(4) P. L. Hall, B. L. Kaiser, and E. T. Kaiser, *J. Am. Chem. Soc.*, **91**, 485 (1969).
(5) D. S. Auld and B. L. Vallee, *Biochemistry*, **9**, 4352 (1970).
(6) D. S. Auld and B. L. Vallee, *Biochemistry*, **10**, 2892 (1971).
(7) D. S. Auld and B. Holmquist, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **31**, 435 Abs (1972).
(8) J. W. Bunting, J. Murphy, C. D. Myers, and G. G. Cross, *Can. J. Chem.*, **52**, 2648 (1974).
(9) E. P. Kang, C. B. Storm, and F. W. Carson, *J. Am. Chem. Soc.*, **97**, 6723 (1975).
(10) J. Suh and E. T. Kaiser, *J. Am. Chem. Soc.*, **98**, 1940 (1976).
(11) J. W. Bunting and S. S.-T. Chu, *Biochemistry*, **15**, 3237 (1976).
(12) E. T. Kaiser, T.-W. Chan, and J. Suh in "Protein-Metal Interactions", M. Friedman, Ed., Plenum Press, New York, N.Y., 1974, pp 49–80.
(13) For a full discussion of this problem see ref 8. The most recent reasonable assignments of pK_a values are given in ref 10.
(14) M. L. Bender and F. J. Kezdy, *Annu. Rev. Biochem.*, **34**, 49 (1965).
(15) J. F. Riordan, *Biochemistry*, **12**, 3915 (1973).
(16) D. S. Auld and B. Holmquist, *Biochemistry*, **13**, 4355 (1974).
(17) L. G. Lange, D. S. Auld, and J. F. Riordan, *Biochemistry*, **13**, 1983 (1974).
(18) J. W. Bunting and C. D. Myers, *Can. J. Chem.*, **53**, 1993 (1975).
(19) Glycolic, L-lactic, D- and DL-mandelic, and L-phenyllactic acids were obtained commercially. DL-3-Phenyllactic and DL-2-hydroxy-4-phenylbutanoic acids were prepared by acidic hydrolysis of the cyanohydrins of phenylacetaldehyde and 3-phenylpropionaldehyde, respectively. Other 2-hydroxy acids were prepared from the commercially available 2-bromo acids.[20]
(20) J. Murphy and J. W. Bunting, *Can. J. Chem.*, **53**, 283 (1975).
(21) Our previous report[8] that the sodium salt of **2** (R = C_6H_5) is insoluble in water at these concentrations is in error. The acetone procedure described herein was not used previously. Our earlier work was complicated by the very low aqueous solubility of the free acid of this ester.
(22) J. W. Bunting and J. Murphy, *Can. J. Chem.*, **52**, 2640 (1974).
(23) G. N. Wilkinson, *Biochem. J.*, **80**, 324 (1961).
(24) J. W. Bunting and C. D. Myers, *Can. J. Chem.*, **51**, 2639 (1973).
(25) J. W. Bunting and C. D. Myers, *Can. J. Chem.*, **52**, 2053 (1974).
(26) J. W. Bunting and C. D. Myers, *Can. J. Chem.*, **53**, 1984 (1975).
(27) S. S.-T. Chu, M.Sc. Thesis, University of Toronto, 1974.
(28) D. S. Auld and B. L. Vallee, *Biochemistry*, **9**, 602 (1970).
(29) L. D. Byers and R. Wolfenden, *Biochemistry*, **12**, 2070 (1973).
(30) C. Hansch, *Acc. Chem. Res.*, **2**, 232 (1969).
(31) A. Leo, C. Hansch, and D. Elkins, *Chem. Rev.*, **71**, 525 (1971).
(32) C. Tanford, *J. Am. Chem. Soc.*, **84**, 4240 (1962).
(33) P. Mukerjee, *Adv. Colloid Interface Sci.*, **1**, 241 (1967).
(34) J. Murphy and J. W. Bunting, *Can. J. Chem.*, **52**, 3829 (1974).
(35) W. N. Lipscomb, G. N. Reeke, Jr., J. A. Hartsuck, F. A. Quiocho, P. H. Bethge, M. L. Ludwig, T. A. Steitz, H. Muirhead, and J. C. Coppola, *Brookhaven Symp. Biol.*, **21**, 24 (1968).
(36) W. N. Lipscomb, G. N. Reeke, Jr., J. A. Hartsuck, F. A. Quiocho, and P. H. Bethge, *Philos. Trans. R. Soc. London, Ser. B*, **257**, 177 (1970).
(37) The current status of the acyl-enzyme intermediate hypothesis for carboxypeptidase A is summarized by Suh and Kaiser[10] and Breslow and Wernick.[38]
(38) R. Breslow and D. Wernick, *J. Am. Chem. Soc.*, **98**, 259 (1976).

Intramolecular Catalysis of Acylation and Deacylation in Peptides Containing Cysteine and Histidine

Michael J. Heller, Joseph A. Walder, and Irving M. Klotz*

Contribution from the Department of Biochemistry and Molecular Biology, and the Department of Chemistry, Northwestern University, Evanston, Illinois 60201.
Received August 27, 1976

Abstract: A series of peptides having various arrangements of cysteine and histidine was synthesized to determine the potential of thiol–imidazole intramolecular systems for catalysis of hydrolytic reactions. With substituted phenyl acetates as substrates, no cooperative effect of histidine was observed for the acylation of the cysteine residue. In the deacylation of the S-acylated peptides, again the intramolecular histidine had little or no effect. However, in the presence of the thiol specific reagents, 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) and *N*-ethylmaleimide, the rates of appearance of free SH from S-acylated peptides were much greater than the observed deacylation rates in the absence of these reagents. For the peptide N-Ac-Gly-Arg-Phe-Cys(Ac)-Phe-His-Gly-COOH, the rate constant for the appearance of free SH in the presence of Ellman's reagent was 3.1×10^{-2} min^{-1} under conditions for which the observed deacylation rate was 3.2×10^{-4} min^{-1} (pH 8.1, 25 °C). This trapping effect was not observed for cysteine peptides lacking histidine or for mixtures of *N,S*-diacetylcysteine and imidazole free in solution. These observations indicate the existence of a rapid, reversible intramolecular transfer of the acetyl group between the cysteine and histidine residues which greatly favors cysteine. A mechanism inhibiting back-attack by cysteine of the acyl histidine would allow efficient deacylation to occur through intramolecular nucleophilic catalysis by the imidazole group. The cysteine proteases may have evolved such a mechanism.

