Affinity Labeling of the Active Site of Staphylococcal Nuclease

REACTIONS WITH BROMOACETYLATED SUBSTRATE ANALOGUES

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SUMMARY

The reaction of staphylococcal nuclease with stoichiometric amounts of 3'- (N-14C-bromoacetyl-p-aminophenylphosphoryl)-deoxythymidine 5'-phosphate, an alkylating reagent that is a derivative of a strong competitive inhibitor, deoxythymidine 3',5'-diphosphate, results in inactivation of enzymatic activity. No reaction occurs in the presence of the competitive inhibitor, or when Ca++, which is required for binding of substrates and inhibitors, is omitted from the reaction mixture. The modified enzyme derivative was readily purified and separated from unreacted enzyme since it did not adsorb to a column containing Sepharose to which the competitive inhibitor, deoxythymidine 3'-p-aminophenylphosphoryl 5'-phosphate, was covalently bound. The radioactivity of this protein derivative was accounted for by N-carboxymethyllysine 48 (36%) N-carboxymethyllysine 49 (40%), and O-carboxymethyltyrosine 115 (15%) in the primary structure of the protein. Only one of these residues is alkylated in a given protein molecule. These residues are presumed to be located in the region of the tertiary structure of the enzyme which affords specific affinity for substrates and inhibitors, but which does not determine the hydrolytic rate constants.

Staphylococcal nuclease is also inactivated, stoichiometrically, in the presence of Ca++, by the 14C-bromoacetyl derivative of the poorly hydrolyzed substrate, deoxythymidine 3'-p-aminophenylphosphoryl. This reagent selectively alkylates tyrosine 85, which is thought to occupy a position near the hydrolytic portion of the active site. The reaction occurs either by alklylation followed by hydrolysis, or by both processes occurring in a concerted fashion, possibly through the initial formation of a phosphorylated enzyme-substrate intermediate. The enzyme derivative modified by alklylation at tyrosine 85 is adsorbed to the specific nuclease-inhibitor Sepharose column, indicating that it possesses residual binding function. The derivative is catalytically active, although its affinity for DNA and synthetic substrates is severely depressed.

Reaction of staphylococcal nuclease with the bromoacetyl derivative of deoxythymidine 3'-p-aminophenylphosphosphate, a weak inhibitor of the enzyme, results in considerable non-specific and random alklylation. Reaction nevertheless occurs selectively with a residue important for catalysis (lysine 24). Although the amount of this specific reaction is small compared to the total reaction with the protein, as judged by incorporation of 14C, no other single residue appears to be as highly labeled.

The principles and techniques of affinity labeling have recently been illustrated by the specific covalent tagging of "contact" amino acids (1) in the hapten binding sites of antibodies (2-5) as well as in the active sites of chymotrypsin and trypsin (6-11). The specific, reversible protein-ligand complex results in an increase in the local concentration of ligand such that a chemically reactive group attached to this ligand may react preferentially with a properly oriented amino acid functional group in the binding region of the protein. By proper selection of reagents and reaction conditions considerable information may be obtained concerning the stereochemistry and orientation of residues in or near the active sites of proteins.

This report presents the results of studies which probe chemically the substrate binding region of staphylococcal nuclease by reacting the enzyme with bromoacetyl derivatives of various competitive inhibitors (Fig. 1). The specificity of the labeling reaction was determined (a) by following the stoichiometry of inactivation, (b) by studies of the prevention of inactivation (as well as alkylation of the specific residue) by the addition of strong competitive inhibitor, pdTp (12, 13), (c) by studies of protection which results by omitting the divalent cation, Ca++, which is required for proper binding of substrates and inhibitors (13, 14), (d) by contrasting the qualitative patterns of reaction found with structurally different inhibitors having identical activities.

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reactive groups (bromoacetyl), and (e) by purification of the affinity-labeled enzymes, followed by identification of their uniquely labeled residues.

