Mechanism of Action of Cutinase: Chemical Modification of the Catalytic Triad Characteristic for Serine Hydrolases†

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ABSTRACT: Cutinase from *Fusarium solani* f. sp. *pisi* was inhibited by diisopropyl fluorophosphate and phenylboronic acid, indicating the involvement of an active serine residue in enzyme catalysis. Quantitation of the number of phosphorylated serines showed that modification of one residue resulted in complete loss of enzyme activity. One essential histidine residue was modified with diethyl pyrocarbonate. This residue was buried in native cutinase and became accessible to chemical modification only after unfolding of the enzyme by sodium dodecyl sulfate. The modification of carboxyl groups with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide in the absence of sodium dodecyl sulfate did not result in inactivation of the enzyme; however, such modifications in the presence of sodium dodecyl sulfate resulted in complete loss of enzyme activity. The number of residues modified was determined by incorporation of [^{14}C]glycine ethyl ester. Modification of cutinase in the absence of sodium dodecyl sulfate and subsequent unfolding of the enzyme with detergent in the presence of radioactive glycine ester showed that one buried carboxyl group per molecule of cutinase resulted in complete inactivation of the enzyme. Three additional peripheral carboxyl groups were modified in the presence of sodium dodecyl sulfate. Carbethoxylation of the essential histidine and subsequent incubation with the esterase substrate *p*-nitrophenyl [1-^{14}C]acetate revealed that carbethoxycutinase was about 10^5 times less active than the untreated enzyme. The acyl-enzyme intermediate was stabilized under these conditions and was isolated by gel permeation chromatography. The results of the present chemical modification study indicate that catalysis by cutinase involves the catalytic triad and an acyl-enzyme intermediate, both characteristic for serine proteases.

Cutinase, an extracellular esterase excreted by phytopathogenic fungi, catalyzes the hydrolysis of cutin, the structural polymer of the cuticle of higher plants. Cutin is a polyester composed mainly of hydroxy and epoxy fatty acids (Kolattukudy, 1980a,b, 1981). Cutinase, first isolated from *Fusarium solani* f. sp. *pisi* in homogeneous form (Purdy & Kolattukudy, 1975a), was recently purified from various phytopathogenic fungi (Soliday & Kolattukudy, 1976; Lin & Kolattukudy, 1980). This enyzme is involved in the penetration of fungal infection structures into plant cells and thus plays a crucial role in pathogenesis (Shaykh et al., 1977; Maiti & Kolattukudy, 1979; Köller et al., 1982).

Cutinase purified from *F. solani pisi* was suggested to be a serine esterase (Purdy & Kolattukudy, 1975b) on the basis that it contained an active serine residue exhibiting unusually high reactivity to compounds such as diisopropyl fluorophosphate. However, the presence of a catalytic triad, involving the hydroxyl group of serine, the imidazole group of histidine, and a carboxylic group, characteristic of serine hydrolases (Blow, 1976), has not been established. The basic mechanistic features established for the well-characterized serine proteases (Kraut, 1977) have been frequently suggested for serine esterases. However, in the absence of three-dimensional structures, which are available for the proteases, and in the absence of reliable chemical evidence for the presence of a catalytic triad, the participation of such a triad in serine esterases has not been established (Krisch, 1971; Rosenberry, 1975; Semeriva & Desnuelle, 1979). In this paper we present evidence for the existence of a catalytic triad consisting of an active serine, one essential histidine, and one essential carboxyl residue in cutinase and for the involvement of an acyl-enzyme intermediate in the catalytic mechanism of this enzyme.

Materials and Methods

Materials. [1-^{3}H(N)]Diisopropyl fluorophosphate (0.9 Ci/mmol), [1-^{14}C]glycine ethyl ester (50 Ci/mol), and [1-^{14}C]acetic anhydride (4 Ci/mol) were obtained from New England Nuclear. $NaDodSO_4$[1] was obtained from Pierce Chemical Co. All other chemicals were purchased from Sigma Chemical Co.

p-Nitrophenyl [^{14}C]acetate (PNA) was synthesized by esterification of *p*-nitrophenol (0.7 mmol) with 0.15 mmol of [1-^{14}C]acetic anhydride (4 mCi/mmol) in 5 mL of dry benzene containing 2% (v/v) pyridine for 15 h at 25 °C. PNA was purified by repeated thin-layer chromatography on silica gel G as described (Chapus et al., 1976). About 50 μmol of the ester with a specific radioactivity of 2 Ci/mol was obtained.

Cutinase. Cutinase was purified from the extracellular fluid of *F. solani* f. sp. *pisi* (isolate T-8 obtained from Professor H. D. van Etten of Cornell University) grown on cutin by a modification of the procedure previously described (Purdy & Kolattukudy, 1975a). Two serine esterases with cutinase activity, cutinase A (M_r 24 000) and cutinase B (M_r 21 500), were separated by hydrophobic interaction chromatography with octyl-Sepharose. Both enzymes were electrophoretically homogeneous after ion-exchange chromatography on SP-Sephadex (Purdy & Kolattukudy, 1975a). Cutinase B, which had a much higher specific activity than cutinase A with

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[1] Abbreviations: BSA, bovine serum albumin; cmc, critical micelle concentration; Dip-F, diisopropyl fluorophosphate; DEP, diethyl pyrocarbonate; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PNB, *p*-nitrophenyl butyrate; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; SP, sulfopropyl.

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radioactive cutin as a substrate, was very similar to cutinase I previously described (Purdy & Kolattukudy, 1975a,b). Cutinase B was used throughout this study.

Enzyme Assays. Esterase activity was assayed spectrophotometrically with *p*-nitrophenyl butyrate (PNB) as a model substrate (Purdy & Kolattukudy, 1975a). The assay mixture contained 0.13 M sodium phosphate buffer (pH 8.0), 0.4% (w/v) Triton X-100, and 1.3 mM PNB in a final volume of 3 mL. Cutinase activity was assayed with [^{14}C]cutin and [^{3}H]cutin according to the methods previously described (Purdy & Kolattukudy, 1973, 1975a).

