stirred solution of 50 ml of methylene chloride containing 7.25 g (50 mmoles) of phenylisoxazole and 108 g (100 mmoles) of ethyl bromide. The reaction mixture was protected from light. After the initial release of heat subsided, the reaction mixture was heated to reflux and vigorously stirred for an additional 16 hr. Completion of reaction was determined by shaking several drops of the solution with 1 ml of water and then testing the aqueous layer for the presence of silver ions with sodium chloride. If the test was positive then an additional 1.08 g of ethyl bromide was added and reflux was continued until a test for silver was negative. The reaction mixture was then cooled to room temperature and the solid residues were removed by suction filtration. The residue was extensively washed with methylene chloride and the washings were combined with initial filtrate. Just enough anhydrous ethyl ether to produce permanent turbidity was added to this clear, light yellowish solution, which was then placed in a freezer for 2 days. A crystalline product was collected and recrystallized twice from methylene chloride–ether and then twice from acetonitrile–ether, yielding 7.5 g (29 mmoles, 52%) of EPI, mp 100–101°, in agreement with the literature (Woodward and Olofson, 1966). In the infrared spectrum, strong bands were found at 3.4, 6.55, 6.8, 7.26, 9.6 (broad), 12.9, and 14.6 μ. Spectral data and melting point exactly matched that of an authentic sample.

Reaction of Isoxazolium Salts with Trypsin. The isoxazolium salt of choice (10–20 mg/ml) was added to 0.02 M calcium chloride containing β-trypsin (5 mg/ml), at the desired pH. The pH was maintained by automatic titration (Radiometer pH-Stat) with 0.10 M sodium hydroxide. Reactions were carried out in a temperature-controlled room at 21–23°. Reaction time depended on the pH of the reaction and extent of protein modification desired (for specific conditions see text). The reaction was terminated by adjusting pH to 2.5 with formic acid, and the sample was then gel filtered through a Bio-Gel P-2 column with 10^{-3} M HCl. Columns made up from 100-ml burets were suitable for complete separation of protein from unreacted and hydrolyzed reagent molecules when the sample size applied to the column was 1–5 ml. Analytical data were obtained from these gel-filtered samples.

When subsequent reaction of the modified protein with nucleophiles was desired, the gel filtration was carried out at 4° to minimize possibilities of rearrangement and hydrolysis, prior to lyophilization. The lyophilized samples were usually used within 24 hr; however, storage in a freezer for 2–3 days did not seem to cause any noticeable changes in the results.

Displacement Reaction of Modified Trypsin with Nucleophiles. β-Trypsin which had been modified with an isoxazolium salt was dissolved (15 mg/ml) in a solution containing 10^{-2} M benzamidine (to retard autolysis; Mares-Guia and Shaw (1965)) plus the nucleophilic reagent (1–2 M) at the desired pH. The reaction mixture was gently stirred at room temperature (23–25°) for 3–6 hr. Specific conditions given in Table V for each nucleophile have produced the best results. The reaction was terminated by adjusting the pH to 2.5 with formic acid and the solution was then dialyzed for 48 hr at 4° against 10^3 M HCl. Analytical data were obtained from these dialyzed samples.

Amino Acid Analysis. Samples were hydrolyzed in 6 N HCl in evacuated tubes at 109° for 24 hr. Analyses of the protein hydrolysates were performed on a Beckman Model 120 amino acid analyzer. If protein hydrolysates were expected to contain methyl- or ethylamine, then a 7.0-cm column of PA-35 Beckman spherical resin was employed, under conditions described for operation of normal short columns (Spackman *et al.*, 1958) with a buffer flow rate of 40 ml/hr. Mean values for ninhydrin constants with standard deviations obtained for 20 determinations and retention times were as follows: lysine: 117 ± 2.1, 53 min; histidine: 105.2 ± 2.5, 65 min; ammonia: 78 min; methylamine: 68.7 ± 3.8, 90 min; ethylamine: 32.6 ± 2.6, 109 min; arginine: 106.6 ± 2.6, 122 min.