There exists in nature a homologous series of proteolytic enzymes having cysteine and histidine at their active sites. In these enzymes, such as papain, the thiol group of cysteine serves as the primary nucleophile, cleaving the acyl linkage of the substrate with the resultant formation of an acyl-thiol intermediate. The imidazole moiety of histidine is thought to assist in this process as well as in the deacylation of the acyl-enzyme required to regenerate the catalyst.[1]

A number of model thiol–imidazole intramolecular systems have been studied previously in efforts to demonstrate the catalytic ability of this combination for hydrolytic reactions. With cysteine–histidine peptides,[2-6] little or no assistance by imidazole has been found for the nucleophilic attack of the SH group on *p*-nitrophenyl acetate. With the same substrate, only a sixfold increase in rate was observed[6] for the reaction with 4-(2-mercaptoethyl)imidazole over that with glutathione.

For the deacylation step the largest acceleration reported was 30-fold, found for S-Ac-*cyclo*-(Cys-His) compared with ethyl thiolacetate.[6] However, as shown below, when comparisons are made to more appropriate models lacking imidazole, little if any of this effect can be attributed to the imidazole group.

Thus, there has yet to be demonstrated any significant cooperative effect in the catalysis of hydrolytic processes by thiol–imidazole intramolecular systems as is thought to occur in the cysteine proteases. Since in certain of these model systems the imidazole and SH groups are in extremely close proximity, interactions with other functional groups must be required for the imidazole moiety to exert its catalytic effects. With this in mind we studied a series of cysteine–histidine peptides having other functional groups, looking not only for overt catalytic efficacy, but also for potential catalytic effects with thiol-specific trapping reagents. Our results show the nature and magnitude of the intramolecular effects that do

occur within the peptides and explain why no significant cooperative effects were observed in the systems studied by other workers.

Experimental Section

Materials. *N*-*t*-Boc-N^{im}-Tos-L-His, *N*-*t*-Boc-*S*-*p*-MeOBzl-L-Cys, *N*-*t*-Boc-N^{g}-NO_2-L-Arg as well as all other Boc-L-amino acids were obtained from Beckman Instruments, Inc. Bio-Beads S-X-1 chloromethylated resin was purchased from Bio-Rad Laboratories. *N*-Acetylcysteine, imidazole, *p*-nitrophenyl acetate, and *N*-ethylmaleimide were the products of Sigma Chemical Co. Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid)] was obtained from Pierce Chemical Co., and phenyl acetate from Aldrich Chemical Co.

p-Chlorophenyl acetate was prepared from the reaction of 1 equiv of *p*-chlorophenol, 1 equiv of acetyl chloride, and 1 equiv of pyridine for 1 h at 25 °C. Cold ethyl ether was added and the precipitated pyridinium chloride was filtered off. The ether was evaporated under reduced pressure. The product, *p*-chlorophenyl acetate, revealed only one component in chromatographic analysis on silica gel plates with the solvent mixture, benzene–dioxane–acetic acid (18:2:1). Spectrophotometric analysis at 280 nm, before and after complete hydrolysis, indicated the product was >95% *p*-chlorophenyl acetate.

Peptide Synthesis. The peptides, N-Ac-Gly-Arg-Phe-Cys-Phe-His-Gly-COOH (I), N-Ac-Gly-His-Phe-Cys-Phe-His-Gly-COOH (II), N-Ac-His-Pro-Cys-Pro-His-Gly-COOH (III), N-Ac-Cys-Gly-His-Gly-COOH (IV), and N-Ac-Gly-Cys-Gly-COOH (V), were synthesized by the Merrifield solid-phase method.[7a] Boc-glycine, the carboxyl terminal amino acid, was esterified to S-X-1-chloromethylated resin (1.3 mequiv of Cl/g of resin) by refluxing in ethanol with triethylamine for 8 h. The amount of Boc-glycine esterified was 0.1 mequiv of amino acid/g of resin. *tert*-Butoxycarbonyl (*t*-Boc) groups were removed by treatment with 30% (v/v) of trifluoroacetic acid in chloroform for 30 min. Couplings of successive amino acids were done in methylene chloride using a six- to eightfold excess of Boc-amino acid and of dicyclohexylcarbodiimide above the amount of the first amino acid coupled to the resin. Each coupling step was checked for completeness by mixing a small amount of peptide–resin with 2,4,6-trinitrobenzenesulfonic acid in buffer solution (Bistris, 0.02 M, pH 8.0). If no orange color was detectable within 2 min, coupling was assumed complete. The N-terminal group of all completed peptides was acetylated by dicyclohexylcarbodiimide coupling with acetic acid.

The completed N-acetylated peptides were cleaved from the resin with CoF_3-dried hydrogen fluoride. About 15 mL of HF and 2 g of peptide/resin were mixed for 30 min at 0 °C. Anisole was added to protect sensitive amino acids during the HF cleavage. The cleaved peptide/resin mixture was thoroughly dried by vacuum to remove HF and anisole. The peptide/resin was then washed several times with ethyl acetate to remove organic impurities and again dried in vacuum. The peptides were extracted from the resin with a small amount of 1% acetic acid–water solution. The resin was then removed by filtration and the resulting solution lyophilized.

