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The Chemical Modification of Papain with 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide†

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ABSTRACT: The reaction of the water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), with active papain in the presence of the nucleophile ethyl glycinate results in an irreversible inactivation of the enzyme. This inactivation is accompanied by the derivatization of the catalytically essential thiol group of the enzyme (Cys-25) and by the modification of 6 out of 14 of papain's carboxyl groups and up to 9 out of 19 of the enzyme's tyrosyl residues. No apparent irreversible modification of histidine residues is observed. Mercuripapain is also irreversibly inactivated by EDC/ethyl glycinate, again with the concomitant modification of 6 carboxyl groups, up to 10 tyrosyl residues, and no histidine residues; but in this case there is no thiol derivatization. Treatment of either modified native papain or modified mercuripapain with hydroxylamine results in the complete regeneration of free tyrosyl residues but does not restore any activity. The competitive inhibitor benzamidoacetonitrile substantially protects native papain against inactivation and against the derivatization of the essential thiol group as well as 2 of the 6 otherwise accessible carboxyl groups. The inhibitor has no effect upon tyrosyl modification. These findings are discussed in the context of a possible catalytic role for a carboxyl group in the active site of papain.

A recent comprehensive review (Glazer and Smith, 1971) of the literature concerning the structure and function of the sulfhydryl proteinase papain (EC 3.4.22.2) cites extensive evidence for the following generally accepted features of the mechanism of papain-catalyzed hydrolyses of amides or esters of α-*N*-acyl-L-amino acids. After an initial reversible substrate-binding step, the carbonyl group of the scissile amide or ester linkage of the substrate undergoes a nucleophilic attack by the thiol group of Cys-25 in the papain active site. This leads to the displacement of the amine or alcohol leaving group from the amide or ester substrate and the concomitant formation of a thiol ester, the so-called acyl-enzyme intermediate. Hydrolysis of this intermediate then occurs, completing the acylation–deacylation cycle of catalysis. The pH dependence of the acylation step rate constant is generally attributed to two catalytically essential functional groups in the papain active site with apparent pK_a's of about 4 and about 8. The latter pK_a is most readily assigned to the aforementioned thiol group of Cys-25, and the former pK_a is usually attributed to a group which functions as a general base (Brubacher and Bender, 1966) in the acylation step.

Opinion varies as to the identity of this putative general base. Early speculation (Smith and Kimmel, 1960; Whitaker and Bender, 1965) centered on a carboxyl group, a reasonable assignment based upon the normally expected pK_a values of aspartate or glutamate residues in proteins (Tanford and Hauenstein, 1956) and supported by the finding (Smith and Parker, 1958) that a low apparent heat of ionization, typical of carboxylic acid dissociation, is associated with the acidic limb of the pH–rate profile for papain catalysis. On the other hand, much recent speculation (see, for example, Lowe and Whitworth, 1974) has focused upon the imidazole group of His-159 in the active site of papain, an assignment first suggested by Lowe and Williams (1965) on the basis of chemical modification studies. X-ray crystallography (Drenth et al., 1968, 1971) tends to support the proponents of the imidazole group, showing that at least in the crystal the imidazole moiety of His-159 is in fact immediately adjacent to the thiol group of Cys-25 in the papain active site, whereas the only free carboxyl group of the active site (Asp-158) is several angstroms farther away in a position which makes direct interaction with the thiol group difficult to visualize. Be that as it may, recent evidence has

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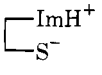
been published (Okimura and Murachi, 1975) which indicates that His-159 has a "normal" pK_a of ~6.7 rather than a perturbed pK_a near 4. And in another recent paper (Drenth et al., 1975) the hypothesis is advanced that, within the pH range 5–7, the imidazole and thiol groups of the active site of native papain exist predominantly as an ion pair,



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in which the imidazolium group has a pK_a of 8.5 and the thiol group a pK_a of 4.2. It does seem likely that the imidazole group may indeed participate in papain catalysis, but the evidence associating this group with the acidic limb of the pH–rate profile for acylation is not compelling. Furthermore the possibility remains that the apparent pK_a of ~4 might be ascribed to a carboxyl group such as that of Asp-158.

In the investigation reported here, we have employed the water-soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC[1]), a reagent which has been widely used to modify carboxyl groups in enzymes (Means and Feeny, 1971), in an attempt to further assess the possibility that one or more carboxyl groups may assume a significant role in papain catalysis.

Experimental Section

Materials. Papain (EC 3.4.22.2) was purchased from Worthington Biochemical Corp. (two-times recrystallized, No. PAP33K857 and PAP34D677) and was further purified by the method of Blumberg et al. (1970), using a glycylglycyl-*O*-benzoyl-L-tyrosyl-L-arginine-Sepharose affinity column. The purified, fully active papain was converted to its more stable inactive mercury derivative for storage at 4 °C as a solution (about 3.5 mg/ml) in distilled water. EDC was purchased from Sigma Chemical Co. and was used without further purification, mp 114–115 °C (lit. mp 113.5–114.5 °C (Sheehan et al., 1961)). Glycine ethyl ester hydrochloride (sublimed before use), L-cysteine (free base), EDTA (disodium salt), dithiothreitol, 2,2′-dipyridyldisulfide (2,2′-dithiodipyridine), and α-*N*-benzoyl-L-arginine ethyl ester hydrochloride were also purchased from Sigma Chemical Co. 2-Mercaptoethanol was obtained from Eastman Kodak Co. and was distilled before use. [^{14}C]Glycine ethyl ester hydrochloride was obtained from New England Nuclear (No. 600-281). L-Histidine and *tert*-butoxycarbonyl azide were purchased from Aldrich Chemical Co. Benzamidoacetonitrile was prepared by the method of Klages and Haak (1903), mp 140 °C (lit. mp 139–141 °C (Lucas and Williams, 1969)). Triethylamine hydrochloride was prepared by mixing predistilled triethylamine (J. T. Baker) with constant boiling 6 N HCl and lyophilizing. The white crystalline salt melted at 255–256 °C (lit. mp 253–254 °C (Harris, 1965)). All other chemicals employed were reagent grade and were used without further purification. Oxygen-free, nitrogen-purged, glass-distilled water was used throughout.