Inactivation with Diisopropyl Fluorophosphate (Dip-F). Cutinase (50 μM) was incubated at 25 °C with Dip-F in 100 mM sodium phosphate (pH 7.5). Aliquots were withdrawn at specified intervals and assayed immediately for esterase activity.

Estimation of Number of Serine Residues Modified. Cutinase (15 μM) was incubated at 25 °C with [^{3}H]Dip-F (0.9 Ci/mol, 35 μM), and aliquots were withdrawn at time intervals and diluted into 8% Cl_3CCOOH. The precipitated protein was recovered by centrifugation, and the precipitate was washed 3 times with 10% Cl_3CCOOH. Protein-bound radioactivity was assayed after dissolving the precipitate in NCS tissue solubilizer (Amersham) in ScintiVerse (Fisher). Alternatively, cutinase (70 μM) was incubated with different concentrations of unlabeled Dip-F. After incubation at 25 °C for 10 min, Dip-F was removed by Sephadex G-25 column chromatography equilibrated with 100 mM sodium phosphate buffer (pH 7.5). The number of unmodified serine residues remaining after the treatments with unlabeled Dip-F was determined by the amount of radioactivity incorporated upon complete inactivation of the esterase activity with [^{3}H]Dip-F. Unbound [^{3}H]Dip-F was removed by Cl_3CCOOH precipitation as described above.

Inactivation with Diethyl Pyrocarbonate (DEP). An ethanol solution of DEP was added to a cutinase solution (50 μM) in 100 mM Mes buffer (pH 7.0) containing various amounts of $NaDodSO_4$. The ethanol concentration, not exceeding 5% (v/v), had no effect on the enzyme stability. After appropriate incubation periods at 30 °C the reaction was stopped by 20-fold dilution of the reaction mixture with 100 mM imidazole buffer (pH 8.3). Esterase activity was assayed in the presence of Triton X-100.

Spectroscopic Studies on Inactivation by DEP. The time course of histidine modification under identical conditions as described above was followed spectrophotometrically at 240 nm. The number of histidine residues modified was calculated from the molar extinction coefficient of 3200 cm^{-1} M^{-1} (Miles, 1977). The UV spectrum was taken after complete inactivation of enzymatic activity against a blank containing the same amount of unmodified enzyme.

Inactivation of Cutinase with 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC). Modification of cutinase (10 μM) with EDC was carried out at 25 °C in 100 mM sodium phosphate buffer (pH 5.8) containing 2 mM $NaDodSO_4$. The reaction was started by addition of EDC from a stock solution in the same buffer. Aliquots of the reaction mixture were diluted (1500-fold) into the assay mixture containing Triton X-100 and immediately assayed for esterase activity.

Stoichiometry of EDC Modification. The number of carboxyl groups modified was estimated by the EDC-promoted incorporation of [^{14}C]glycine ethyl ester (Carraway & Koshland, 1972). Cutinase (35 μM) was incubated in 100 mM phosphate buffer (pH 5.8) containing 2 mM $NaDodSO_4$, 50 mM [1-^{14}C]glycine ethyl ester, and 50 mM EDC at 25 °C. Aliquots of the reaction mixture were removed, and the reaction was stopped by a 5-fold dilution into 1 M sodium acetate buffer, pH 5.0. After 10 min an aliquot of the mixture was diluted into 200 mM phosphate buffer (pH 8.0) containing 0.5% Triton X-100, incubated for 10 min, and assayed for enzymatic activities. The protein-bound radioactivity was determined after Cl_3CCOOH precipitation (10%) as indicated above.

In an alternate approach, cutinase was preincubated in the presence of EDC (100 mM) and unlabeled glycine ethyl ester (100 mM), in the absence of $NaDodSO_4$, for 30 min at 25 °C. The incubation mixture was diluted with an equal volume of 4 mM $NaDodSO_4$ solution containing the radioactive ester. Enzyme activity and protein-bound radioactivity were determined as described above.

Preparation of [1-^{14}C]Acetylcutinase. Cutinase (100 μM) was inactivated with DEP (1 mM) in 100 mM Mes–NaOH (pH 7.0) and 4 mM $NaDodSO_4$. The enzyme solution was incubated at 30 °C for 30 min and mixed with the same volume of buffer containing 1% (w/w) Triton X-100. A control sample was treated the same way except for the inactivation step with DEP. After a 30-min incubation in the Triton solution the enzyme preparations were incubated with *p*-nitrophenyl [1-^{14}C]acetate (7 mM) for 10 min. The reaction was stopped by filtration of the solution (0.7 mL) through a Sephadex G-25 column (0.8 × 13 cm) equilibrated with 100 mM Mes–NaOH (pH 7.0) containing 0.5% (w/w) Triton X-100, and the protein was eluted with the same buffer; 0.35-mL fractions were collected and assayed for radioactivity and esterase activity.

Determination of the Critical Micelle Concentration (cmc) of NaDodSO$_4$. The cmc of $NaDodSO_4$ in 100 mM Mes, pH 7.0, was determined by the dye binding method with Rhodamine 6G according to Carey & Small (1969).

Protein and Radioactivity Determination. Protein was measured by the method of Lowry et al. (1951). Radioactivity was determined by liquid scintillation spectrometry using ScintiVerse (Fisher) as the scintillation fluid in a Packard Tri-Carb liquid scintillation spectrometer 3255.

Immunological Cross-Reactivity. Antiserum against cutinase was prepared as described elsewhere (Soliday & Kolattukudy, 1976). Immunodiffusions were carried out in 1% agar gels (Nobel, Difco) at room temperature for 24 h with 100 mM veronal (pH 8.5) containing 0.01% thimerosal and 0.9% NaCl as buffer. The agar gels contained 1 mM $NaDodSO_4$ and 0.5% (w/w) Triton X-100.