The reagents used in these studies (Fig. 1) are all bromoacetylaminoo alkylating reagents derived from the appropriate p-nitrophenylphosphoryl thymidine derivatives (15). Reagent I is derived from pdTp-nitrophenol, which, because of the free 5'-phosphate group, is a potent competitive inhibitor, having a dissociation constant of $1.1 \times 10^{-6}$ M (15, 16). Reagent II is derived from nitropheryl-pdT, a compound having a dissociation constant of $2.2 \times 10^{-5}$ M, which is slowly cleaved by staphylococcal nuclease to yield p-nitropherylphosphate and thymidine (15). Reagent III is a derivative of the inhibitor, dTp-nitrophenol, which has a dissociation constant of $2.2 \times 10^{-5}$ M; no hydrolytic cleavage of dTp-aminophenol by staphylococcal nuclease could be detected (15). The bromoacetyl derivatives of p-aminophenylphosphate (Reagent IV) and of p-aminophenol (Reagent V) were designed as controls for the other reagents.

**EXPERIMENTAL PROCEDURE**

**Materials**

Staphylococcal (Foggi strain) nuclease was obtained by a modification (17) of techniques described by Fuchs, Cuatrecasas, and Anfinsen (18). pdTp was purchased from Calbiochem, bromoacetic anhydride from K and K Laboratories, snake venom diesterase from Worthington, Sepharose 4B from Pharmacia, bromoacetic-14C acid (4.88 mCi per mmole) from New England Nuclear. Deoxythymidine 3',5'-di-p-nitrophenylphosphate was synthesized as previously described (15). dTp-nitrophenol and nitrophenyl-pdT were obtained from Raylo Chemicals Ltd., Alberta, Canada. Royal Blue X-ray film was obtained from Eastman Kodak Company.

**Methods**

Ribonuclease and deoxyribonuclease assays were performed spectrophotometrically as described earlier (12). Assays with the synthetic, small molecular weight substrate, deoxythymidine 3',5'-di-p-nitrophenylphosphate, were performed in 0.05 M borate buffer, pH 8.8, with 0.01 M CaCl$_2$; the rate of release of p-nitrophenylphosphate at 25° was measured with a Gilford model 2000 multiple sample absorbance recorder (15). Ultraviolet and visible spectra were obtained with a Cary model 15 spectrophotometer.

The Sepharose-inhibitor gel used to separate mixtures of partially reacted nuclease by affinity chromatography was prepared by coupling 3'-(4-aminophenylphosphoryl)deoxythymidine 5'-phosphate to cyanogen bromide-activated Sepharose 4B as described previously (19).

Amino acid analyses were performed according to the procedure of Spackman, Moore, and Stein (20) with the use of a Spinco model 120 amino acid analyzer. Samples were hydrolyzed in constant boiling HCl in evacuated, sealed tubes at 112° for 20 hours. The DNS-chloride end group and sequential Edman DNS-chloride procedures were performed as described by Gray (21).

The reaction mixtures used for the affinity labeling experiments contained low concentrations of staphylococcal nuclease (about $10^{-3}$ M). Protein concentration was determined spectrophotometrically (280 nm) using $E_{1\text{cm}}^{1\text{cm}}$ of 9.30 (18). The molecular weight used in the calculations was 16,800 (22). The concentration of protein to which the affinity reagent had been covalently attached was determined by amino acid analysis, or from the absorbance at 280 nm after subtracting the absorbance contribution at this wave length of the reagent nucleotide. The concentration of the latter was determined from radioactivity measurements, which were determined at 60% efficiency with 15 ml of Bray's solution (23) in a liquid scintillation spectrometer.

Tryptic digests of nuclease were obtained by incubating samples with trypsin (1% by weight) for 3 hours at 37° in 0.05 M ammonium bicarbonate, pH 8.0. Peptide mapping was performed on tryptic digests from 0.1 mmole of nuclease, with the use of Whatman No. 3MM paper. These were subjected to chromatography for 16 hours in n-butyl alcohol-acetic acid-water (4:1:5), followed by electrophoresis in pyridine-acetate-water (1:10:200), pH 3.6, for 60 to 70 min. Phenol red served as
a reference standard in the chromatographic dimension, and free lysine released during trypic digestion served as a marker in the electrophoretic dimension. The radioactive peptides were identified by autoradiography by exposing the maps to X-ray paper (Royal Blue) for 24 to 48 hours. Peptides were then located by dipping in 25% ninhydrin in ethyl alcohol.