Hydrolysis times of modified proteins beyond 24 hr did not significantly change analyses for methyl- or ethylamine.

Scintillation Counting. This was performed on a Nuclear-Chicago Model 6801 scintillation counter. Samples of 0.50 ml were added to 10 ml of scintillation fluid which consisted of 120 g of naphthalene, 7.0 g of 2,5-diphenyloxazole, and 0.5 g of *p*-bis[2-(5-phenyloxazolyl)]benzene dissolved in 1 l. of dioxane which had been passed through an alumina column just before use.

Results

Isoxazolium Salts. In order to retain the unique reactivity of isoxazolium salts, it is necessary to leave the 3 position of the isoxazole ring unsubstituted. This, however, still permits a great deal of flexibility, allowing use of a variety of structurally different reagents since one is free to choose substituents for the 2, 4, and 5 positions. We have not, thus far, taken full advantage of these possibilities and have limited ourselves to a study of the usefulness of several of the more readily available isoxazolium salts (Table I).

Synthetic procedures previously published involved use of trialkyloxonium fluoroborate (Woodward and Olofson, 1966; Kemp and Woodward, 1965) or methyl sulfates (Wilson and Burness, 1966) for alkylation of the isoxazole nitrogen. In addition, we describe a new method which provides fairly high yields of *N*-ethyl- and *N*-methylisoxazolium fluoroborates through the reaction of the isoxazole precursor with an alkyl halide in the presence of silver fluoroborate as described in

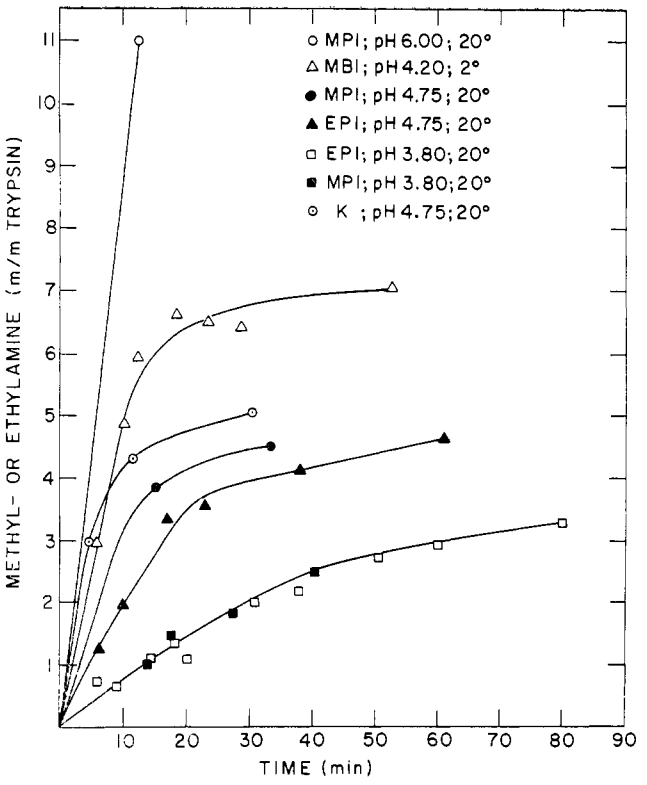
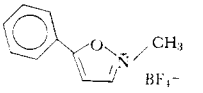
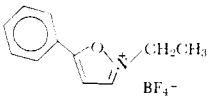
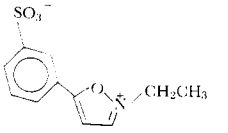
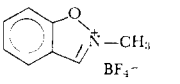


FIGURE 1: Reaction of isoxazolium salts with β-trypsin at various pH values. In all reactions, the initial reagent and protein concentrations were 10 and 5 mg per ml, respectively, except for the reaction with K reagent in which the respective concentrations were 5 and 1.5 mg per ml. Each point was obtained by removing an aliquot from the reaction mixture and adjusting its pH to 2.5 with formic acid at the indicated times. Amino acid analyses were then performed on the gel-filtered samples to determine methyl- or ethylamine content per mole of trypsin.