The peptides were analyzed by thin-layer chromatography on silica gel plates in butanol–acetic acid–water (8:2:2) and in 2-butanone–acetic acid–water (1:3:2.5). The chromatograms were sprayed with Pauly reagent (for histidine) to visualize the peptides. The peptides were pure by chromatographic analysis. Chromatograms of peptides were also sprayed with ninhydrin, and the results established that N-terminal acetylation was complete. Histidine content of the peptides was determined spectrophotometrically, with a Beckman DU spectrophotometer set at 500 nm, by means of the Pauly reaction.[7b] Cysteine assays used Ellman's reagent[8] and the spectrophotometer set at 412 nm. Arginine was determined qualitatively by the Sakaguchi test.[7b] Amino acid analyses, with a Durrum D-500 analyzer, were carried out on acid (HCl) hydrolysates of peptides I–IV. The observed values were in good agreement with those expected from the sequence shown in Table I. Cysteine was not determined in the amino acid analyzer.

Kinetics. For kinetic determinations all buffers were made fresh and degassed. All samples and reaction mixtures were kept under argon to minimize oxidation of the thiol group.

The pK_a of the cysteine SH group within the peptides was determined from the difference spectra at 230 nm as a function of pH, according to the method of Donovan.[9]

The catalyzed hydrolysis of substituted phenyl acetates was studied with imidazole, with *N*-acetylcysteine and with each of the peptides I–IV. The substrates employed were *p*-nitrophenyl acetate, *m*-nitrophenyl acetate, *p*-chlorophenyl acetate, and phenyl acetate. Their cleavage reactions were followed with a Beckman DU spectrophotometer measuring the release of the corresponding phenol at 400, 400, 280, and 280 nm, respectively. Second-order rate constants for the acylation process were determined from the initial rates according to

$$k_a = \frac{\text{initial rate} - \text{spontaneous rate}}{[\text{catalyst}][\text{substrate}]} \quad (1)$$

The nitrophenyl esters were examined in the pH range 7.5–8.5 (in 0.01 M Bistris buffer with 0.1 M NaCl), *p*-chlorophenyl acetate and phenyl acetate in the pH range 9.0–9.5 (in 0.01 M Tris buffer with 0.1 M NaCl). The concentration of the ester was 6–8 × 10^{-4} M and the concentration of potential catalyst 3–5 × 10^{-4} M. All experiments were performed at 25 °C.

The deacylation studies were carried out in 0.02–0.07 M borate buffer containing 0.1 M NaCl and in 0.02 M Tris buffer with 0.1 M NaCl, buffers covering the pH range of 8 to 10. The peptides, at a concentration of approximately 5 × 10^{-5} M, were acetylated with a 10- to 15-fold excess of acetic anhydride (at the pH at which deacylation was to be followed), injected as a 10% solution in acetonitrile. The volume of acetonitrile in the final solution was <0.2%. The acetylations of the imidazole group and the thiol group were observable at 270 and 235 nm, respectively (Figure 1). The subsequent deacylation of the acetylimidazole was followed to completion with observations at 270 nm. The excess acetic anhydride was hydrolyzed sufficiently faster than the acetylimidazole to permit calculation of

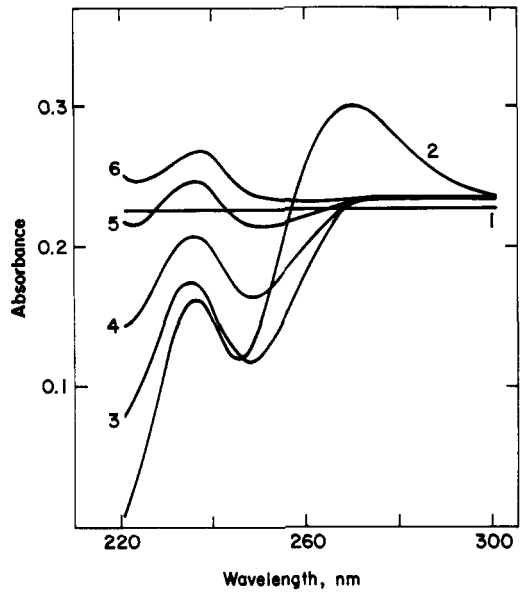


Figure 1. (1) Ultraviolet difference spectrum with peptide I at about 1 × 10^{-4} M in both sample and reference cells (1 cm, 3 mL volume); 0.07 M borate–0.1 M NaCl buffer, pH 9.6, at 25 °C; (2) difference spectrum recorded about 30 s after injection of 4 μL of 10% acetic anhydride (in acetonitrile) into the sample cell; (3) same solution as in (2) recorded 30 min after acetylation; the absorbance near 260 nm was completely gone after about 10 min; (4) 80 min after acetylation; (5) 155 min after acetylation; (6) 215 min after acetylation.

Table I. Amino Acid Composition of Peptides

	I		II		III		IV	
	Obsd	Theor	Obsd	Theor	Obsd	Theor	Obsd	Theor
Gly	2.0	2.0	2.0	2.0	1.0	1.0	2.0	2.0
His	1.0	1.0	2.2	2.0	2.1	2.0	1.1	1.0
Phe	1.9	2.0	2.1	2.0				
Arg	1.2	1.0						
Pro					2.2	2.0		

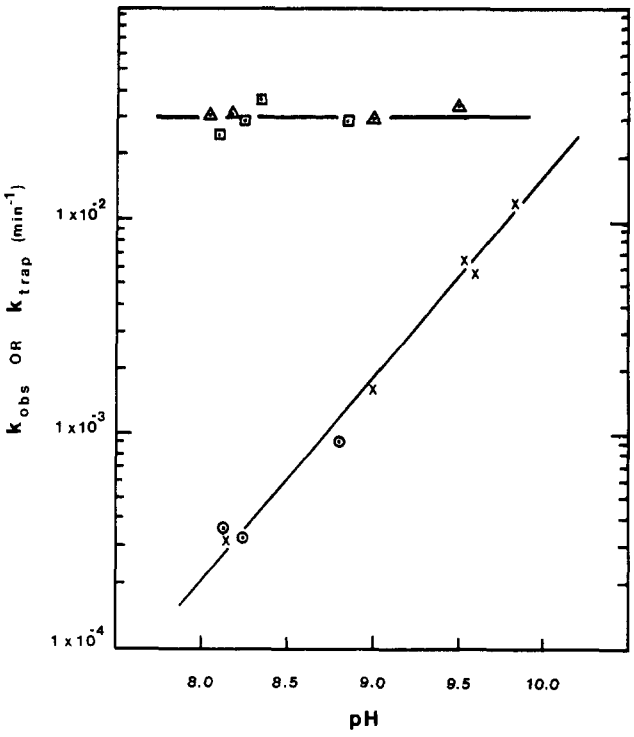


Figure 2. Sloped line shows k_{obsd} for the deacylation of S-acetylated peptide I as a function of pH, determined in 0.02 M borate–0.1 M NaCl buffer (X) and in 0.02 M Tris–0.1 M NaCl buffer (⊙) at 25 °C. The upper line shows k_{trap} for S-acetylated peptide I in the presence of Ellman's reagent as a function of pH, determined in borate–NaCl buffer (△) and in Tris–NaCl buffer (⊡) at 25 °C.