Results

Inhibition of Cutinase by Diisopropyl Fluorophosphate (Dip-F). Organophosphorus compounds are known to inactivate a number of serine proteases as well as serine esterases by phosphorylation of the active serine hydroxyl group (Cohen et al., 1967). The treatment of cutinase with Dip-F resulted in rapid loss of esterase activity, and this inactivation appeared to be a pseudo-first-order reaction (data not shown). The second-order rate constant of inactivation was found to be 810 M^{-1} s^{-1} at 25 °C and pH 7.0. This rate constant compares well with the value found for acetylcholinesterase (770 M^{-1} s^{-1}) (Cohen et al., 1967).

[^{3}H]Dip-F was used to follow the time course of phosphorylation. Figure 1A shows the relationship between inactivation and the number of serine groups modified with time. Modification of 0.96 residue resulted in the loss of 98% esterase activity. For determination of the stoichiometry of this reaction for cutin hydrolysis, the free Dip-F was removed by Sephadex

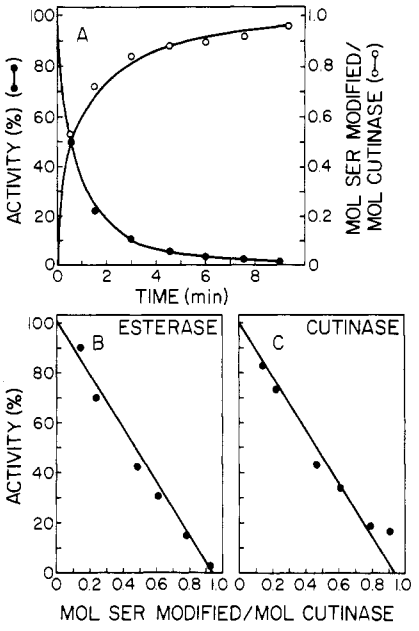

FIGURE 1: Time course of inactivation of cutinase and number of serine residues modified with [^{3}H]Dip-F. Esterase activity was measured with PNB and cutinase activity with [^{3}H]cutin as a substrate.

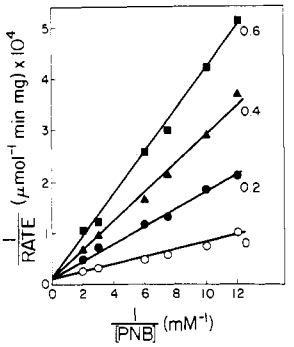

FIGURE 2: Inhibition of cutinase by phenylboronic acid. The assay mixture contained variable concentrations (millimolar) of inhibitor.

G-25 chromatography after incubation of cutinase with various concentrations of Dip-F. The correlation between the number of modified residues and remaining enzymatic activity with the model substrate PNB and cutin as substrates is shown in parts B and C of Figure 1, respectively. Extrapolation to zero enzymatic activity shows that phosphorylation of 0.95 group/mol of cutinase would result in complete inactivation of esterase as well as cutinase activity. These results provide strong evidence for the presence of one "active serine" per molecule of cutinase.

Inhibition of Cutinase by Phenylboronic Acid (PBA). Organic boronic acid derivatives are known to be reversible inhibitors of serine hydrolases like proteases (Kraut, 1977), acetylcholinesterase (Koehler & Hess, 1974), and pancreatic lipase (Garner, 1980). The inhibition of cutinase by phenylboronic acid was competitive in nature with a K_i value of 140 mM at pH 7.5 and 30 °C (Figure 2). A kinetic study of cutin hydrolysis is complicated by the fact that the substrate is an insoluble polymer. The rate of cutin hydrolysis, however, assayed at pH 8.5 and remaining linear for 40 min, showed 30% and 75% inhibition at PBA concentrations of 200 and 600 mM, respectively (data not shown). This result indicates that the K_i values for the inhibition of esterase and cutinase activity by PBA are similar. If PBA inhibits cutinase by formation of a reversible complex with the active serine, this inhibitor might be expected to protect this serine from modification by

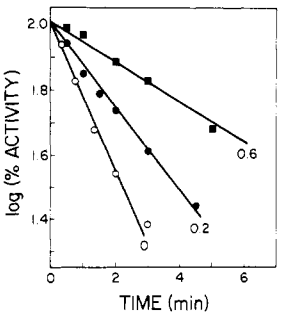

FIGURE 3: Time course of inactivation of cutinase with Dip-F in the presence of phenylboronic acid at concentrations (millimolar) as indicated in the figure.

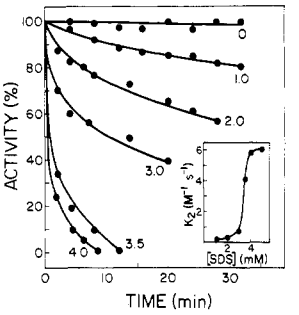

FIGURE 4: Time course of inactivation of cutinase with DEP (2 mM) with variable concentrations (millimolar) of $NaDodSO_4$ as indicated in the figure. The inset shows the dependence of the second-order rate constant of inactivation on the $NaDodSO_4$ concentration. The second-order rate constant was calculated from the pseudo-first-order rate constant for inactivation of cutinase.

Dip-F. The result shown in Figure 3 shows that PBA did in fact afford such protection.

Inhibition of Cutinase by Diethyl Pyrocarbonate (DEP). DEP is known to be a relatively specific reagent for histidine modification in proteins (Miles, 1977). All modification studies with cutinase were done in Mes buffer, which appeared to be especially suitable for this chemical modification (Meyer & Cromartie, 1980).