TABLE I: Isoxazolium Salts

Structure	Nomenclature	Abbreviation
	*N*-Methyl-5-phenylisoxazolium fluoroborate	MPI
	*N*-Ethyl-5-phenylisoxazolium fluoroborate	EPI
	*N*-Ethyl-5-phenylisoxazolium-3′-sulfonate Woodwards K reagent)	K
	*N*-Methylbenzisoxazolium fluoroborate	MBI

the Methods section. This procedure should facilitate the introduction of radioactive alkyl groups.

Reaction with Protein. Results from several exploratory studies of the reaction of isoxazolium salts with β-trypsin at various pH's are summarized in Figure 1 in which incorporation of reagent as indicated by methylamine or ethylamine content is plotted with respect to reaction time. The rate of reagent incorporated into the protein was largely a function of the rate of reagent hydrolysis. The decreasing rate of incorporation with time is, therefore, due to decreasing reagent concentration. 5-Phenylisoxazolium salts hydrolyze extremely rapidly above pH 5 at room temperature and are relatively stable at or below pH 3. The benzisoxazolium salts, however, are much more reactive, hydrolyzing rapidly at pH 4, even at low temperature (2°).

Normal amino acid compositions were obtained for trypsin which had undergone limited modification, *i.e.*, analyzed for less than 5 equiv of alkylamine, in reactions with 5-phenylisoxazolium salts carried out below pH 4.75, However, a significant loss of lysine was observed in proteins analyzing for more than five alkylamines. For example, β-trypsin which had been treated for 72 min with a 250-fold molar excess of MPI at pH 4.75 analyzed for 10.9 equiv of methylamine and only 11.2 residues of lysine. This is 2.8 short of the expected 14 residues of lysine in trypsin. However, it is remarkable that determinations of all other residues were within the average of those obtained for β-trypsin itself. Analytical data summarized in Table II for EPI-β-trypsin are characteristic of all reagents except MBI. Data presented for MBI-β-trypsin reveals larger lysine losses, even under mild reaction conditions, probably reflecting the greater reactivity of the reagent.

During those reactions which yielded highly modified proteins, analyzing for more than 5 equiv of alkylamine, a large percentage of the starting protein, up to 50%, precipitated. This phenomenon was independent of the type of reagent used. The precipitated protein usually analyzed for at least 2–3 equiv of amine more than found for the protein remaining in solution.[2]

Stability of Reagent–Protein Bond in the Carboxyl-Activated Intermediate. Modified protein, irrespective of derivatizing reagent, exhibited very little loss of alkylamine after incubation in 10^{-3} M HCl at room temperature for several hours or at 5° for several days. For example, following dialysis for 36 hr at 5° against 10^{-3} M HCl, samples showed generally no more than 15% loss of alkylamine. Samples which were incubated under the same conditions for 6 days and then gel filtered at room temperature exhibited less than 25% loss of initial alkylamine content. At pH values above 7, however, loss of alkylamine content was quite rapid. For example, one sample, following incubation at pH 8.3 for 1 hr, analyzed for only 50% of the initial alkylamine content. However, incubation for longer periods of time at pH's above 7 did not result in 100% loss of alkylamine.

[2] All data presented in this paper are from the modified protein remaining in solution.

Rearrangement of the Carboxyl-Activated Intermediate. The tendency of enol esters, derived from the reaction of carboxylic acids with isoxazolium salts, to undergo rearrangement to the imide form has already been noted (Scheme I). Since the ultraviolet spectra of these two species are very different (Woodward and Olofson, 1966), such processes, occurring in the modified proteins, should give rise to changes in the ultraviolet spectrum of the protein. Observation of such changes, associated with the rearrangement process, would provide further evidence for protein modification having taken place at the carboxylic acid side chains and also for the presence of the reagent in the various states.