the deacylation rate for the acetylimidazole. The deacylation of the acyl-thiol (which had progressed <5%) was then followed at 235 nm. Deacylation of the acyl-thiol was also followed with Ellman's reagent which reacts rapidly with free thiol groups. Aliquots of the S-acetylated peptides were removed every 30 min and a fourfold excess of Ellman's reagent over the total thiol concentration was added. Immediately thereafter the absorbance at 412 nm, due to the highly colored thionitrobenzoate anion, was measured. When the pH was greater than 9, first-order deacylation rate constants were determined from an appropriate semilogarithmic plot of optical density difference vs. time. When the pH was less than 9, deacylation was slow and could not be followed to completion because of oxidation of the free SH. Under these conditions, rate constants were determined from the initial rates. In addition, since the endpoints could be accurately determined from reaction of the free peptide with Ellman's reagent, first-order plots were constructed. These were found to be linear, even beyond the first half-life.

In the trapping studies, rates for the appearance of SH from the S-acetylated peptides in the presence of either Ellman's reagent or *N*-ethylmaleimide were determined as follows. Acetylation of the peptides by acetic anhydride was carried out as described above. After the hydrolysis of the acetylimidazole was complete, either Ellman's reagent (in 5–30-fold excess over total thiol) or *N*-ethylmaleimide (in 2–3-fold excess) was added. With Ellman's reagent, the increase in absorbance at 412 nm was followed, with the maleimide the decrease at 232 nm. *N*-Ethylmaleimide was not used for studies at pH greater than 8.0 because of the spontaneous hydrolysis of this reagent to *N*-ethylmaleamic acid. The trapping rates were first order in S-acetylated peptide. With *N*-ethylmaleimide the first-order rate constant, k_{trap}, was determined from semilogarithmic plots of optical density differences vs. time. With Ellman's reagent, k_{trap} had to be determined from initial rates as the thionitrobenzoate anion tends to be autooxidized,[8] and the absorbance at 412 nm is reduced. Nevertheless, since the endpoints could be determined accurately by reaction of the free peptide with Ellman's reagent, first-order plots were made, and these were found to be linear past the first half-life.

Results

The reactions of each of the peptides I–IV, of *N*-acetylcysteine, and of imidazole with the substrates *p*-nitrophenyl acetate, *m*-nitrophenyl acetate, *p*-chlorophenyl acetate, and phenyl acetate were studied over a pH range of 7.5 to 9.5. None of the peptides was acetylated at a rate significantly greater than that of *N*-acetylcysteine with any of the above substrates. The second-order rate constants for the acetylation of peptides I–IV, *N*-acetylcysteine, and imidazole by *p*-chlorophenyl acetate at pH 9.0 are given in Table II, along with the pK_a values for the cysteine SH groups.

Figure 1 shows the spectral changes associated with the acetylation of peptide I with acetic anhydride. The absorption maxima at 270 and 235 nm are due to the formation of the acetylimidazole and the *S*-acetylcysteine moieties, respectively. The deacylation rate constants for peptide I (at pH 9.6 and 25 °C), obtained by following the decrease in intensity at the absorption maxima, were 0.2 min^{-1} for the acetylimidazole

Table II. Second-Order Rate Constants for the Reaction of *p*-Chlorophenyl Acetate with Peptides, *N*-Acetylcysteine, and Imidazole

Compound	Concn,[a] M × 10^4	k_a, M^{-1} min^{-1} [b]	pK_a (SH)[c]
Imidazole	5.0	2	
N-Acetylcysteine	4.7	14	9.5
N-Ac-Gly-Arg-Phe-Cys-Phe-His-Gly-COOH (I)	3.6	22	8.8
N-Ac-Gly-His-Phe-Cys-Phe-His-Gly-COOH (II)	3.6	16	8.8
N-Ac-His-Pro-Cys-Pro-His-Gly-COOH (III)	3.6	17	8.8
N-Ac-Cys-Gly-His-Gly-COOH (IV)	3.6	12	8.7

[a] Concentration of peptides and of *N*-acetylcysteine was determined spectrophotometrically at 412 nm from reaction with Ellman's reagent. Concentration of substrate was 6 × 10^{-4} M. All reactants were in 0.02 M Tris–0.01 M NaCl buffer, pH 9.0, at 25 °C. [b] Determined from initial rate of appearance of *p*-chlorophenol at 280 nm. Spontaneous rate of hydrolysis for *p*-chlorophenyl acetate under these conditions was 1.45 × 10^{-3} min^{-1}. Rate constant for alkaline hydrolysis was calculated to be 145 M^{-1} min^{-1}, in good agreement with 132 M^{-1} min^{-1} reported by Kirsch and Jencks.[11] k_a calculated for total thiol. [c] N-acetylcysteine pK_a from Friedman et al.;[12] those for peptides determined by spectrophotometric titration method of Donovan.[9]

Table III. Rate Constants for the Deacylation (Hydrolysis) of *S*-Acetyl-Peptides, Thiol Esters, and Intramolecular Model Systems