In the preparative purification of the radioactive affinity-labeled peptides, about 0.06 pmole of sample from a trypic digest was applied per cm of paper. After chromatography on Whatman No. 3MM paper for about 30 hours, in the above solvent, autoradiographs were obtained and the radioactive region was cut out and sewn to another sheet of Whatman No. 3MM paper, which was then subjected to electrophoresis (90 min) with the use of pyridine-acetate buffer, pH 3.6. Radioautographs were again prepared, and the central portions of the radioactive streaks were cut out. The maps were then stained with ninhydrin. If the peptide seemed pure (by ninhydrin) it was eluted with 50% aqueous pyridine; if incomplete separation was indicated or suspected, the radioactive regions were cut out, sewn to another sheet of Whatman No. 3MM paper, and subjected to electrophoresis (90 min) in pyridine-water (1:19) titrated to pH 6.5 with acetic acid. After radioautography, the radioactive peptides were eluted with 50% aqueous pyridine.

Preparation of O-carboxymethyltyrosine—A solution containing 2.5 mm BOC-tyrosine, 20 mm iodoacetic acid, and 43 mm magnesium oxide, at pH 10, was stirred in the dark for 3 days at 37°C. After filtering and adjusting the solution to pH 8.0, cysteine was added (40 mm), and the pH was maintained at 8.0 with NaOH. The solution was then acidified to pH 3.0 with citric acid and extracted with ethyl acetate. The ethyl acetate solution was dried over anhydrous Na2SO4 and the solvent was then removed in a vacuum. The mixture of BOC-tyrosine and BOC-O-carboxymethyltyrosine was unproctected at room temperature with 4 x HCl-dioxane for 15 min. The precipitated, unprotected compounds were washed with ether and dried. The tyrosine derivative was separated from free tyrosine by elution from a column of Dowex 50, equilibrated with 0.2 M pyridine acetate buffer, pH 3.5, using the same buffer at pH 4.0. The first fraction eluted, containing O-carboxymethyltyrosine, was lyophilized. The resulting solid was again lyophilized, first from 0.1 N HCl solution and then from water. The O-carboxymethyltyrosine was recrystallized from methanol-water. No contamination was detected by thin layer chromatography, and a single peak appeared in the long column of the amino acid analyzer, located between valine and the breakthrough of the second buffer, as described by Plummer and Lawson (24).

\[ \text{C}_3\text{H}_6\text{O}_2\text{NH} \]

Calculated: C 55.2, H 5.4, N 6.9, Cl 0
Found: C 54.0, H 5.5, N 6.1, Cl 0.1

After drying over phosphorus pentoxide for 3 days, the following ultraviolet absorption properties were determined: in acid, \( \lambda_{max} 273.5 \text{ nm}, E_m 1060 \); in base, \( \lambda_{max} 275.0 \text{ nm}, E_m 1230 \).

**Synthesis of Affinity Labeling Reagents**

**Preparation of O-\(^{14}\text{C}\) Bromoacetyl-N-hydroxysuccinimide Ester**—In 3 ml of dioxane 87 mg (630 \( \mu \)moles) of bromoacetyl-1-\(^{14}\text{C}\) acid (1.54 mCi per mmole) and 86 mg (750 \( \mu \)moles) of \( N \)-hydroxysuccinimide were dissolved. To this solution 132 mg (700 \( \mu \)moles) of dicyclohexycarbodiimide were added. The urea precipitated immediately; after 1 hour the urea was removed and the solution brought to 5 ml with dioxane. The compound was used without further purification. Bromoacetyl anhydride was used for the preparation of the nonradioactive compounds.