Early in these investigations we noted that the ultraviolet spectrum of modified trypsin was different from that of normal β-trypsin. Thus, the ratio OD_{280}/OD_{250} is usually close to 2.70 for β-trypsin, whereas trypsin which had been modified with isoxazolium reagents tended to exhibit much lower ratios, usually less than 2.00, depending upon the degree of modification.

Woodward and Olofson (1966) reported a maximum at 267 mμ (ε 18,700) in the ultraviolet spectrum of the enol ester, β-acetoxy-*N*-methylcinnamamide, derived from a reaction of MPI and sodium acetate. The corresponding rearranged imide, *N*-acetylmethylbenzoylacetamide, exhibited a maximum at 242 mμ (ε 12,300). Both of these chromophores, if present in trypsin, would be expected to decrease the OD_{280}/OD_{250} ratios. Furthermore, in the spectrum of freshly modified trypsin in which this ratio was initially lower than that of trypsin itself due to contributions from enol ester chromophores, a rearrangement of the latter to the imide form would be expected to result in a further decrease of the initial low ratio due to the lower wavelength maximum of the imide chromophore. On the other hand, loss of bound reagent due to hydrolysis or nucleophilic displacement should result in a restoration of the OD_{280}/OD_{250} ratio toward a value characteristic of trypsin.

These expectations were borne out by observation on the carboxyl-activated intermediate. The OD_{280}/OD_{250} ratio, however, was not useful for the direct quantitation of incorporated isoxazolium residues since, although the ratio was generally lower the greater the protein was modified, the relationship was not simple and not very reproducible. However, the spectral properties were useful in interpreting the behavior of the modified protein. In particular, for eventual replacement of the activating agent by nucleophiles, it was essential to avoid rearrangement to the less reactive imide form. Also, the extent of rearrangement could be gauged as a competing process in the nucleophilic displacement.

The OD_{280}/OD_{250} ratio depended upon the history of the sample, that is, the pH at which the reaction with trypsin was carried out, total length of time in solution, and temperature of solution. We also noted that derivatized trypsin, when dissolved in 10^{-3} M HCl, exhibited decreasing OD_{280}/OD_{250} ratios with time. For example, an MPI-derivatized sample of β-trypsin which analyzed for 2.85 moles of methylamine/mole of trypsin exhibited an initial ratio of 1.54. After dialysis against 10^{-3} M HCl at 5° for 4 days the ratio dropped to 1.24, and after 7 days to 1.21. Results of this type indicated that the rate of change of the OD_{280}/OD_{250} ratio diminished with time, becoming almost negligible after 4–5 days. Further, the rate of change was found to be larger for MPI derivatives than for EPI or K derivatives.

TABLE II: Analytical Data for EPI- and MBI-β-trypsin of Different Degrees of Modification.[a]

Reagent[b]	pH	Moles/Mole of Trp		
		$MeNH_2$ or $EtNH_2$	Lys	Arg
		1.10	13.66	1.85
EPI	4.75	3.50	13.57	1.91
		4.60	13.11	1.79
		5.28	12.96	1.71
		2.44	13.52	1.86
EPI	4.20	4.36	13.73	1.69
		5.98	13.96	1.88
		2.08	13.67	1.87
EPI	3.80	2.62	14.35	1.88
		5.06	13.80	2.01
		5.33	13.76	1.91
		2.53	12.83	1.80
MBI	4.25	4.14	12.63	1.84
		5.19	12.27	1.75
	Control		13.86	1.94
	Theory		14.00	2.00

[a] Analytical values were related to 3.0 moles of histidine. Hydrolysis was for 24 hr. [b] Reactions with EPI were carried out at room temperature at the indicated pH values. Ratios of starting reagent:protein concentrations in mg/ml were 10:5 except for the reaction at pH 3.8 in which case the ratio was 20:5. Reactions with MBI were carried out at 2° with the reagent:protein ratio being 5:10.