Compounds	k_d, M^{-1} min^{-1} [a]	Conditions[b]
N-Ac-Gly-Arg-Phe-Cys(Ac)-Phe-His-Gly-COOH (I)	180	pH 9.5, 25 °C
N-Ac-Gly-His-Phe-Cys(Ac)-Phe-His-Gly-COOH (II)	170	pH 9.5, 25 °C
N-Ac-Cys(Ac)-Gly-His-Gly-COOH (IV)	75	pH 9.5, 25 °C
N-Ac-Gly-Cys(Ac)-Gly-COOH (V)	31	pH 9.4, 25 °C
N,S-Diacetylcysteine	12	pH 10.5, 25 °C
$CH_3COSC_2H_5$[6,13]	1.5	20 °C
CH_3COSCH_3[6,13]	2.4	20 °C
Imidazole-CH_2SCOCH_3[6]	9.5	pH 11.0, 24 °C
Imidazole-$(CH_2)_2SCOCH_3$[6]	6.0	pH 11.1, 24 °C
S-Ac-*cyclo*-(Cys-His)[6]	47.0	pH 11.0, 24 °C

[a] k_d is $k_{obsd}/(OH^-)$, k_{obsd} being determined by Ellman's reagent. [b] Deacylation rates of *S*-acetyl-peptides, and of *N,S*-diacetylcysteine were determined in 0.07 M borate–0.1 M NaCl buffer. Conditions and methods for thiol esters given by Rylander and Tarbell,[13] and those for imidazole–SH model systems and the cyclic peptide given by Schneider.[6]

Table IV. Rate Constants for Trapping Reactions with Ellman's Reagent [5,5′-Dithiobis(2-nitrobenzoic acid)] Compared with Deacylation (Hydrolysis) Rates in Absence of Trapping Reagent

S-Acetylated compound	k_{trap}, min^{-1} [a]	k_{obsd}, min^{-1}	Conditions[b]
N-Ac-Gly-Arg-Phe-Cys(Ac)-Phe-His-Gly-COOH (I)	3.1×10^{-2}	3.6×10^{-4}	pH 8.05, 25 °C
N-Ac-Gly-His-Phe-Cys(Ac)-Phe-His-Gly-COOH (II)	3.5×10^{-2}	3.5×10^{-4}	pH 8.05, 25 °C
N-Ac-Cys(Ac)-Gly-His-COOH (IV)	9.0×10^{-3}	1×10^{-4}	pH 8.4, 25 °C
N-Ac-Gly-Cys(Ac)-Gly-COOH (V)	$<1 \times 10^{-4}$ [c]	$\sim 6 \times 10^{-5}$	pH 8.1, 25 °C
N,S-Diacetylcysteine + imidazole	$<1 \times 10^{-4}$ [c]	$\sim 2 \times 10^{-5}$	pH 8.4, 25 °C

[a] k_{trap} determined from the rate of appearance of thionitrobenzoate anion at 412 nm. [b] Trapping reactions were carried out in 0.02 M Tris–0.1 M NaCl buffer with $2-3 \times 10^{-4}$ M Ellman's reagent, $5-7 \times 10^{-5}$ M S-acetylated peptides, and 8×10^{-5} M *N,S*-diacetylcysteine and imidazole. Spontaneous deacylation, k_{obsd}, determined under approximately the same conditions. [c] In these cases, the trapping rates were no different than the rate observed for spontaneous hydrolysis of Ellman's reagent under the same conditions. This was about $\sim 3 \times 10^{-4}$ min^{-1}. The limits of experimental error in the trapping experiments were about $\pm 1 \times 10^{-4}$ min^{-1}; therefore, values for V and *N,S*-diacetylcysteine were $\leq 1 \times 10^{-4}$ min^{-1}.

group and 6×10^{-3} min^{-1} for the *S*-acetylcysteine moiety. At pH 8.1 (in 0.02 M Tris buffer with 0.1 M NaCl, at 26 °C), the deacylation rate constant of acetylimidazole in peptide I was 4.6×10^{-2} min^{-1}.

The deacylation rate of the S-acetylated peptide I was determined from pH 8 to 10 in both Tris and borate buffers. The pseudo-first-order rate constants, k_{obsd}, were linear in (OH^-) over this entire pH range (Figure 2). Table III presents the second-order rate constants, $k_{obsd}/(OH^-)$, for the S-acetylated peptides I–IV and for several reference thiol esters and other S-acetylated thiol–imidazole intramolecular systems. The rate constants for deacylation as determined by following the absorbance at 235 nm and with Ellman's reagent are in good agreement.

The results of the sulfhydryl trapping experiments for the S-acetylated peptides with Ellman's reagent are given in Table IV. The first-order rate constants, k_{trap}, for the reaction with Ellman's reagent are compared with k_{obsd} for the deacylation of the S-acetylated peptides under the same conditions but in the absence of Ellman's reagent. In the trapping studies the concentration of Ellman's reagent was varied from 5- to 30-fold above the peptide concentration, and the trapping rates were independent of concentration of this reagent. The trapping effect was observed with all histidine–cysteine peptides, but not with *N,S*-diacetylcysteine, *S*-acetyl peptide V without histidine, or mixtures of imidazole and *N,S*-diacetylcysteine. The upper line in Figure 2 shows a plot of k_{trap} determined with Ellman's reagent for peptide I in both Tris and borate buffers from pH 8.1 to 9.5 at 25 °C. The trapping rate was independent of pH over this entire range. To rule out the possibility that the trapping effect was specific to Ellman's reagent, *N*-ethylmaleimide, another thiol specific reagent, was also used. The results with this reagent are given in Table V. Again the trapping effect was observed, and only with the cysteine–histidine peptides.

Table V. Rate Constants for Trapping Reactions with *N*-Ethylmaleimide (NEM) and S-Acetylated Peptides

S-Acetylated compounds[a]	k_{trap}, M^{-1} min^{-1} [b]
N-Ac-Gly-Arg-Phe-Cys(Ac)-Phe-His-Gly-COOH (I)	1.3×10^{-2}
N-Ac-Cys(Ac)-Gly-His-Gly-COOH (IV)	5.0×10^{-3}
N,S-Diacetylcysteine + imidazole	$<1 \times 10^{-3}$ [c]

[a] Concentration of NEM was $\sim 1.5 \times 10^{-4}$ M, S-acetylated peptides $\sim 7-8 \times 10^{-5}$ M, and *N,S*-diacetylcysteine and imidazole $\sim 1 \times 10^{-4}$ M. All reactants were in 0.05 M Bistris–0.1 M NaCl buffer at pH 7.7 and 25 °C. [b] k_{trap} determined from rate of decrease in absorption at 232 nm. [c] Trapping rate for *N,S*-diacetylcysteine and imidazole was not different from rate of spontaneous hydrolysis of *N*-ethylmaleimide which was $\sim 1.5 \times 10^{-3}$ min^{-1}.