L-Histidine methyl ester was prepared by the method described by Greenstein and Winitz (1961), mp 212–214 °C (lit. mp 200–201 °C (Hay and Morris, 1971)). *Anal.* Calcd for $C_7H_{13}Cl_2N_3O_2$: C, 34.71; H, 5.37; N, 17.32. Found: C, 34.56; H, 5.32; N, 17.32. *N-tert*-Butyloxycarbonyl-L-histidine methyl ester was prepared by the method of Schröder and Gibian (1962), mp 126–128 °C (lit. mp 127–130 °C (Hofmann et al., 1965)).

Methods. Ir spectra were taken on a Beckman IR 20A. NMR spectra were run on a JEOL-PS-100 NMR spectrometer. Melting points (uncorrected) were taken in open capillary tubes in a Thomas-Hoover melting point apparatus. Uv spectra and spectrophotometric rate assays were recorded in standard 1-cm quartz cuvettes using a Cary 14 recording spectrophotometer equipped with a thermostated cell compartment and cuvette holder. pH measurements and pH-stat titrimetric rate assays were carried out with a Radiometer PHM-26 pH meter equipped with a Radiometer GK 2321-c combination electrode and interfaced with a Radiometer TTT-11 titrator, SBR2 recorder, and SBU1 syringe buret. Radioactivity was determined using a Beckman LS-133 liquid scintillation counter.

Papain Assays. Total papain concentration (total protein) was determined spectrophotometrically using the published parameters $A_{278nm,1cm}^{1\%}$ = 25.0 (Glazer and Smith, 1961) and mol wt = 23 430 (Husain and Lowe, 1969). The concentration of activatable papain in freshly purified (see Materials) mercuripapain stock solutions was determined by spectrophotometric rate assay using the substrate Bz-ArgOEt as described by Whitaker and Bender (1965) under conditions and using kinetic parameters which relate this assay to absolute papain concentrations determined by direct active-site titration. Comparison of the rate-assay data with concentrations determined from protein absorbance at 278 nm established that these stock solutions contained no inactivatable enzyme.

Determinations of relative papain activity during the course of inactivation reactions were carried out titrimetrically. Each assay was initiated by adding an appropriate small aliquot of the inactivation reaction mixture to a 2.5-ml titration vessel thermostated at 25 °C and containing 1 mM EDTA, 50 mM BzArgOEt, 0.23 M KCl, and water to a final volume of 2.5 ml. For those assays performed on mercuripapain, the 2.5-ml assay mixture was made 5 mM in cysteine. All assays were carried out at pH 6.6, delivering freshly standardized 0.05 N NaOH titrant from a 0.5-ml syringe buret. The calculated final protein concentration in the assay mixture was either 0.85 or 1.44 μM. Initial rates, recorded as percent of syringe capacity per minute, were normalized to an averaged control rate using fully active papain and are reported as percent active enzyme.

Kinetic parameters characterizing modified papain were determined from Lineweaver–Burk plots of initial rate data in which BzArgOEt was used as the substrate at pH 6.6, 25 °C. Details of the procedures employed have been described previously (Hall et al., 1972).

Determinations of the free-thiol (-SH) content of papain samples (either before, during, or after inactivation reactions) were performed after removing any low molecular weight thiol activator compounds by Sephadex G-25 gel filtration. The method employed was spectrophotometric titration using 2,2′-dipyridyl disulfide (Brocklehurst and Little, 1973).

Inactivations of Papain with EDC. All inactivations were carried out at pH 4.75, 25 °C, using either mercuripapain or freshly activated, activator-free papain. In experiments requiring mercuripapain, an aliquot of stock solution was first dialyzed for 24 h at 4 °C against 3 × 500 ml of 10 mM

[1] Abbreviations used are: EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; BzArgOEt, α-*N*-benzoyl-L-arginine ethyl ester; GlyOEt, glycine ethyl ester.

KCl to remove excess mercuric ions. Active, activator-free papain was prepared by adding dithiothreitol (to a final concentration of 2.9 mM) to a 2.5-ml aliquot of mercuripapain stock solution and stirring at room temperature for 30 min. The activator and complexed mercuric ions were then removed by gel filtration on a Sephadex G-25 (medium) column (1 × 37 cm). The elution solvent was oxygen-free, nitrogen-purged, 10 mM KCl. The activator-free enzyme usually appeared between 15 and 18 ml of eluate volume and was used immediately.

Inactivation reaction mixtures, thermostated at 25 °C were made up in 10 mM KCl to contain 0.103 mM enzyme in the presence or absence of the desired added reagents. These added reagents, when present, had the following initial concentrations in the reaction mixture (after addition of EDC): glycine ethyl ester, 0.5 M; triethylamine, 0.5 M; benzamidoacetonitrile, 18.7 mM (when this competitive inhibitor was present, the reaction mixture was also 1% v/v acetonitrile); [^{14}C]glycine ethyl ester, 0.5 M (in these experiments the initial enzyme concentration was only 0.061 mM). The initial pH was adjusted to 4.75 and maintained at that value by adding 0.2 N HCl throughout the course of inactivation. Inactivation was initiated by addition of EDC in the prescribed molar excess over the amount of papain present. The carbodiimide was added either as the pure crystals or as an aqueous solution at pH 4.75. In some experiments a second addition of EDC was made after the reaction had been allowed to proceed for some time. Aliquots of the reaction mixture were removed at timed intervals for papain assays. Parallel control reaction mixtures, identical in every respect with the inactivation reaction mixtures without EDC, were assayed periodically during all inactivations. In no case was any activity loss or reduction in thiol titer observed in these controls. After inactivation, the reaction mixture was dialyzed for 20 h against 5 × 500 ml oxygen-free, nitrogen-purged, 10 mM KCl before a final assay for enzymatic activity and free sulfhydryl content and/or amino acid analysis.