The rearrangement of enol ester to imide was reported to be base catalyzed (Woodward and Olofson, 1966). It was therefore not surprising that treatment of modified trypsin with 0.1 M Tris, at pH 7.1, resulted in a very rapid decrease in the OD_{280}/OD_{250} ratio in the ultraviolet spectrum of the protein. A rapid loss of part of the alkylamine content accompanied this spectral change but was not responsible for it, since processes involving only loss of enol esters, such as hydrolysis or nucleophilic displacement, should result in an increase in the OD_{280}/OD_{250} ratio from values near 2.0 or lower, usually observed for modified protein, toward a value near 2.70, characteristic of β-trypsin. These changes, caused by Tris, took place chiefly within the first 5 hr of incubation as shown with EPI-β-trypsin (Table III). Subsequent changes in optical properties or ethylamine content during an additional 19 hr were minimal, presumably reflecting the greater stability of that portion of reagent which had rearranged to imide. Tris may be involved in several competing processes: catalyzing rearrangement, catalyzing hydrolysis, and nucleophilic displacement on the enol ester. Tris has been observed to be an effective reagent for the cleavage of reactive ester linkages (Gregory and Bruice, 1957), a property explaining large initial losses of ethylamine content accompanying rearrangement. However, the possibility of ethylamine loss due to displacement by a suitably oriented, reactive amino acid residue, resulting in cross-linking, cannot be ruled out.

Similar observations were made for MPI-derivatized samples. However, these underwent rearrangement faster

TABLE III: Effect of Incubating EPI-β-trypsin in Tris Buffer, at pH 7.1, on Content of Isoxazolium Reagent and Ultraviolet Spectrum.[a]

Incubn time (hr)	$EtNH_2$ (moles/mole of trypsin) Initial (t = 0 hr)	Final	OD_{280}/OD_{250} Initial (t = 0 hr)	Final
5	5.33	3.89	1.51	1.09
24	5.33	3.55	1.51	0.90
24	3.11	1.88	1.57	1.25
48	3.11	1.97	1.57	1.33

[a] At time zero modified protein (5 mg/ml) was introduced into the buffered solution which was also 10^{-2} M in benzamidine (to retard autolysis). Aliquots were removed from the reaction mixture, and their pH was adjusted to 2.5 at the indicated times. These samples were then gel filtered through Bio-Gel P-2 columns with 10^{-3} M HCl. Analytical data were obtained from the eluted samples.

TABLE IV: Reaction of EPI- and MPI-β-trypsin with Nucleophiles. Comparison of Displaceability of Carboxyl-Activating Reagent.[a]

Nucleophile (1.0 M)	pH	Me- or $EtNH_2$ (moles/mole of trypsin) Initial	Final
EPI-β-trypsin			
NH_2OH[b]	6.3	4.24	Trace[c]
NH_2OH	5.4	1.20	Trace[c]
CH_3NHOH	5.4	1.20	Trace[c]
NH_2OCH_3	5.0	1.20	0.52
$NH_2CH_2CO_2CH_2CH_3$	6.2	1.20	0.65
MPI-β-trypsin			
NH_2OH	5.5	1.92	0.25
CH_3NHOH	5.5	1.92	0.63
NH_2OCH_3	5.2	1.92	0.90
$NH_2CH_2CO_2CH_2CH_3$	6.4	1.92	1.03

[a] Reactions were initiated by introducing the modified protein (15 mg/ml) into a solution, which was 1.0 M in nucleophile and 10^{-2} M in benzamidine (to retard autolysis) at the indicated pH. The reaction was allowed to proceed at room temperature for 6 hr except where indicated. [b] Reaction time was 2.75 hr. [c] This refers to analyses which revealed the presence of methyl- or ethylamine in amounts which were too small to be measured; that is in a range from >0 to <0.1 mole per mole of trypsin.

and the resulting imide seemed to be more susceptible to attack by Tris, as evidenced by a continued drop in methylamine content, even after rearrangement.