Treatment of cutinase with DEP had no effect on the enzymatic activity. However, such a treatment in the presence of $NaDodSO_4$ inactivated the esterase activity (Figure 4). This inactivation was not a result of $NaDodSO_4$ denaturation. Cutinase is indeed inactivated by $NaDodSO_4$ but rapidly and completely reactivated with Triton X-100 (W. Köller and P. E. Kolattukudy, unpublished results), a detergent that was always present in the assay mixture. This unique feature of cutinase was a prerequisite for all chemical modification studies described below. The rate of inactivation with DEP was strongly dependent on the $NaDodSO_4$ concentration employed. It increased slowly as the $NaDodSO_4$ concentration was raised up to about 3 mM, and a subsequent increase in detergent concentration up to 4 mM caused a dramatic increase in the inactivation rate, with very little further increase above 4 mM. The cmc of $NaDodSO_4$, known to be strongly dependent on the ionic strength of the solvent (Helenius et al., 1979), was 0.5–1 mM under the present conditions as determined by dye binding of Rhodamine 6G. Thus the drastic increase in the rate of inactivation by DEP occurred well above the cmc of $NaDodSO_4$. All subsequent studies were done at 5 mM $NaDodSO_4$. Control samples were incubated under exactly the same conditions but without DEP.

DEP treatment in the presence of $NaDodSO_4$ resulted in a rapid loss of esterase activity in a pseudo-first-order reaction (Figure 5). The second-order rate constant was found to be 5.9 M^{-1} s^{-1} at pH 7 and 30 °C. Although DEP is a fairly

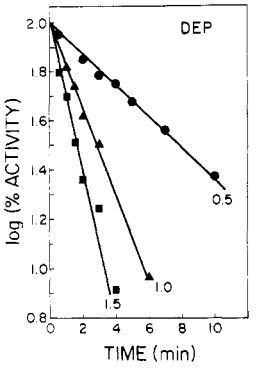

FIGURE 5: Time course of inactivation of cutinase by DEP. The inactivation was carried out in the presence of 5 mM $NaDodSO_4$ with variable concentrations of DEP (millimolar) as indicated in the figure.

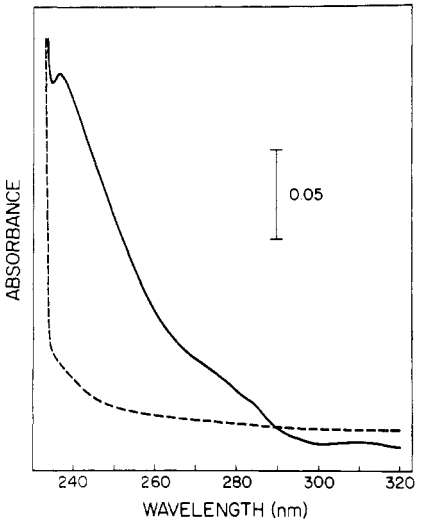

FIGURE 6: Difference spectrum of cutinase inactivated with DEP against untreated cutinase. The dashed line shows the difference spectrum of a control sample.

selective acylating agent for histidine, reactions with other functional groups in proteins have been reported (Miles, 1977). The reaction of DEP with histidine is known to give rise to *N*-carbethoxyhistidine with an absorption maximum near 240 nm (Miles, 1977). The UV difference spectrum of cutinase obtained after complete inactivation with DEP showed an increase in absorbance with a λ_{max} value of 237 nm (Figure 6). A small increase at 280 nm indicated that no tyrosine residue was modified (Miles, 1977). Modification of a cysteine residue can be ruled out since cutinase has no free SH groups (W. Köller and P. E. Kolattukudy, unpublished results). Inactivation of cutinase with DEP was reversed by hydroxylamine, but this treatment resulted in a severe activity loss of unmodified control samples. Cutinase was found to be stable at alkaline pH conditions under which *N*-carbethoxyhistidine is known to be unstable (Miles, 1977). Incubation of cutinase at pH 10 for 2 h resulted, as expected, in 85% recovery of esterase as well as cutinase activity.

Further evidence for the modification of an essential histidine residue was obtained from the study of the inactivation rate as a function of pH. With the assumption that the unprotonated imidazole reacts substantially faster than the protonated, the rate of inactivation can be expressed as in eq 1 (Cousineau & Meighen, 1976); k_2 is the second-order rate

$$1/k_2(\text{obsd}) = 1/k_2 + [H^+]/(k_2K_a) \qquad (1)$$

constant for modification of the unprotonated residue with a dissociation constant of K_a for the protonated form. From a

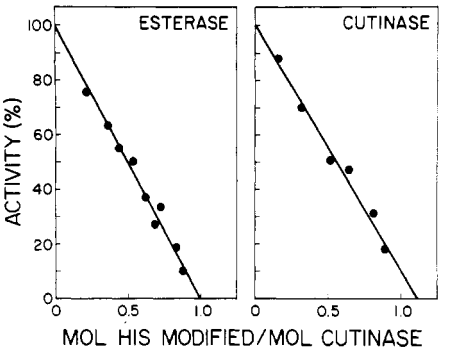

FIGURE 7: Stoichiometry of modification of cutinase by DEP. Esterase activity was determined with PNB and cutinase activity with [^{3}H]cutin as a substrate.

Table I: pH Dependence of Cutinase Inactivation by EDC[a]

pH	k_2 (M^{-1} s^{-1})
5.3	6.7
5.8	1.3
6.8	0.5
7.3	0.2

[a] Determined with *p*-nitrophenyl butyrate as the substrate.

rectilinear plot of $1/k_2$(obsd) vs. $[H^+]$ (not shown), k_2 was calculated to be 22.7 M^{-1} s^{-1}, and the pK_a was calculated to be 7.52. Thus, inactivation of cutinase with DEP was dependent on the unprotonated form of the modified residue with an apparent pK_a value of 7.5. This value is slightly higher than that found for histidine residues in most proteins (Schneider, 1978), but pK_a values up to 8 have been reported (Narita, 1980). Furthermore the rate constant for modification of the unprotonated residue (21.3 M^{-1} s^{-1}) is very close to the rate constant for DEP modification of *N*-acetylhistidine and imidazole (Holbrook & Ingram, 1973).