Incorporation of [^{14}C]*Glycine Ethyl Ester.* EDC inactivations carried out in the presence of [^{14}C]glycine ethyl ester (specific activity approximately 9 μCi/mmol) resulted in the irreversible incorporation of radioactivity by papain. To determine the stoichiometry of incorporation of label by the protein in a given experiment, the final reaction mixture was first dialyzed under nitrogen pressure at 25 °C against oxygen-free, nitrogen-purged, 10 mM KCl on an Amicon Micro Ultrafiltration System Model 8MC using an Amicon PM 10 Diaflow membrane. In experiments involving the competitive inhibitor, benzamidoacetonitrile, this dialysis step was preceded by a 1:1 dilution of the final inactivation reaction mixture with 1 M acetic acid buffer, pH 4.75, to destroy any remaining EDC. When the dialysis eluate no longer contained any radioactivity, the dialysate was concentrated to approximately 1 ml and its protein concentration was determined spectrophotometrically at 278 nm. Aliquots of this dialysate were then assayed with BzArgOEt for residual enzyme activity and counted in duplicate in Bray's solution (Bray, 1960). The stoichiometry of label incorporation was determined from the specific activity of the [^{14}C]glycine ethyl ester employed, as determined by counting aliquots of standard solutions of that labeled ester.

Regeneration of Free Tyrosyl Residues by Hydroxaminolysis of Modified Papain. After EDC modification and dialysis as described above, an appropriate aliquot of the modified papain solution was treated with hydroxylamine (0.5 M) at pH 7.0. The reaction mixture was allowed to stand for 5 h at 25 °C, after which it was dialyzed once more and assayed for enzymic activity and total protein concentration prior to preparation for amino acid analysis. It was established in independent control experiments that the hydroxaminolysis procedure did not affect the activity of unmodified papain.

Amino Acid Analysis. The amino acid composition of hydrolyzed samples of various papain preparations were determined using a Beckman Model 121 automatic amino acid analyzer equipped with a Beckman System AA computing integrator, using standard procedures. Duplicate lyophilized samples, each containing approximately 1 mg of protein, were hydrolyzed with constant boiling HCl (Pierce Chemical Co., Sequanal Grade) in evacuated sealed tubes for 24, 48, and 72 h. Values obtained for Thr and Ser were extrapolated to zero hydrolysis time to correct for destruction of these amino acids.

Results and Discussion

The primary conclusion to which this investigation has led is that the modification of carboxyl groups, brought about by the treatment of mercuripapain with the water-soluble carbodiimide EDC in the presence of GlyOEt, is directly responsible for an irreversible loss of enzymatic activity. This in turn is consistent at least with the *possibility* that one or more carboxyl groups participate actively in papain catalysis and thus might conceivably be responsible for the "kinetic" pK of about 4 in papain concerning which, as discussed in the introduction to this paper, there has been so much speculation. Having stated this conclusion at the outset, let us now consider in more detail the interpretation of the actual results.

The use of water-soluble carbodiimides in conjunction with reactive nucleophilic species as a technique for the modification of carboxyl groups in enzymes and other proteins was introduced several years ago (Hoare and Koshland, 1967; Reihm and Scheraga, 1966) and the versatility and usefulness of the technique have since been amply demonstrated (Means and Feeny, 1971). Unfortunately, however, these carbodiimides are not specific for carboxyl groups alone. Under the conditions employed in this investigation, it was anticipated that EDC would react also with free sulfhydryl groups (Carraway and Triplett, 1970) as well as accessible phenolic hydroxyl groups (Carraway and Koshland, 1968) in papain. Thus, after it became apparent that EDC did in fact inactivate papain (Figure 1, Table I), it remained to be determined which sorts of groups were modified, and then which of those were responsible for the loss of enzymatic activity. The results show, not unexpectedly, that all three potentially reactive groups are in fact derivatized when papain is treated with EDC at pH 4.75 in the presence of GlyOEt.

Derivatization of -SH groups was determined by the difference between the total protein concentration (assayed spectrophotometrically, A_{278}; see Experimental Section) and the concentration of free thiol groups (assayed by spectrophotometric titration using 2,2′-dipyridyl disulfide according to the method of Brocklehurst and Little, 1973). For unmodified control papain, the total protein assays and free thiol assays have the expected 1:1 correspondence (Brocklehurst and Little, 1973). But, as shown in Table I, treatment of active papain with EDC, either alone or in the presence of 0.5 M GlyOEt, results in the irreversible loss of titrable thiol groups.

Table I: Inactivation of Active Papain by EDC at pH 4.75, 25 °C, in 10 mM KCl.

Initial [EDC]/[Enzyme]	Second Addition of EDC after 60 min[a]	[GlyOEt] (M)	[Benzamidoacetonitrile] (M)	% Activity Remaining[b]	% Thiol Remaining[b]
600	−			26	28
600	+	0.5		17	21
600	+	0.5	0.0187	80	80

[a] Plus sign indicates that a second portion of EDC, equal in amount to the initial portion, was added after 60 min and the reaction was allowed to proceed for an additional 30 min. Total reaction time before dialysis was otherwise (minus sign) 60 min. [b] After modification followed by exhaustive dialysis. See Experimental Section for description of assays for enzymatic activity and thiol.