These preliminary observations are understandable in terms of the chemical properties expected of enol esters as outlined in Scheme I, with particular emphasis on hydrolysis or nucleophilic displacement as competing reactions with base-catalyzed rearrangement to the relatively inert imide.

Nucleophilic Displacement on the Carboxyl-Activated Intermediate. The most convincing evidence for the formation of enol ester derivatives of trypsin carboxyl groups would be the further reaction of these with suitable nucleophiles to yield new trypsin derivatives. As a preliminary effort, we were interested in determining the susceptibility of the reagent–trypsin bond to the action of several nucleophiles, *i.e.*, hydroxylamine, *N*-methylhydroxylamine, *O*-methylhydroxylamine, and glycine ethyl ester. Modified trypsin, treated with such nucleophiles, should exhibit large, if not quantitative, loss of the initial alkylamine content (Scheme I). Results of such experiments are given in Table IV for EPI- and MPI-derivatized β-trypsin. As had been anticipated, these reactions did cause large losses of alkylamine in the protein.

In order to establish whether these nucleophiles were actually being incorporated into the protein during the displacement reaction, nucleophiles with radioactive labels were used. Results summarized in Table V revealed that all except *N*-methylhydroxylamine were indeed being incorporated in high yield. Reasons for the unique behavior of this nucleophile will be discussed. Conditions under which these reactions were performed represent those which have given us the best results thus far.

Cross-Linking. A question of considerable importance was whether any inter- or intramolecular cross-linking had taken place, either during the initial reaction of trypsin with isoxazolium salts or during the subsequent reaction with nucleophiles. Indeed the use of K reagent for the cross-linking of synthetic polypeptides has previously been reported (Marfey *et al.*, 1965; Bláha *et al.*, 1965). However, the reaction of modified trypsin with nucleophiles, which resulted in high yields of protein-bound nucleophiles suggested that cross-linking, if any, occurred only during the initial reaction with isoxazolium salts.

In order to check for the presence of products arising from intermolecular cross-linking, a modified β-trypsin sample was gel filtered on a calibrated Sephadex G-100 column. This sample was derived from an initial reaction of EPI and β-trypsin at pH 3.8 and a subsequent reaction with 2.0 M glycine ethyl ester at pH 7.5. The initial and final products analyzed for 3.36 and 0.54 moles of ethylamine per mole of trypsin, respectively. The only protein peak, other than monomeric trypsin, was an extremely small peak which eluted in a position expected for a dimeric form of trypsin, and amounted to about 3% of total protein.

Evidence for the presence or absence of intramolecular cross-linking is much more difficult to obtain. However, some preliminary observations might be taken to indicate the presence of a maximum 15–20% of intramolecular cross-linking between the N-terminal and C-terminal chain of α-trypsin. EPI-α-trypsin itself, or derivatives formed by the subsequent reaction with glycine ethyl ester of hydroxylamine, when subjected to chain separation (Schroeder and Shaw, 1968) gave a third product (besides the two expected chains) which had the characteristics of an intact trypsin chain as evident from amino acid composition and elution position on

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

Reaction Conditions			Incorp Isoxazolium Reagent		Incorp Nucleophile
Nucleophile (2.0 M)	pH	Time (hr)	Initial[b]	Final[b]	^{14}C (moles bound/mole of trypsin)
$^{14}CH_2(NH_2)CO_2CH_2CH_3$	7.5	4	3.18	0.34	2.00
$^{14}CH_2(NH_2)CO_2CH_2CH_3$	6.7	3	1.97	0.36	1.51
$^{14}CH_3ONH_2$	6.0	6	2.19	0.39	1.83
$^{14}CH_3NHOH$	5.5	6	2.24	Trace	0.43
$^{14}CH_3NHOH$	5.7	5	1.27	Trace	0.31