Discussion

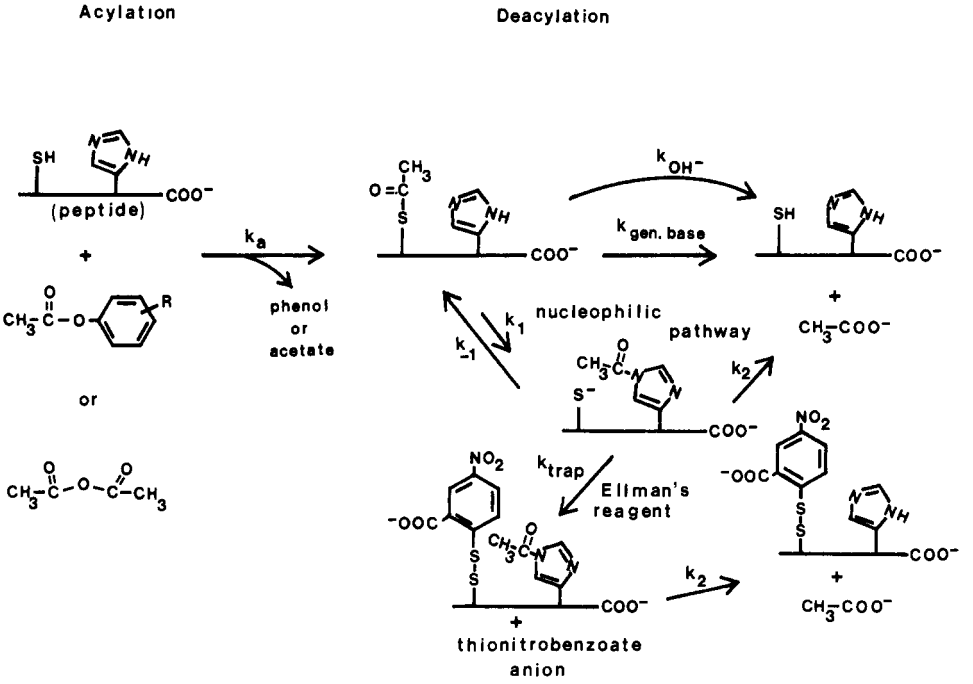
The invariance of histidine at the active site of the cysteine proteases, such as papain,[1,14,15,16] ficin,[17] and bromelain,[18] indicates that imidazole plays an important role in the catalytic processes performed by these enzymes. The failure of earlier investigations[2-6] to demonstrate a significant cooperative effect between the thiol group and the imidazole in even extremely well-approximated intramolecular systems can only reflect the fact that interactions with several functional groups are required for imidazole to exert its catalytic effects. In all the peptides we have examined the imidazole moiety of histidine is adjacent to the C-terminal carboxyl group of glycine, similar in its relationship to that of histidine and aspartic acid in the serine protease, chymotrypsin.[19] In addition, peptide I contains the cationic guanidinium group of arginine, peptide II has histidine on either side of the thiol group, and peptide III has proline which produces a bend in the secondary structure of the polypeptide chain to give a better approximation of the SH and imidazole groups.

None of these arrangements manifested a kinetically significant general base assistance by imidazole of the nucleophilic attack of the thiol group on substituted phenyl acetates.

These results were not totally unexpected for the following reason. The rate of the general base catalyzed reaction of the SH group must be less than the rate of the reaction of S^-. At most, therefore, general base catalysis can increase that rate of the nucleophilic attack of the thiol group by a factor of $1/\alpha$ where α is the fraction of the peptide in the S^- form. Since the pK_a of the SH group within the peptides is approximately 8.7, the largest possible acceleration in the pH range we examined, 7–10, is less than 100-fold. Below this pH range the imidazole group becomes protonated and its catalytic effect is abolished. To obtain a value for the acceleration in rate of the general base catalyzed pathway over that for some reference thiol in the fully protonated state, the rate data would generally be extrapolated to zero ionization of the thiol ($\alpha = 0$). However, as such extrapolated rates are very small and the nucleophilic contribution of imidazole not resolvable, this procedure is quite unreliable. Furthermore, such a calculated acceleration would not be a contributing acceleration in the pH range of interest, 7–8.

This same argument raises doubts about the general base role assigned to imidazole in the acylation step of the cysteine proteases.[1] Rather the major function of the imidazole moiety in acylation may be to properly align the SH group, instead of to assist in the deprotonation of the SH in the transition state.

Scheme I



In any event, the interactions necessary for imidazole to assist in the acylation of a thiol have yet to be demonstrated in model systems.

In the deacylation process (Scheme I), we have found for the S-acetylated peptides accelerations as large as approximately 120-fold above the deacylation rate reported for ethyl thiolacetate (Table III). Schneider[6] observed an acceleration of about 30-fold for the deacylation of S-Ac-*cyclo*-(Cys-His) compared with the same reference compound. However, the amide bonds in cysteine peptides are electron withdrawing and in themselves should labilize the thiol ester. Consequently a reference compound more appropriate than ethyl thiolacetate is required. For such, we have chosen the peptide N-Ac-Gly-Cys(Ac)-Gly-COOH(V). The deacylation rate of peptide V was found to be 20 times greater than that of ethyl thiolacetate. With peptide V as the reference compound, tha largest possible acceleration attributable to the imidazole moiety in S-Ac-*cyclo*-(Cys-His)[6] is only 1.5-fold, and in even our best peptide, I, only 5.6-fold. Thus, large variations in peptide structures show only small differences in the deacylation rates.