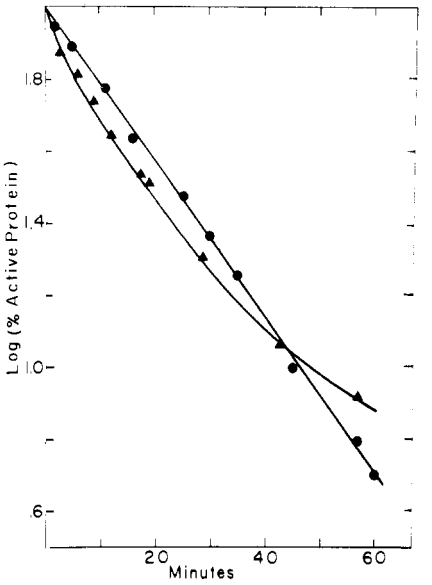


FIGURE 1: The inactivation of active, activator-free papain in the presence (●) or absence (▲) of 0.5 M GlyOEt. The initial EDC concentration was 61.8 mM, a 600-fold molar excess over initial active papain concentration. For other reaction conditions, see Table I.

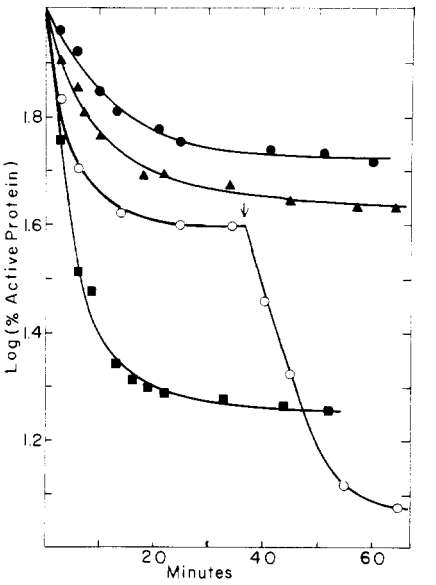


FIGURE 2: The inactivation of mercuripapain by EDC in the absence of added nucleophile or base. The carbodiimide was added initially in 300-fold (●), 600-fold (▲), 780-fold (○), or 1200-fold (■) molar excess over enzyme. The arrow indicates the addition of a second 780-fold excess of EDC to the reaction mixture. For other reaction conditions, see Table II.

Any derivatization of the catalytically essential thiol group of Cys-25 should be expected to lead to the inactivation of papain, as has been amply demonstrated in a variety of ways (Finkle and Smith, 1958; Light et al., 1964; Husain and Lowe, 1965, 1968; Bender and Brubacher, 1966; Shaw et al., 1965; Wallenfels and Eisele, 1968; Whitaker and Perez-Villaseñor, 1968; Anderson and Vasini, 1970). And indeed, our results show (Table I) a close correspondence between activity loss and thiol derivatization when active papain is treated with EDC. Furthermore, in the presence of benzamidoacetonitrile, a good competitive inhibitor[2] of papain, both the extent of activity loss and the extent of thiol derivatization are substantially reduced. Since the only free thiol group of papain belongs to Cys-25 in the active site, this clear example of a "protective effect" (see, for example, Singer, 1967) confirms that benzamidoacetonitrile must indeed bind at the active site of the enzyme.

Clearly, since the reactivity of Cys-25 in active papain toward EDC is so high, its rapid derivatization would mask any effects upon enzymic activity which the chemical modification of other less reactive groups might have. Thus, our attention shifted from the active enzyme to mercuripapain, a derivative obtained by treating papain itself with mercuric chloride which reversibly metallates the single thiol group of the enzyme. Mercuripapain as such has no enzymatic activity. However, when it is placed in the presence of an excess of some appropriate thiol compound (e.g. cysteine, 2-mercaptoethanol, dithiothreitol), the mercurienzyme is completely and instantaneously reconverted to active papain. In the context of the investigation reported here, when it is noted that mercuripapain is subject to "inactivation" under stated conditions, the tacit implication is that the mercurienzyme is rendered *nonactivatable* by thiol activator species in the usual manner.

Figure 2 and Table II show that mercuripapain is indeed subject to inactivation by EDC. Admittedly, inactivation of the mercurienzyme is less rapid and, ultimately, less complete than inactivation under comparable conditions of the native enzyme. But most significantly, little or no irreversible derivatization of the critical Cys-25 residue accompanies this inactivation. Even under forcing conditions, when the ultimate extent of irreversible inactivation is similar to that achieved with active papain (see Table I), the reaction of EDC with mercuripapain does *not* result in significantly lowered titers of free thiol. It is perhaps worth noting here that mercuration is not universally effective in protecting papain against derivatization of its thiol group by

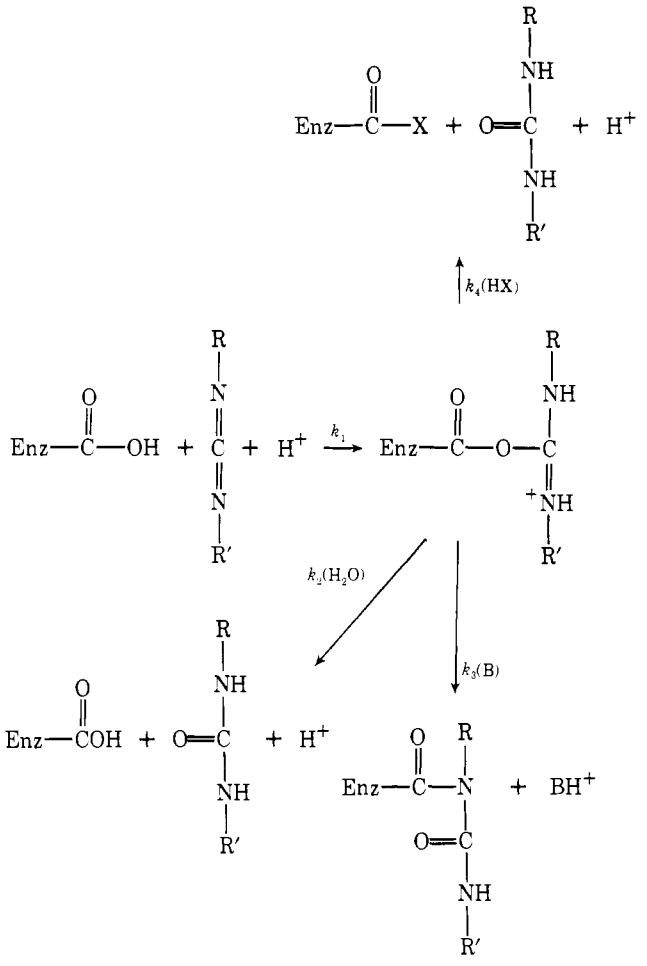
[2] In kinetic studies carried out by Sluyterman and Wijdenes (1973), the K_i values (presumably net dissociation constants) for benzamidoacetonitrile as a competitive inhibitor of papain were determined to be 2.4×10^{-4} M at pH 3.8 and 1.3×10^{-4} M at pH 6.0.