[a] The reaction was initiated by adding EPI-β-trypsin (15 mg/ml) to a solution which was 2.0 M in nucleophile, 10^{-2} M in benzamidine and which had been adjusted to the desired pH. The EPI-β-trypsin samples used in these experiments were derived from the reactions of EPI (20 mg/ml) and β-trypsin (5 mg/ml) at pH 3.8 for 10–15 min. [b] Moles per mole of trypsin.

gel filtration. Control experiments with the parent α-trypsin itself produced only the two chains and no product corresponding in size to intact trypsin.[3]

Discussion

Our goal in this work was to evaluate the usefulness of isoxazolium salts as reagents for modifying carboxyl groups in proteins. The chemical behavior of this class of reagent was elucidated by Woodward and his colleagues (cited above) who applied it successfully to peptide synthesis in anhydrous medium. Our results indicate that the essential features of this chemistry (Scheme I) apply to the reactions of isoxazolium reagents with proteins in which carboxyl groups can be selectively modified near pH 4.

All of the isoxazolium salts studied, *i.e.*, EPI, MPI, MBI, and K, readily reacted with trypsin under mild conditions in aqueous solutions, and the extent of modification could be followed by analyzing for methyl- or ethylamine in the hydrolysates of the protein on the amino acid analyzer (Figure 1). Protein hydrolysates exhibited amino acid composition expected for trypsin when reactions were carried out with EPI, MPI, or K reagents, below pH 4.75, such that the resulting protein analyzed for less than 5 alkylamines (moles per mole of trypsin). MBI exhibited less specificity as revealed by a tendency for the lysine content to be low for proteins derivatized with this reagent (Table II). Only the 5-phenylisoxazolium salts were therefore further studied.

As pointed out in the preceding section (Results), the initially formed enol ester (III) may rearrange to an imide (IV) which is relatively stable. This could, in some cases, be a useful end form of the modification. However, our work was directed toward the introduction of smaller substituents on the modified carboxyl groups; therefore the isomerization of III to IV was considered an unfavorable property of the reagent or conditions under examination.

Although rearrangement has not been directly utilized for the preparation of more stable protein derivatives, an understanding of this process was necessary for optimal use of isoxazolium salts, especially when subsequent reactions with nucleophiles was desired.

Reaction of Nucleophiles with the Carboxyl-Activated Intermediate. EPI and MPI derivatives of β-trypsin were treated with several nucleophiles, *i.e.*, hydroxylamine, *N*-methylhydroxylamine, *O*-methylhydroxylamine, and glycine ethyl ester. In preliminary experiments, the extent of reaction was followed only by the decreasing methyl- or ethylamine content of the protein (Table IV). The effectiveness of these nucleophiles was found to be in the order expected from studies of reaction involving nucleophilic attack on esters with good leaving groups: hydroxylamine $\simeq$ *N*-methylhydroxylamine $\gg$ *O*-methylhydroxylamine $\simeq$ glycine ethyl ester (Bruice and Benkovic, 1966). Further the displacement reactions were more efficient with EPI- than with MPI-derivatized trypsin. These results can be very well understood in terms of the model (Scheme I). The initially formed enol ester derivatives could undergo four possible reactions under the conditions of the subsequent reaction with nucleophiles: nucleophilic displacement resulting in a new trypsin derivatives; base-catalyzed rearrangement of enol esters to the less reactive imide; hydrolysis of the ester, regenerating the carboxylic acid side chain; intramolecular nucleophilic displacement, resulting in cross-linking.