The imidazole group could, in principle, participate in the deacylation of the acyl-thiol through either a general base or nucleophilic mechanism (Scheme I). The deacylation rate of peptide I was found to be linear in (OH^-) between pH 8.0 and 10 (Figure 2) and was zero within experimental error when extrapolated to zero (OH^-) concentration. Since a general base pathway with imidazole should be independent of (OH^-) in this pH region, its contribution to the observed rate must be kinetically insignificant.

The contribution of the nucleophilic pathway to the observed deacylation rate can be best appreciated in the light of the trapping experiments with Ellman's reagent and *N*-ethylmaleimide. Both compounds are thiol specific reagents and have not been observed to react with acylated thiols. For every one of our S-acetylated thiol-imidazole peptides, however, the rate of appearance of free SH, k_{trap}, in the presence of either of these agents was much greater than the observed deacylation rate in the absence of the thiol specific reagent, k_{obsd} [approximately 100-fold greater at pH 8 (Tables IV and V)]. This trapping effect thus demonstrates the existence of a rapid transfer of the acetyl group from cysteine, not reflected in the observed rate of deacylation in solutions without the sulfhydryl reagents. Since a corresponding effect was not observed for N-Ac-Gly-Cys(Ac)-Gly-COOH(V) or for *N,S*-diacetylcysteine, the transfer in peptides I–IV must represent the intramolecular nucleophilic attack by imidazole on the acetylated thiol.

The rate (V_N) for the nucleophilic mechanism based on a steady-state assumption applied to the concentration of the imidazole-acetylated peptide (A) is given by

$$V_N = \frac{k_2 k_1}{k_{-1} + k_2}(\text{S-acetylated peptide}) \quad (2)$$

$$V_N = k_N(\text{S-acetylated peptide}) \quad (3)$$

where k_N is the effective first-order rate constant for the nucleophilic pathway and the other rate constants are defined in Scheme I. The term k_1(S-acetylated peptide) gives the rate of formation of A, and $k_2/(k_{-1} + k_2)$ represents the probability that A will hydrolyze to products rather than transacetylate to regenerate the S-acetylated peptide. Once the acetyl group is transferred to the imidazole moiety, the trapping reagent can react rapidly with the free thiol (Scheme I). Since the trapping rate was independent of the concentration of Ellman's reagent, k_{trap} with Ellman's reagent must equal k_1. Accordingly, k_{trap} with Ellman's reagent was found to be independent of (OH^-) from pH 8.0 to 10 (Figure 2).[20] For the S-acetylated peptide I, $k_1 = k_{trap} \simeq 3 \times 10^{-2}$ min^{-1}. Similar trapping rates were obtained for the other S-acetylated thiol-imidazole peptides (Table IV).

From eq 2 and 3, one can show that

$$k_{-1} = \frac{k_2(k_1 - k_N)}{k_N} \quad (4)$$

From eq 4 we can obtain a lower limit for k_{-1}. The rate of hydrolysis of the acetylimidazole moiety in thiol-imidazole diacetylated peptide I at pH 8.1 is 4.6×10^{-2} min^{-1}. Since the thiol group in this peptide is also acetylated, this observed rate is not rigorously k_2, but if it is at all different it should be less than k_2, as the free thiol might be able to serve as a general acid-base catalyst. The value for k_N must be less than the observed deacylation rate constant, 3×10^{-4} min^{-1} at pH 8.1, for peptide I (Figure 2). Substituting these values into eq 4, we obtain about 5.0 min^{-1} as a lower limit for k_{-1} at pH 8.1.

From this result it is also possible to estimate the equilibrium constant $K_{SH\text{-}Im}$ for the transfer of the acetyl group from

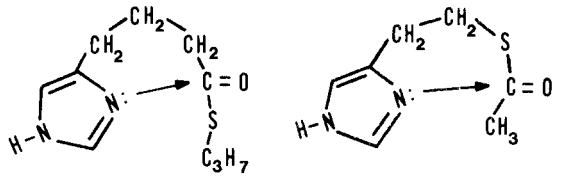
cysteine to histidine:

$$K_{SH\text{-}Im} = k_1/k_{-1} < 4.4 \times 10^{-3} \quad (5)$$

This value is consistent with that of about 2.7×10^{-4} calculated on the basis of the free energies of hydrolysis of ethyl thiolacetate and acetylimidazole.[21] Thus, the preequilibrium for acylimidazole formation is highly unfavorable. With increasing pH, $K_{SH\text{-}Im}$ increases attendant to the ionization of the SH group. Accordingly if k_{obsd} were equal to k_N the titration of the thiol group ($pK_a \sim 8.7$) would be manifested in the pH-rate profile for peptide I (Figure 2). Since it is not, k_N cannot contribute appreciably to k_{obsd}. In large measure, then, k_{obsd} appears to simply represent the hydroxide ion attack, k_{OH^-}, on the acyl-thiol, with subtle structural and electronic factors contributing to the slightly higher deacylation rates that are observed for the cysteine-histidine peptides over peptide V lacking histidine.

The trapping studies clearly demonstrate that if the back-attack of the thiol group were inhibited in cysteine-histidine peptides, intramolecular imidazole would be able to function effectively as a nucleophilic catalyst for the deacylation process. This conclusion is strongly supported by results from Bruice's[22a] study of the model compound *N*-propyl-γ-(4′-imidazolyl)thiolbutyrate (structure **1** in Scheme II). The rate

Scheme II



1

2

of hydrolysis of **1** at pH 7.0 is $\sim 10^5$ times faster than that of methyl thiolacetate and is limited by the hydrolysis of the intermediate lactam. In compound **1**, the thiol moiety is the leaving group and cannot reattack the acylimidazole that is formed.[22b] For the comparable intramolecular system in which back-attack is possible, the *S*-acetyl derivative of 4-(2-mercaptoethyl)imidazole (structure **2** in Scheme II), Schneider[6] observed little if any increase in the rate of deacylation over simple thiol esters. To inhibit the back attack of the thiol in an intramolecular catalytic system such as the cysteine-histidine peptides, one needs other functional groups, properly positioned to stabilize the thiol in an unreactive conformation while the imidazole is deacylated and to enhance the rate of hydrolysis of the acylimidazole. Although this type of mechanism might be difficult to attain synthetically, the cysteine proteases, such as papain, may have evolved a mechanism that embodies this effect.