Table II: Inactivation of Mercuripapain by EDC at pH 4.75, 25 °C, in 10 mM KCl.

Initial [EDC]/[Enzyme]	Second Addition of EDC after 60 min[a]	[Gly/OEt] (M)	[Benzamidoacetonitrile] (M)	% Activity Remaining[b]	% Thiol Remaining[b]
300	−			68	100
600	−			71	94
600[c]	−			54	90
600	+			27	100
1200	+			32	100
600	−	0.5		17	92
600	+	0.5		10	98
600	+	0.5	0.0187	17	100

[a] See Table I. [b] See Table I. [c] Inactivation carried out in the presence of 0.5 M triethylamine.

Scheme I: Carboxyl Group Derivatization by Water-Soluble Carbodiimides.



other reagents.[3] But its effectiveness in this investigation appears to have been excellent.

The nature of the actual chemical reactions involved in carboxyl group modifications using water-soluble carbodiimides has been outlined in some detail by Hoare and Koshland (1967). This chemistry is summarized here in Scheme I. In the first step of the reaction, the carboxyl group adds to the carbodiimide, forming a very labile *O*-acylisourea intermediate. The intermediate then reacts further to give stable products in one or more of the ways shown. In the absence of any added nucleophilic species (designated HX in Scheme I), most of the intermediate undergoes hydrolysis, which simply regenerates the original carboxyl group. However, there may be some isomerization of the intermediate to a stable *N*-acylurea via the k_3 step of Scheme I, a reaction which is catalyzed by bases (Kurzer and Douraghi-Zadeh, 1967). On the other hand, when a nucleophilic species HX is present in high concentration the intermediate *O*-acylisourea is efficiently captured in the k_4 step of Scheme I, thus producing the stable "labeled" derivative shown. If, as in our case for example, the nucleophilic species were GlyOEt, the final product would have the glycinamide structure II.

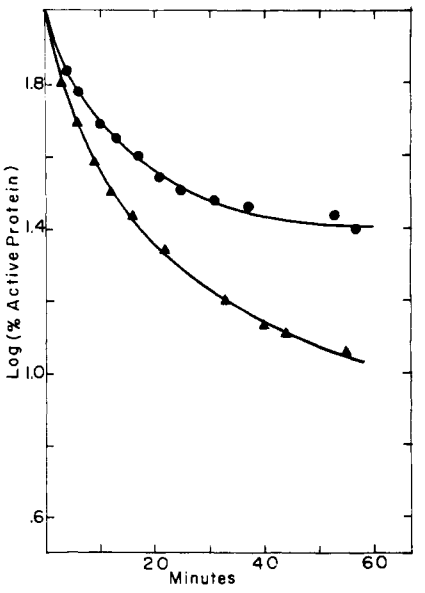
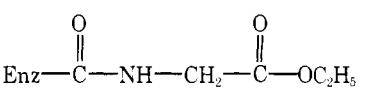


FIGURE 3: The inactivation of mercuripapain by EDC (in 600-fold molar excess over enzyme) in the presence of 0.5 M triethylamine (●) or 0.5 M GlyOEt (▲). For other reaction conditions, see Table II.



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Returning now to the results of this investigation, the data of Table II and Figures 2 and 3 can be nicely accounted for in terms of Scheme I. In the absence of GlyOEt (Figure 2) the initial relatively rapid rate of inactivation is presumably due to the buildup of the labile (but nonetheless enzymatically inactive) *O*-acylisourea derivative in the k_1

[3] The thiol sulfur of mercuripapain has been found to be readily modified by *N*-bromosuccinimide (Lowe and Whitworth, 1974), by *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (Perfetti, 1975), and by *N*-ethylbenzisoxazolium tetrafluoroborate (R. B. Perfetti and P. L. Hall, unpublished results).

Table III: Inactivation of Papain by EDC in the Presence of [^{14}C]Glycine Ethyl Ester.[a]

Enzyme	[Benzamidoacetonitrile] (M)	No. of Gly Residues[c]	% Activity Remaining[b]
Mercuripapain		6.0	15
Active papain		6.0	17
Active papain	0.0187	4.1	71

[a] Two 600-fold molar excess portions of EDC added at 0 and at 60 min to a reaction mixture containing enzyme (61 μM), labeled GlyOEt (0.5 M, specific activity 9 μCi/mmol), and KCl (10 mM) at pH 4.75, 25 °C. Each numerical entry in the table represents the mean result of several experiments. [b] After modification followed by exhaustive "diafiltration". See Experimental Section for assay procedure and other details. [c] Number of glycine residues incorporated per enzyme molecule.