The dependence of the extent of reaction upon the particular nucleophile used, and also on the initial carboxyl-activating reagent, suggests that rearrangement is a competing process, and that residual alkylamine content remaining after nucleophilic displacement reaction can be taken as a measure of rearrangement to the imide. Observations of nearly invariant alkylamine content after the first 3 hr of reaction with nucleophiles provide further basis for this assumption since imides are expected to be poor acylating agents. Results in Table IV, then, indicate that the rearrangement is virtually negligible during reaction of hydroxylamine and *N*-methylhydroxylamine with EPI-β-trypsin but competes more effectively with nucleophilic displacement when weaker nucleophiles are used, and when reactions were carried out with MPI-β-trypsin derivatives. This corresponds to the greater tendency for β-acyloxy-*N*-methylcinnamamide to undergo rearrangement compared with the *N*-ethyl analog, and therefore sug-

[3] We recognize that this observation does not constitute proof of cross-linking nor that it reveals anything about possible intrachain cross-links but does suggest a maximum possible value for the extent of cross-linking between N-terminal and C-terminal chains.

gests that *N*-ethyl-5-phenylisoxazolium salts are more suitable when subsequent reaction with nucleophiles is desired.

In order to estimate the importance of hydrolysis and cross-linking as possible competing reactions, it was necessary to ascertain whether nucleophiles were becoming covalently bound to the protein. In these experiments ^{14}C-labeled nucleophiles were treated with EPI-β-trypsin derivatives (Table V). High yields of carbon-14 covalently bound in the case of *O*-[^{14}C]methylhydroxylamine and [^{14}C]glycine ethyl ester indicate that nucleophilic displacement on the enol ester is indeed the primary process. These results not only provide strong evidence that carboxyl groups of the protein were modified but also provide a method for the synthesis of new, stable protein derivatives.

Reaction of EPI-β-trypsin with *N*-[^{14}C]methylhydroxylamine was unqiue. Although treatment resulted in nearly quantitative loss of ethylamine, the yield of nucleophile incorporated into the protein was only 20% of theory (Table V). Hydrolysis of the enol ester was therefore the predominant pathway. This result can be interpreted in terms of a mechanism advanced for the nucleophilic displacement by hydroxylamine on esters with good leaving groups (Jencks, 1958). An initial reaction was reported to result in the formation of an unstable *O*-acylhydroxylamine which then reacted with additional hydroxylamine to yield hydroxamic acid. However, in the case of *N*-methylhydroxylamine, this second step may be retarded by the steric interference of the *N*-methyl group. Hydrolysis of initially formed *O*-acyl-*N*-methylhydroxylamine is therefore faster than formation of the hydroxamic acid. The over-all effect of this nucleophile was to catalyze the hydrolysis of the enol ester.

Side chains of trypsin which were derivatized with isoxazolium salts under suitable conditions have exhibited all the expected chemical properties of products obtained from reactions of isoxazolium salts with carboxylic acids. Results of our preliminary investigation therefore provide strong evidence that isoxazolium salts can be used for the specific modifications of carboxylic acid groups in proteins.

Among the various procedures previously developed for the modification of carboxylic acid side chains of proteins, the most promising is one developed by Hoare and Koshland (1967), using a water-soluble carbodiimide in the presence of a suitable nucleophile. The procedure is particularly suitable for the quantitative modification and estimation of carboxyl groups in proteins.

We have noted the tendency for extensively modified trypsin, *i.e.*, containing more than five activated carboxyl groups, to precipitate. Unless this behavior is a peculiarity specific to trypsin, isoxazolium salts would seem to be unsuitable for use in the estimation of total carboxyl groups in proteins. However, the use of isoxazolium salts as carboxyl-activating agents does supplement the previous procedures, and may well allow greater possibilities in the type of modification at a specific carboxyl group. The unique features of the present procedure are as follows: the activated carboxyl groups are sufficiently stable to allow isolation of the modified protein; the extent of modification can be easily determined; and the isolated, modified protein can be reacted with nucleophilic reagents in a subsequent step. Such a separation of the activation from nucleophilic displacement allows one to choose conditions which are optimal for each step. This situation is not possible in the procedure with water-soluble carbodiimide, since the activation and displacement steps must be carried out simultaneously.

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The Modification of Essential Carboxylic Acid Side Chains of Trypsin*

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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.

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