The time course of histidine modification was followed spectrophotometrically and correlated with the rate of inactivation measured under identical conditions. Extrapolation to zero enzymatic activity indicated that the modification of one histidine residue per molecule of cutinase is required for inactivation of the esterase activity as well as cutinase activity (Figure 7). It should be mentioned that this minimal number of one excludes the possible formation of dicarbethoxyhistidine, a reported side reaction of DEP with histidine, which leads to an overestimation of the number of modified residues due to an increased molar extinction at 240 nm (Miles, 1977). Since this protein contains only one histidine per molecule of the enzyme, it seems reasonable to conclude that this single histidine is involved in catalysis.

Inhibition of Cutinase by EDC. Water-soluble carbodiimides like EDC have been used for modification of carboxyl groups in proteins (Carraway & Koshland, 1972). Treatment of cutinase with EDC at pH 5.8 and in the presence of $NaDodSO_4$ resulted in a rapid loss of activity. This reaction followed pseudo-first-order kinetics (data not shown). $NaDodSO_4$ at concentrations above the cmc increased the rate and extent of inactivation by EDC substantially (data not shown). The pH dependence of this inactivation is shown in Table I. The second-order rate constant increased about 35-fold as the pH was decreased from 7.3 to 5.3, indicating the modification of carboxyl groups (Kurzer & Douraghi-Zadeh, 1967). Furthermore, the UV absorbance of the modified cutinase was not diminished as would be expected from tyrosine modification. Both λ_{max} (278 nm) and the molar extinction coefficient ϵ_{278} (10 700 M^{-1} cm^{-1}) were not changed by modification with EDC. The inactivation of cutinase was

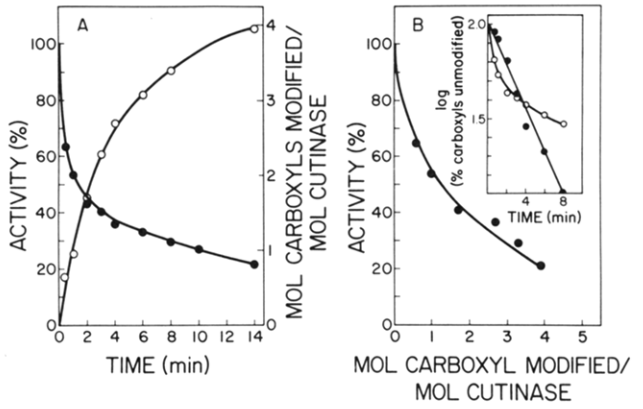

FIGURE 8: Time course of inactivation of cutinase by EDC and number of carboxyl groups modified (A) and the correlation of enzyme activity with number of modified carboxyl groups (B). The inset shows the theoretical time course of carboxyl-group modification on the assumption that three groups are nonessential for the enzyme activity (●) and that the modification of one essential group results in inactivation of cutinase (○).

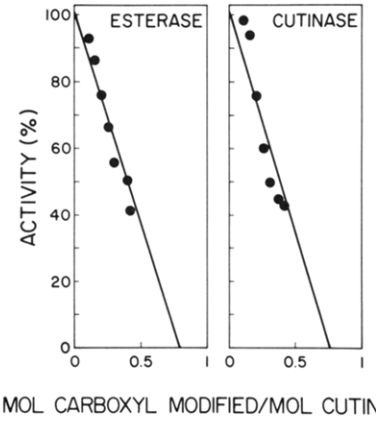

FIGURE 9: Stoichiometry of modification of cutinase with EDC. Cutinase was preincubated with unlabeled glycine ethyl ester and subsequently mixed with a $NaDodSO_4$ solution (5 mM final concentration) containing [1-^{14}C]glycine ethyl ester. Esterase and cutinase activities were determined with PNB and [^{3}H]cutin, respectively.

not dependent on the presence of a nucleophile, indicating the formation of an *N*-acylurea derivative (Carraway & Koshland, 1972). The stoichiometry of this reaction was estimated by modification of the enzyme in the presence of the nucleophile [1-^{14}C]glycine ethyl ester. The esterase activity of cutinase was reduced to 20% with the incorporation of 4 mol of ester/mol of enzyme (Figure 8). The method (i plot) frequently used to relate the inactivation of an enzyme to the number of groups essential for catalysis (Paterson & Knowles, 1972) was not applicable in the present case, indicating that the carboxyl groups reacted at different rates. An analysis of these data revealed, however, that three groups were modified in a pseudo-first-order reaction if it is assumed that the modification of only one carboxyl group resulted in inactivation of the enzymatic activity (Figure 8).

The presence of one buried essential carboxyl group was confirmed by an alternate modification approach. Cutinase was incubated in the presence of EDC and unlabeled glycine ethyl ester in the absence of $NaDodSO_4$. The enzyme was not inactivated under these conditions. Addition of $NaDodSO_4$ together with [1-^{14}C]glycine ethyl ester resulted in enzyme inactivation. The correlation of the remaining enzyme activity with the number of carboxyl groups modified under these conditions is shown in Figure 9. Extrapolation to zero enzymatic activities showed that modification of 0.8 carboxyl group/molecule of cutinase resulted in complete inactivation. These results strongly suggest that three nonessential peripheral carboxyl groups were modified in the absence of $NaDodSO_4$ and that one buried essential carboxyl group became accessible to carbodiimide only in the presence of $NaDodSO_4$.