Table IV: Changes in Amino Acid Composition of Papain upon Reaction of the Enzyme with EDC in the Presence of Glycine Ethyl Ester.[a]

	Amino Acid Composition[c]			
Expt[b]	Tyr	His	Gly	% Activity Remaining[d]
A	19.26 (19)	1.74 (2)	27.88 (28)	100
B	10.20 (10)	1.78 (2)	32.31 (32)	80
C	10.03 (10)	1.81 (2)	34.09 (34)	10
D	19.98 (20)	1.82 (2)	27.56 (28)	100
E	19.74 (20)	2.05 (2)	33.75 (34)	6
F	19.99 (20)	1.73 (2)	27.56 (28)	100
G	13.17 (13)	1.95 (2)	27.50 (28)	32
H	15.49 (15)	2.06 (2)	34.26 (34)	Tr

[a] Reaction conditions as described in notes for Table III, except that the GlyOEt was not radioactive. [b] Experiment: A, unmodified stock Hg-papain; B, active papain modified in the presence of 18.7 mM benzamidoacetonitrile; C, modified Hg-papain; D (control for experiment C), Hg-papain treated as in experiment C but with EDC omitted; E, Hg-papain modified with EDC-GlyOEt followed by exhaustive hydroxaminolysis to regenerate tyrosyl residues; F (control for experiment E), Hg-papain treated as in experiment E but with EDC omitted; G, Hg-papain modified with EDC alone (two additions of EDC in 1200-fold molar excess over enzyme); H, major fraction (inactive) from affinity chromatography of modified Hg-papain. See text for details. [c] Values tabulated are residues per molecule of papain calculated relative to an assumed value for alanine of 14.00 (Mitchel et al., 1970). For reference, values for these amino acids from the most recent published primary sequence of papain (Mitchel et al., 1970) are Tyr, 19; His, 2; Gly, 28. [d] After modification (if any) followed by exhaustive dialysis. See Experimental Section for assay procedure and other details.

step. The concentration of this species reaches a maximum at the point in time when its second-order rate of formation is equaled by its pseudo-first-order rate of disappearance via the k_2 and k_3 steps. Subsequent inactivation proceeds at a very slow rate with the irreversible formation of the *N*-acylurea derivative of the enzyme in the k_3 step. Further consistent with Scheme I is the observation (Figure 3 and the third entry of Table II) that triethylamine, a tertiary amine which, unlike the primary amine GlyOEt, cannot serve as a nucleophile in the k_4 step, apparently does catalyze the inactivation of mercuripapain by EDC, presumably by enhancing the k_3 step.

In the presence of added GlyOEt, the rates and extents of mercuripapain inactivation by EDC are, as expected, greater than in its absence. Furthermore, although it has been pointed out that carboxyl groups are not the only groups in proteins which are susceptible to modification by water-soluble carbodiimides, they are the only groups which react with added GlyOEt or other such nucleophiles to give stable derivatives under the conditions of the investigations reported here. One can thus accurately assess the extent of carboxyl-group modification by determining the extent of incorporation of glycine residues into the protein as a result of modification. This is conveniently done either by conventional amino acid analysis or by the use of ^{14}C-labeled GlyOEt followed by determinations of radioactivity in the modified protein (Hoare and Koshland, 1967). Both procedures have been employed in this investigation, and the results are presented in Tables III and IV.

Table IV summarizes the results of analyses of various modified and unmodified papain preparations for their amino acid composition. Values are given only for tyrosine, histidine, and glycine. Values obtained for all other amino acids were unaffected by modification under any of the conditions noted in Table IV, and in all cases their nearest integral values (normalized relative to an assumed value of 14 for alanine) agreed with the established composition of native papain (Mitchel et al., 1970). The data in Table III show the results of the experiments employing ^{14}C-labeled GlyOEt.

Looking first at the extent of carboxyl-group modification, it is quite apparent in Table III that just six groups are involved, either in native papain or in mercuripapain. Comparison of data in the Glycine column of Table IV for Experiments C and D confirms (for mercuripapain) that indeed six additional glycine residues are incorporated by the enzyme in its reaction with EDC-GlyOEt. The native enzyme has, altogether, 14 carboxyl groups: 6 Asp and 7 Glu residues plus the carboxyl terminus of the single polypeptide chain (Mitchel et al., 1970). So clearly, more than half of these groups remain inaccessible to the modifying reagents—a finding which might have been anticipated from some of the features of the papain structure (intrachain hydrogen bonds, etc.) as described by Drenth et al. (1971). Of somewhat greater interest and importance is the finding that the protection afforded active papain by benzamidoacetonitrile against inactivation and against thiol modification (see Table I) includes substantial protection against carboxyl-group modification as well.[4] Only four glycine residues are incorporated by active papain in the presence of the inhibitor as opposed to six residues in its absence (see Table III and experiment B in Table IV). This finding would ordinarily be interpreted to imply that the competitive inhibitor benzamidoacetonitrile, which must bind at the active site of papain since it affords protection against thiol modification, also prevents two active-site carboxyl groups from reacting with EDC-GlyOEt. From the active site description by Drenth et al. (1971) one can tentatively identify one of the two protected carboxyl groups as that of Asp-158. However, if residue-64 of papain, also known to belong to the active site (Drenth et al., 1971), is indeed an aspara-

[4] Benzamidoacetonitrile does not protect mercuripapain against inactivation. This is presumably the result of the failure of this inhibitor to associate appreciably with the metallated active site of the enzyme. It is known that mercuripapain does not bind to a tetrapeptide competitive inhibitor ligand attached to agarose (Blumberg et al., 1970). On the other hand, oligopeptide substrates have been found which apparently do bind at the extended active site of mercuripapain (Lowbridge and Fruton, 1974). It might be informative to determine to what extent such substrates protect mercuripapain against modification by EDC, but experiments along these lines have yet to be carried out.

gine rather than an aspartic acid residue (Husain and Lowe, 1970), it is difficult to assign a tentative identity to the second protected carboxyl group. It may be that the binding of benzamidoacetonitrile to the active site of papain results indirectly, through conformational changes that involve amino acid residues without as well as within the active site, in a reduction of carboxyl-group accessibility at some other locus on the protein surface. Or it may be that the inhibitor binds significantly at one or more alternate sites, in addition to the active site, where it could provide protection against EDC attack. Indeed evidence exists (though none specifically for benzamidoacetonitrile) which suggests that effectors of papain catalysis may in fact bind at remote sites on the enzyme (Hall and Anderson, 1974; Fink and Gwyn, 1974).