That such reaction mechanisms can be achieved has been demonstrated by Fersht and Kirby[23] with the hydrolysis of aspirin-like compounds. They have pointed out[24] that in aspirin itself back-attack by the phenolate group on the intermediate anhydride prevents the ortho carboxyl group of aspirin from functioning effectively as a nucleophilic catalyst for aspirin hydrolysis. On the other hand, incorporation of a second carboxyl group to that of aspirin, giving 3-acetoxyphthalate,[23] allowed the nucleophilic pathway to function efficiently.

Prevailing theory holds that imidazole functions as a general base catalyst for the deacylation of the cysteine proteases.[1,25] However, significant general base catalysis by imidazole in the deacylation of an acyl-thiol has never been demonstrated in an intramolecular model system. On the other hand, it is clear from Bruice's studies[22a] of structure **1** (Scheme II) that in a well-approximated system in which back-attack by the thiol is inhibited, in this case because of the leaving group effect, nucleophilic catalysis by imidazole would result in a deacylation rate of approximately the same order of magnitude as occurs in the cysteine proteases. The main evidence which suggests general base catalysis in the proteases is a deuterium isotope effect of approximately 3 for the deacylation process.[26,27] However, this is also compatible with the nucleophilic mechanism: the nucleophilic attack by imidazole or the deacylation of the acylimidazole, or both, may be subject to general acid-base catalysis. Although one must proceed cautiously in extrapolating from model systems to an enzyme, the existing evidence would certainly favor nucleophilic catalysis over general base catalysis for the role of imidazole in the deacylation of the cysteine proteases.

Acknowledgment. This investigation was supported in part by grants from the National Science Foundation (No. BMS72-02204) and from the National Institute of General Medical Sciences, U.S. Public Health Service (No. GM 09280).

References and Notes

(1) G. Lowe, *Tetrahedron*, **32**, 291 (1976).
(2) K. Nakajima and K. Okawa, *Bull. Chem. Soc. Jpn.*, **46**, 1881 (1973).
(3) I. Photaki, V. Bardokos, A. W. Lake, and G. Lowe, *J. Chem. Soc.* C, 1860 (1968).
(4) I. Photaki and S. Moschopedis, *Experientia*, **25**, 903 (1969).
(5) F. Schneider, E. Schaich, and H. Wenck, *Hoppe-Seyler's Z. Physiol. Chem.*, **349**, 1525 (1968).
(6) F. Schneider, *Hoppe-Seyler's Z. Physiol. Chem.*, **348**, 1034 (1967).
(7) (a) J. M. Steward and J. D. Young, "Solid Phase Peptide Synthesis", W. H. Freeman, San Francisco, Calif., 1969; (b) *ibid.*, p 56.
(8) G. L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).
(9) J. W. Donovan, *Biochemistry*, **3**, 67 (1964).
(10) E. Roberts and G. Rouser, *Anal. Chem.*, **30**, 1291 (1958).
(11) J. F. Kirsch and W. P. Jencks, *J. Am. Chem. Soc.*, **86**, 837 (1964).
(12) M. Friedman, J. F. Cavins, and J. S. Wall, *J. Am. Chem. Soc.*, **87**, 3672 (1965).
(13) P. N. Rylander and D. S. Tarbell, *J. Am. Chem. Soc.*, **72**, 3021 (1950).
(14) J. Drenth, J. N. Jansonius, R. Koekoek, and B. G. Wolthers, "The Enzymes", Vol. III, P. D. Boyer, Ed., Academic Press, New York, N.Y., 1971, pp 485–499.
(15) S. S. Husain and G. Lowe, *Biochem. J.*, **108**, 855 (1968).
(16) S. S. Husain and G. Lowe, *Biochem. J.*, **108**, 861 (1968).
(17) S. S. Husain and G. Lowe, *Biochem. J.*, **117**, 333 (1970).
(18) S. S. Husain and G. Lowe, *Biochem. J.*, **117**, 341 (1970).
(19) D. M. Blow, *Acc. Chem. Res.*, **9**, 145 (1976). Although papain, like chymotrypsin, does have a carboxyl group (aspartate-158) in close proximity to the active-site histidine, the imidazole moiety of this histidine appears to be more closely approximated to an asparagine residue (see ref 14).
(20) k_{trap} with *N*-ethylmaleimide, pH 7.7, was approximately one-half the value for k_{trap} with Ellman's reagent, pH 8.0–10 (Tables IV and V). Although this difference may be due to differences in reaction conditions, saturation of the trapping rate was not demonstrated with *N*-ethylmaleimide, whereas it was with Ellman's reagent, and hence k_{trap} with *N*-ethylmaleimide may be less than k_1. Attack by Ellman's reagent of the acetylated thiol does not occur and hence cannot account for this difference.
(21) J. Gerstein and W. P. Jencks, *J. Am. Chem. Soc.*, **86**, 4655 (1964). The equilibrium constant $K_{SH\text{-}Im}$ was calculated using a value of 8.7 for the pK_a of the thiol group, as occurs in the peptides.
(22) (a) T. C. Bruice, *J. Am. Chem. Soc.*, **81**, 5444 (1959). (b) Bruice showed in this study, that if thiol at very high concentration is added to the reaction, the hydrolysis of compound **1** could be completely inhibited.
(23) A. R. Fersht and A. J. Kirby, *J. Am. Chem. Soc.*, **90**, 5833 (1968).
(24) A. J. Kirby and A. R. Fersht, *Prog. Bioorg. Chem.*, **1**, 11 (1971).
(25) A. N. Glazer and E. L. Smith, "The Enzymes", Vol. III, P. D. Boyer, Ed., Academic Press, New York, N.Y., 1971, pp 533–535.
(26) J. R. Whitaker and M. L. Bender, *J. Am. Chem. Soc.*, **87**, 2728 (1965).
(27) L. J. Brubacher and M. L. Bender, *J. Am. Chem. Soc.*, **88**, 5871 (1966).