Immunological Tests for Renaturation of Modified Cutinase. Since the modification of histidine and the essential carboxyl group required $NaDodSO_4$, the effect of such modification on renaturation was tested immunologically. The presence of $NaDodSO_4$ prevented immunoprecipitin line formation, but Triton X-100 fully restored the cross-reactivity, giving fusion of precipitin lines (Figure 10). The immunological cross-reactivity of the enzyme, in which the essential histidine was carbethoxylated in the presence of $NaDodSO_4$, was fully restored in the presence of Triton X-100, resulting in fusion of the immunoprecipitin lines (Figure 10). Also the enzyme in which the peripheral carboxyl groups were modified by EDC treatment in the absence of $NaDodSO_4$ retained full immunological cross-reactivity. However, modification of the carboxyl group essential for catalytic activity resulted in the loss of immunological cross-reactivity. This result indicates that modification of the buried carboxyl group interfered with the Triton-mediated conformational recovery of $NaDodSO_4$-treated cutinase.

Isolation of an Acyl-Enzyme Intermediate. The results described above indicate the presence of a catalytic triad consisting of a serine, a histidine, and a carboxyl residue. Thus, the ester hydrolysis catalyzed by cutinase might be expected to proceed, by analogy with other serine hydrolases, via an acyl-enzyme intermediate as shown by

$$E + S \rightarrow E{\cdot}S \rightarrow ES' + \text{alcohol} \rightarrow E + \text{acid}$$

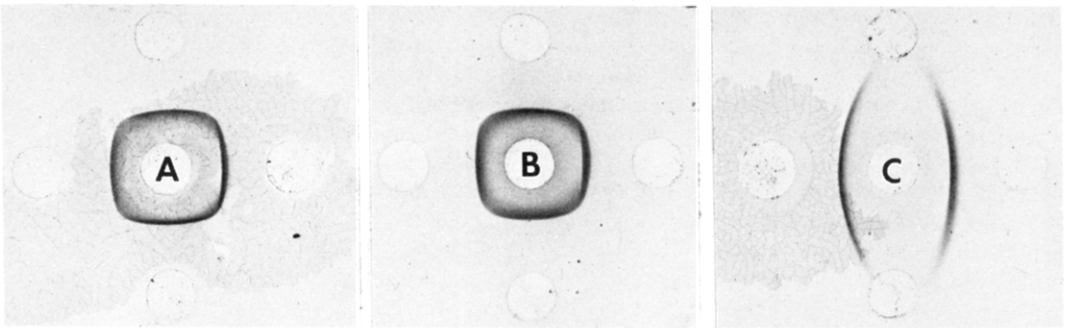

FIGURE 10: Effect of $NaDodSO_4$ and Triton X-100 on immunological cross-reactivity of chemically modified cutinase. The immunodiffusion plates contained 1 mM $NaDodSO_4$ and 0.5% Triton X-100. The central well contained antiserum prepared against cutinase; the wells at the left and right contained cutinase treated with $NaDodSO_4$. The wells at the top and bottom contained (A) cutinase carbethoxylated in the presence of $NaDodSO_4$, (B) cutinase modified with EDC in the absence of $NaDodSO_4$, and (C) cutinase modified with EDC in the presence of $NaDodSO_4$. Cutinase in (B) was treated with $NaDodSO_4$ after free EDC was removed by incubation in 1 M acetate buffer at pH 5.

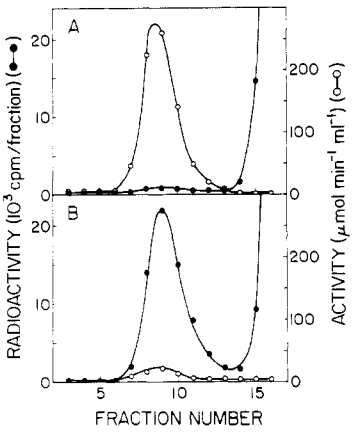

FIGURE 11: Elution profile of cutinase after gel permeation chromatography using Sephadex G-25. Cutinase was inactivated with DEP, renatured with Triton X-100, and incubated with *p*-nitrophenyl [1-^{14}C]acetate prior to gel filtration (A). (B) shows the elution profile of cutinase treated the same way except the inactivation step with DEP.

where E·S is the noncovalent Michaelis–Menten complex and ES′ is the acylated enzyme.

The best direct evidence for an acyl-enzyme mechanism would be the isolation of an acylated intermediate at low pH [e.g., Chapus et al. (1976)]. This experimental approach, however, is restricted to enzymes with fairly low hydrolysis rates, conditions that are not met by cutinase with PNB as a substrate (Purdy & Kolattukudy, 1975b). Chymotrypsin, methylated at the essential histidine residue, was strongly inhibited but retained a small proportion of catalytic power. General base catalysis remained an integral feature of the enzyme mechanism (Henderson, 1971; Byers & Koshland, 1978). The hydrolysis reaction, slowed down by a chemical modification of the essential histidine in cutinase, might lead to a fairly stable acyl-enzyme intermediate. Cutinase was carbethoxylated with DEP in the presence of $NaDodSO_4$ and refolded by a Triton treatment. The modified enzyme preparation was incubated with *p*-nitrophenyl [1-^{14}C]acetate for 10 min and subsequently subjected to gel permeation chromatography in the presence of Triton X-100 in order to remove labeled free acetate and *p*-nitrophenyl acetate. The results of these protein separations are shown in Figure 11. No radioactivity was bound to a control sample of cutinase treated with $NaDodSO_4$ and incubated with Triton X-100 without prior chemical modification. This result strongly argues against the possibility that the acetyl group was attached to some side chain other than the serine hydroxyl. An artificial binding of [1-^{14}C]acetate to the protein or to Triton X-100 micelles can also be excluded. However, the cutinase sample carbethoxylated at the essential histidine residue contained radioactivity. An alkaline hydrolysis revealed that this enzyme-bound radioactivity was not due to simple physical binding of unhydrolyzed *p*-nitrophenyl acetate since no *p*-nitrophenol was photometrically detectable. The number of acetyl groups bound per mole of cutinase was 0.7. That [1-^{14}C]acetate was indeed covalently bound to the modified enzyme was confirmed by the observation that about 75% of the radioactivity remained protein bound after a Cl_3CCOOH precipitation performed immediately after isolation of the protein. An incubation for 1 h at 30 °C, however, resulted in a complete release of enzyme-bound [1-^{14}C]acetate.