Having established that carboxyl groups are chemically modified when papain is inactivated by EDC-GlyOEt, and that no thiol derivatization occurs in the mercurienzyme, what can we say about any other potentially reactive and modifiable groups? Carraway and Koshland (1968) have shown that EDC does in fact convert accessible tyrosine residues in proteins to *O*-arylisourea derivatives which are resistant toward acid hydrolysis. However, they have also shown that hydroxaminolysis of the modified protein quantitatively reverses this tyrosine modification. The results for tyrosine presented in Table IV, obtained using the procedures described by Carraway and Koshland (1968), indicate that indeed 6–10 of the 19 tyrosine residues in papain are modified by EDC when either the native enzyme or its mercury derivative is inactivated. Complete regeneration of free tyrosyls is brought about by hydroxaminolysis of the modified protein, but no enzymatic activity is regained thereby. Furthermore, the competitive inhibitor benzamidoacetonitrile affords no protection of active papain against tyrosyl modification by EDC. Thus, it must be regarded as highly unlikely that any of the observed EDC inactivation of papain or mercuripapain can be attributed to modification of tyrosyl residues.

It is conceivable that EDC inactivation of papain or mercuripapain might be due, at least in part, to a reaction of some kind between the carbodiimide and the imidazole moiety of His-159, a known constituent of the papain active site (Drenth et al., 1968, 1971). Such a reaction must be regarded as unlikely in view of the successful use of carbodiimide reagents in peptide synthesis involving histidine derivatives (Greenstein and Winitz, 1961). Furthermore, histidine residues in ribonuclease were found to be unaffected by treatment with a water-soluble carbodiimide at pH 4.5 (Riehm and Scheraga, 1966). Accordingly in our case, the data for histidine in Table IV reveal no significant loss of histidine in papain or mercuripapain upon treatment with EDC at pH 4.75. In addition, we carried out a number of experiments in which a model compound, *N-tert*-butyloxycarbonyl-L-histidine methyl ester, was treated with EDC under a wide variety of conditions, including high pH. In all of these experiments, the unmodified histidine derivative was recovered intact, and analysis of reaction mixtures by thin-layer chromatography revealed no trace of unidentifiable components which might have been attributable to any product with a modified imidazole moiety.

To this point in the discussion it has been shown that thiol modification in papain by EDC can be prevented by prior mercuration, that tyrosine modification occurs but is without effect on enzymatic activity, that histidine modification does not occur, and thus that the observed inactivation of mercuripapain by EDC-GlyOEt can be attributed to the chemical modification of one or more of six carboxyl groups in the enzyme. The number of carboxyl groups potentially involved in catalysis can tentatively be reduced to just one or two on the basis of the observed protective effect of benzamidoacetonitrile. But there is a recurring feature in the data of Tables I–IV which is disturbing and remains to be dealt with. The inactivations reported are invariably incomplete. Is the residual esterase activity found after treatment of papain with EDC-GlyOEt attributable to an incomplete (or partially reversible) reaction, leaving a small fraction of the enzyme unmodified and active? Or is complete modification in fact occurring but resulting only in partial rather than complete inactivation of each papain molecule?

Table V: Effects of EDC-GlyOEt Modification upon Papain Activity. Kinetic Catalysis Parameters for the Hydrolysis of BzArgOEt at pH 6.6, 25 °C.

Enzyme	% Activity	% Thiol	$(k_{cat})_{app}$[a] (s^{-1})	$(K_m)_{app}$[a] (M × 10^2)
Unmodified mercuripapain[b]	(100)	97	23.1 ± 0.4	1.73 ± 0.04
Modified mercuripapain[c]	10	96	2.7 ± 0.5	3.4 ± 0.8
Control mercuripapain[c]	101	98	24.1 ± 2.9	1.9 ± 0.3

[a] From Lineweaver-Burk plots of initial rate data. Values for unmodified mercuripapain are taken from a previous investigation (Hall and Anderson, 1974). [b] Mercuripapain stock as described in the Experimental Section has a specific activity of 4.46×10^{-3} mmol of BzArg per mg of enzyme per min in a standard assay employing 50 mM BzArgOEt as the substrate at pH 6.6, 25 °C in 0.23 M KCl. This activity is taken as the 100% figure to which all other activities and thiol-content data in the table are referred. [c] Mercuripapain (0.103 mM) was inactivated by two successive 60-min treatments with EDC in 600-fold molar excess in the presence of GlyOEt (0.5 M) at pH 4.75, 25 °C in 10 mM KCl. Control experiment identical except that no EDC was added.

Following an experiment in which a sample of mercuripapain was inactivated as before (two additions of EDC in 600-fold molar excess; see Table II), Michaelis-Menten kinetic parameters were determined for the modified enzyme, as well as for a control in which the EDC additions had been omitted but otherwise was identical. The results are given in Table V. In view of the large drop in $(k_{cat})_{app}$ and the relatively modest increase in $(K_m)_{app}$, it seems likely that this modification resulted in a ~9:1 mixture of totally inactive and (essentially) fully active papain species. Had the modification produced homogeneous partially active enzyme, so small an increase in the observed $(K_m)_{app}$ would be surprising.