**Preparation of Reagent I—pdTp-aminophenyl, 50 \( \mu \)moles**, prepared by previously described procedures (15), was dissolved in 0.3 ml of water, diluted with 0.7 ml of dioxane, and 1 ml of the \( O\, ^{14}\text{C} \)-bromoacetyl-N\'-hydroxysuccinimide ester solution (125 \( \mu \)moles) was added. After 10 min, 3 to 4 eq of triethylamine were added, and the reaction was left for 4 hours. One volume of water was then added, and the mixture was passed through Dowex 50 (hydrogen form) and lyophilized. It was then dissolved in methanol and precipitated with ether. The precipitate was dissolved in a small volume of water. After adjusting the pH to 3.5 with NaOH, the solution was lyophilized. The yield was 90%. The compound was pure on thin layer chromatography using 2-propanol-NH\(_4\)OH-H\(_2\)O (7:1:2) and 1-butanol-acetic acid-water (5:2:4). The \( \lambda_{max} \) was 258 \( \mu \)m; the \( E_m \) at 258 \( \mu \)m was 14,900, and, at 267 \( \mu \)m, 13,900. The specific activity was 1.10 \( \mu \)Ci per \( \mu \)mole.

**Preparation of Reagents II and III**—These were prepared from nitrophenyl-dTp and dTp-nitrophenyl after reduction to the corresponding aminophenyl derivatives by catalytic hydrogenation using palladium on charcoal, as previously described (15). The bromoacetyl derivatives were prepared essentially as described for Reagent I. Fifty micromoles of the aminophenyl ester were dissolved in 0.3 ml of water and 0.7 ml of dioxane, and 1 ml (125 \( \mu \)moles) of the stock solution of \( O\, ^{14}\text{C} \)-bromoacetyl-N\'-hydroxysuccinimide ester was added. After 3 hours, 1 volume of water was added, and the solution was extracted 5 times with ether and concentrated to dryness. The product was dissolved in methanol and precipitated with ether. Yields were 90% and the compounds were chromatographically pure on thin layer chromatography using the solvents described for Reagent I. The \( \lambda_{max} \) of Reagent II was 290 \( \mu \)m, the \( E_m \) (260 \( \mu \)m) was 14,600, and the specific radioactivity was 1.30 \( \mu \)Ci per \( \mu \)mole. For Reagent III \( \lambda_{max} \) was 264 \( \mu \)m, the \( E_m \) (264 \( \mu \)m) was 14,500, and the specific radioactivity was 0.44 \( \mu \)Ci per \( \mu \)mole.

**Preparation of Reagents IV and V**—These were prepared by reacting, under the above conditions, bromoacetyl anhydride with \( \text{p-aminophenylphosphinate} \) (for Reagent IV) and with \( \text{p-aminophenol} \) (for Reagent V). The former was prepared from \( \text{p-nitrophenylphosphinate} \) by catalytic hydrogenation for 2 hours at 35 p.s.i., and room temperature, with palladium on charcoal (25). Of the aminophenyl derivative, 100 \( \mu \)moles were dissolved in 1 ml of water, and 2 ml of dioxane containing 400 \( \mu \)moles of bromoacetyl anhydride were added. One volume of water was added after 1 hour, and the mixture was extracted several times with ether. The aqueous solution was concentrated to dryness, dissolved in methanol, and precipitated by addition of ether. Yields were quantitative, and the compounds were chromatographically pure.

**RESULTS**

**Affinity Labeling with Reagent I**

Reaction of staphylococcal nuclease with small amounts of Reagent I caused significant loss of enzymatic activity (Table I). The amount of radioactivity incorporated into the protein correlated well with the fall in enzymatic activity, indicating...
stoichiometric inactivation. Loss of enzymatic activity, and the incorporation of label, could be prevented by addition of the competitive inhibitor, pdTp. Reaction of Reagent I with nuclease could also be prevented by omitting Ca++. This is further evidence for the specificity of the reaction, since Ca++ is required for binding of substrates and inhibitors to the active site of the enzyme (12–14).

Fig. 2 indicates that enzymatic inactivation increased with increasing pH in the range 7 to 10. This dependence on H+ concentration does not reflect the pH dependence of binding of the inhibitor, since the latter is maximal at pH 7 (13). The results suggested that loss of activity was occurring as a result of nucleophilic attack on the reagent by the unprotonated form of a group on the enzyme of apparent pKₐ around 9 (i.e. tyrosine or lysine).

Low concentrations of protein (about 10⁻⁵ M), and of the affinity reagent, were generally used in these experiments to enhance the likelihood of selective reaction at the inhibitor binding site of the protein. Under these conditions, however, the rates of inactivation were relatively slow (Fig. 3); hence, reactions were allowed to proceed for 24 to 30 hours. With longer incubation times the rate of nonspecific alkylation with residues not in the inhibitor binding site became more significant relative to the continued selective modification of the binding site residue.