These results strongly suggest that the acetyl derivative described above is an intermediate in catalysis and that the chemical modification of an essential histidine residue resulted in a severe inhibition of enzyme activity but not in complete inactivation. Cutinase, with a turnover number of about 30 000 min^{-1} for PNA hydrolysis, will have as its slowest catalytic step the deacylation of the enyzme, with a rate constant of about 0.5×10^4 s^{-1}. The deacylation of carbethoxylated cutinase was completed after 1 h, thus indicating an inhibition factor between 10^5 and 10^6. This factor is in good agreement with the magnitude of inhibition reported for methylchymotrypsin (Byers & Koshland, 1978).

Discussion

Serine hydrolases are a class of enzymes characterized by the presence of a serine residue exhibiting high reactivity to organophosphorus compounds. X-ray crystallography has provided the tertiary structure of serine proteases, and striking similarities of the active site have been reported (Kraut, 1977). The essential structural feature is a catalytic triad involving the hydroxyl group of the active serine, the imidazole side chain of a histidine, and a buried carboxylate side chain (Blow, 1976). In the absence of three-dimensional structures for serine esterases, conclusions about the mechanism of ester hydrolysis are based mainly on kinetic studies and are drawn from analogies with serine proteases. Conclusive evidence for the participation of a histidine and a carboxyl residue in enzyme catalysis is rather limited for this class of serine hydrolases. Pancreatic lipase like cutinase is active at an interface but unlike cutinase is only weakly active on monomeric carboxyl esters. The pancreatic enzyme was inhibited by organophosphates and by chemical modification of histidine residues and carboxyl groups. However, only the histidine residue was found to be essential for catalysis (Semeriva & Desnuelle, 1979). The serine residue phosphorylated by organophosphates was shown to be involved in surface absorption rather than in catalysis (Guidoni et al., 1981), and the carboxyl groups were assumed to be involved in conformational changes of the protein at the interface (Semeriva & Desnuelle, 1979). Krupka (1966) proposed a two-histidine reaction pathway for acetylcholinesterase. However, alternate explanations for these kinetic data were recently discussed (Rosenberry, 1975). The evidence for an essential histidine residue in nonspecific carboxylesterases has been suggested but remains inconclusive (Krisch, 1971; Heymann & Marcussen-Wulff, 1975; Farb & Jencks, 1980). The results presented in this paper strongly suggest the presence of a catalytic triad in cutinase. Specific chemical modifications suggest that one serine, one histidine, and one buried carboxyl residue comprise, like in serine proteases, the essential units in the catalytic mechanism of the enzyme. Cutinase was inhibited by Dip-F, indicating the existence of an active serine residue, and modification of one serine residue per molecule of the enzyme resulted in complete inactivation of the enzyme. The involvement of an active serine was further established by the reversible inhibition of cutinase with phenylboronic acid. Both inhibitors are believed to act as transition-state analogues and serve as a model for the acylation step at the active serine residue (Froede & Wilson, 1971; Kraut, 1977).

The existence of one essential histidine residue was shown by chemical modification of cutinase with DEP. This essential histidine, however, was accessible only in the presence of $NaDodSO_4$ with a sharp increase in reactivity at a $NaDodSO_4$ concentration above the cmc. These results strongly suggest the presence of a buried histidine residue, which becomes accessible to modification only after a conformational change. The existence of a buried essential histidine residue in serine hydrolases has not been reported, although tritium-exchange studies revealed that the solvent accessibility of the essential histidine residue in trypsin was strongly restricted (Narita,

1980). The carboxyl group of the catalytic triad of serine proteases, on the other hand, is known to be buried (Kraut, 1977; Brayer et al., 1979; Sielecki et al., 1979; James et al., 1980). Consequently, this carboxyl group remains unaccessible for chemical modification prior to unfolding of the protein structure (Carraway et al., 1969). In the present study similar results were obtained for cutinase. Carboxyl group modification in the presence of $NaDodSO_4$ revealed that chemical modification of one buried carboxyl group resulted in complete inactivation of the enzyme.

Cutinase is inactivated by $NaDodSO_4$ and it is rapidly and fully reactivated in the presence of Triton X-100 (W. Köller, R. J. Foster, and P. E. Kolattukudy, unpublished results). This Triton-induced refolding was, as shown by immunodiffusion, not affected by the chemical modification of the histidine residue. It is therefore highly unlikely that the carbethoxylation described in this study prevented the refolding rather than inhibited the catalysis of ester hydrolysis. However, the situation is complicated in the case of carboxyl-group modification. The chemical modification of the buried carboxyl group interfered with the Triton-mediated refolding of cutinase. Thus the correlation of enzyme activity loss with the chemical modification of one buried carboxyl group could possibly reflect the inhibition of the refolding process rather than inhibition of catalysis. However, the analogy with serine proteases suggests that the buried carboxyl group of cutinase, modified in the presence of $NaDodSO_4$, is part of the catalytic triad. The model for the catalytic mechanism of serine proteases suggested that the essential aspartic acid is located in a hydrophobic environment (Blow, 1976; Kraut, 1977). This proposal was not confirmed in recent structural studies of microbial serine proteases (Brayer et al., 1979; Sielecki et al., 1979; James et al., 1980). The essential aspartic acid was found, on the contrary, in a strongly polar environment although buried and isolated from direct solvent contact. Brayer et al. (1979) suggested that this controlled polar environment is a fundamental catalytic requirement that is conserved during evolution. A similar highly ordered environment surrounding the buried essential carboxyl group in cutinase might explain the lack of refolding after its chemical modification and, furthermore, might emphasize the importance of this one carboxyl group for the maintenance of a functional conformation of the enzyme.