As a check on the conclusion reached after the kinetic experiments on the modified enzyme, one additional experiment was carried out. Once again mercuripapain (20 mg) was inactivated in 0.5 M GlyOEt with two 600-fold additions of EDC followed by the usual dialysis. Once again the result was a modified enzyme with 11% activity. At this point the solution of modified papain was concentrated several fold by ultrafiltration (Amicon PM-10 Diaflo membrane), incubated with mercaptoethanol (60 mM) and EDTA (50 mM), passed over a Sephadex G-25 column to remove the activator and mercury, and finally subjected to fractionation by affinity chromatography on a small (0.5 × 7 cm) tetrapeptide-Sepharose column (Blumberg et al.

(1970)). All of the applied protein (15 mg) was recovered in two distinct fractions. The larger fraction (13 mg or 87%) eluted earlier at high ionic strength as a sharp band—the behavior expected for inactive papain. The remaining 2 mg of papain was retained by the affinity column, suggesting that it was indeed active enzyme. It eluted as a very broad, dilute peak, quite possibly consisting of a mixture of more than one partially modified papain species. Efforts to concentrate this presumably active papain fraction for further study were accompanied by irretrievable losses of protein, unfortunately precluding its more complete characterization.

The larger fraction from the affinity chromatography was further characterized, however. After its elution from the peptide–Sepharose column, it was dialyzed against 0.1 mM $HgCl_2$ to help stabilize any free thiol groups, and then against water. Although this dialyzed protein was found to have retained at least 30% of its original free thiol content,[5] its enzymatic activity in the standard BzArgOEt esterase assay was, at most, barely perceptible. The amino acid composition of this protein is given in Table IV (experiment H) and, except apparently for the extent of tyrosine loss, is closely comparable to that of freshly modified, unfractionated mercuripapain (experiment C).

Thus it seems clear that, indeed, the residual esterase activity of our modified papain preparations was the result of incomplete reaction rather than partial inactivation. The primary remaining uncertainty surrounding the question of a catalytic role for a carboxyl group in papain stems from a difficulty one almost always faces in evaluating the results of chemical modification studies. The difficulty is that an enzyme can be totally inactivated by the modification of any group in or near the active site, whether or not that group is actually involved in catalysis per se. The likelihood of obtaining inactivation with the modification of a catalytically passive group is especially high when the reaction used results in a significant increase in the steric bulk of the modified group. Certainly the carboxyl-group modification reaction used in this investigation does result in the introduction of considerable steric bulk and, accordingly, our conclusion can only be that one or two carboxyl groups in papain *might* be catalytically essential. More definitive carboxyl-group modification procedures in which protein carboxyl groups are simply reduced to carbinol groups ($-COOH \rightarrow -CH_2OH$) are being actively pursued in our laboratory (Hall and Perfetti, 1974), and it is anticipated that this approach will permit a more confident assessment of the role of carboxyl groups in papain catalysis.

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[5] The low thiol titer in this case is attributed not to any derivatization by EDC, but to irreversible oxidations of the thiol group during the extended manipulations to which the protein had been subjected prior to the thiol assay.

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D-Mannitol Dehydrogenase from *Absidia glauca*. Purification, Metabolic Role, and Subunit Interactions†

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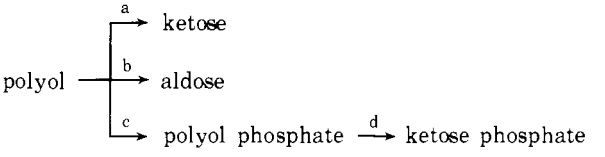
ABSTRACT: When *Absidia glauca* was grown in minimal media with D-mannitol as the only source of carbon, an NAD^+ specific D-mannitol dehydrogenase (EC 1.1.1.67) was induced. The crude extract also gave evidence of mannitol kinase, mannitol-1-phosphate dehydrogenase, phosphofructokinase, and L-iditol dehydrogenase activity. The heat labile purified preparation was judged enzymically homogeneous based on evidence derived from substrate specificity studies and activity staining, following disc gel electrophoresis. The enzymic monomer, with a weight of about 67 000 daltons, slowly polymerizes when stored at −20 °C, giving a multiplicity of protein bands on electrophoresis distributed predominantly across a spectrum from dimer to pentamer, with enzymic activity resident predominantly in even multiples of the monomer. Depolymerization occurred rapidly (hours) when a frozen preparation was brought to and held between 4 and 20 °C. Aggregate fragmentation with sodium dodecyl sulfate showed a time-temperature dependence, terminating in a subunit component of 13 000 daltons. pH optimum for polyol oxidation occurs at 9.6 (NaOH–glycine buffer) while ketose reduction proceeded most rapidly at pH 7.0–7.2 (phosphate buffer). A regulatory role is suggested for this enzyme based on dead-end inhibition by mannitol 1-phosphate, multiple enzyme forms, and its locus at the initiation site for mannitol utilization. The physiological relevance of low-temperature aggregation to regulatory control remains to be established.

While metabolic pathways for polyol utilization and biosynthesis are well established in a variety of plants, animals, and microorganisms (Touster and Shaw, 1962; Touster, 1974) and studies of mannitol utilization have generated key experiments in bacterial genetics and physiology (Berkowitz, 1971), less attention has been directed to the detailed mechanisms of metabolic control which regulate their entrance into the carbohydrate pathway.

As Touster (1974) has indicated, the utilization of polyols by living organisms is initiated by one of the following reactions:



involving (a) oxidation to a ketose; (b) oxidation to an aldose; (c) phosphorylation to the corresponding phosphate. While the direct phosphorylation route c has not been encountered in mammals, the catabolic fate of the phosphate in other tissues is associated frequently with an NAD^+ (or $NADP^+$) linked dehydrogenase (d). However, as Strandberg (1969) has indicated, the presence of two dehydrogenases (a, d) in extracts from mannitol-grown cells of *Aspergillus candidus* has posed difficulties in attempting to assemble a rational picture for mannitol utilization–biosynthesis in this organism.

Boulter and Derbyshire (1957), using conventional and

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