The striking similarities of the essential structural features of cutinase with those of serine proteases discussed above suggest that the hydrolysis of esters by cutinase might proceed via an acyl-enzyme intermediate, a model that is mainly based on kinetic data (Krisch, 1971; Kraut, 1977). The isolation of an acylated serine esterase, the best direct evidence for an acyl-enzyme intermediate, has been reported only for pancreatic lipase (Chapus et al., 1976). In the case of "fast-reacting" enzymes isolation of such intermediates is difficult (Krisch, 1971). Although cutinase is, like pancreatic lipase, active at an interphase (Brown & Kolattukudy, 1978), this enzyme is, in contrast to lipase, highly active with soluble model esters and shares, in this respect, common features with nonspecific esterases (Krisch, 1971). It was recently reported that chymotrypsin N-methylated at the essential histidine is far less active but still retained some catalytic power (Byers & Koshland, 1978). The active site and the binding site of the methylated chymotrypsin remained essentially intact, but the modified enzyme was 10^5 times less active. We present here strong evidence that carbethoxylation of the essential histidine resulted in the formation of an acyl-enzyme intermediate stable enough for isolation and that deacylation of the acylated enzyme, the rate-limiting step in ester hydrolysis, was inhibited by a factor of about 10^5. This factor is in good agreement with the inhibition reported for methylchymotrypsin (Byers & Koshland, 1978). Possible enzyme mechanisms for the methylated chymotrypsin are discussed by Byers & Koshland (1978) in great detail. These authors conclude that the general base catalysis remains intact regardless of the histidine modification. Thus, the isolation of an acylcutinase after carbethoxylation of the essential histidine strongly indicates the involvement of an acylated active serine as an intermediate in the reaction path of ester hydrolysis by cutinase.

With the present evidence for the presence of the characteristic catalytic triad in cutinase and for the involvement of an acyl-enzyme intermediate, cutinase represents an example of a serine esterase sharing the basic catalytic features with serine proteases. Cutinase, with its relatively simple structure, might be a suitable model to study the structure–activity relationships in serine esterases. Even though the catalytic mechanism of cutinase appears to be analogous to that of serine proteases, the binding of cutin to this enzyme must involve some unique structural features. Elucidation of the nature of the binding site for the insoluble polymeric substrate could facilitate development of "suicide substrates" with high affinity for the binding site and high reactivity toward functional groups involved in binding or catalysis. The present results, which elucidate the nature of the functional groups essential for catalysis, would aid in designing molecules with high reactivity for such functional groups. With the recent demonstration that fungal infection of plants can be prevented by inhibition of cutinase (Shaykh et al., 1977; Maiti & Kolattukudy, 1979; Köller et al., 1982), development of such inhibitors could result in their use as effective antipenetrants to protect plants against fungal diseases.

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Trehalase: Stereocomplementary Hydrolytic and Glucosyl Transfer Reactions with α- and β-D-Glucosyl Fluoride†

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ABSTRACT: A new understanding has been obtained of the catalytic capabilities of trehalase, an enzyme heretofore held to be strictly specific for hydrolyzing α,α-trehalose and devoid of transglycosylative ability. Highly purified rabbit renal cortical trehalase and a partly purified *Candida tropicalis* yeast trehalase were found to utilize both α- and β-D-glucosyl fluoride as substrates. In each case, the reactions were competitively inhibited by α,α-trehalose. Both enzymes catalyzed rapid hydrolysis of α-D-glucosyl fluoride to form β-D-glucose (also, of α,α-trehalose to form equimolar α- and β-D-glucose). In addition, digests of β-D-glucosyl fluoride plus α-D-[^{14}C]-glucopyranose with either trehalase (but not controls of enzyme with α-D-[^{14}C]glucopyranose alone) yielded small amounts of radioactive trehalose (α-D-glucopyranosyl α-D-[^{14}C]glucopyranoside) which does not accumulate since it is rapidly hydrolyzed. Trehalase thus catalyzes two stereocomplementary types of glycosylation reactions: (I) α-D-glucosyl fluoride (or α,α-trehalose) + H_2O → β-D-glucose + HF (or α-D-glucose); (II) β-D-glucosyl fluoride + α-D-glucopyranose → α,α-trehalose + HF. Such behavior shows that the catalytic groups of trehalase, as recently found for other glycosylases, are functionally flexible. The results illustrate the inadequacy of conventional views of carbohydrase specificity and the rigor, as a basic guiding principle, of the concept that glycoside hydrolases and glycosyltransferases form a class of glycosylases effecting glycosyl/proton interchange.

The enzyme trehalase, elaborated by a wide range of living forms, has long been considered strictly specific for catalyzing the hydrolysis of α,α-trehalose and certain close analogues (Bourquelot, 1893; Kalf & Rieder, 1958; Saito, 1960; Dahlqvist, 1960; Courtois et al., 1962; Avigad et al., 1965; Guilloux et al., 1968; Nisizawa & Hashimoto, 1970; Sacktor, 1972; Labat et al., 1973; Defaye et al., 1981). From the susceptibility (or lack thereof) of differently modified analogues, Defaye et al. (1981) recently affirmed that trehalase requires a substrate with the steric bulk of α-D-glycopyranosyl α-D-glucopyranoside. Apart from virtual restriction to utilizing α,α-trehalose, trehalase also has invariably been viewed as a strict hydrolase, devoid of the ability to catalyze glycosyl transfer other than to water (Dahlqvist, 1960; Courtois et al., 1962; Avigad et al., 1965; Guilloux et al., 1968; Nisizawa &

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