The affinity-labeled protein derivatives were chromatographed on columns of Sepharose which had been conjugated with the competitive inhibitor, pdTp-aminophenyl (19) (Fig. 4). By these techniques it was possible to separate an early, unretarded peak of inactive and highly radioactive protein from a second peak of weakly labeled and enzymatically active material, which adsorbed very strongly to the inhibitor Sepharose. The latter peak was the predominant species obtained from samples of protein which had been protected from reacting with Reagent 1.

**Table I**

**Affinity labeling of staphylococcal nuclease with Reagent I**

The indicated amount of ^14C Reagent I (1.10 μCi per μmole) was added to a solution containing 1.3 × 10⁻³ M nuclease in 0.05 M borate buffer, pH 9.4. Calcium, when present, was 10 mM, and pdTp was 6.1 × 10⁻⁴ M. Incubations were performed for 30 hours at room temperature.

<table>
<thead>
<tr>
<th>Molar excess of reagent</th>
<th>Additions</th>
<th>DNase activity</th>
<th>^14C per mole of enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>Ca++</td>
<td>59</td>
<td>moles</td>
</tr>
<tr>
<td>3</td>
<td>Ca++</td>
<td>27</td>
<td>0.7</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>90</td>
<td>0.05</td>
</tr>
<tr>
<td>3</td>
<td>Ca++, pdTp</td>
<td>95</td>
<td>0.05</td>
</tr>
<tr>
<td>0</td>
<td>Ca++</td>
<td>17</td>
<td>0.8</td>
</tr>
<tr>
<td>6</td>
<td>None</td>
<td>87</td>
<td>0.05</td>
</tr>
<tr>
<td>6</td>
<td>Ca++, pdTp</td>
<td>93</td>
<td>0.05</td>
</tr>
<tr>
<td>15</td>
<td>Ca++</td>
<td>12</td>
<td>0.85</td>
</tr>
<tr>
<td>15</td>
<td>None</td>
<td>70</td>
<td>0.1</td>
</tr>
<tr>
<td>15</td>
<td>Ca++, pdTp</td>
<td>65</td>
<td>0.1</td>
</tr>
</tbody>
</table>

* Compared to a sample of nuclease maintained in the same conditions without any additions.
* Determined after lyophilization and passage of the sample through a column of fine Sephadex G-25 (0.05 M ammonium acetate, pH 5.5).
I by omitting Ca++ from the incubation mixture (Fig. 4B), or by addition of pdTp (Fig. 4C). The enzymatic activities of these Sepharose-adsorbed samples were identical with that of the native enzyme (Table II). Sample A, which exhibited 26% of native DNase activity before application to the affinity column, was only slightly retarded on this column and emerged early in the elution, as a peak (Fig. 4) devoid of enzymatic activity and containing a radioactive label corresponding to 1.1 residues (Table II). The activity of the material from the second peak of this sample was identical with that of the native enzyme. These studies showed clearly that catalytic activation resulting from reaction of nuclease with Reagent I was complete, specific, and stoichiometric. The modified enzyme, although catalytically inactive, must retain some capacity to bind 3'-phosphoryl nucleotides since it is slightly retarded in its passage through the nuclease-specific Sepharose column (Fig. 4A).

The purified and presumably homogenous preparation of nuclease, alkylated at its active site by 1 eq of Reagent I, was incapable of hydrolyzing DNA, RNA, and the synthetic substrate, nitrophenyl-pdTp-nitrophenyl. No significant DNase activity was detected by modifications of the assay involving pH, Ca++, and substrate concentrations. Return of activity was not observed after treating the modified protein with 0.01 M NaOH or HCl for 5 days at 37°C.

To confirm, in a qualitative manner, the specificity of the reaction of Reagent I with staphylococcal nuclease, and to identify the site of reaction within the primary structure of the protein, a large quantity of 14C-labeled modified enzyme was prepared, cleaved by trypsin, and examined by peptide mapping and radioautography. In these experiments, 265 ml of 0.05 M borate buffer, pH 9.4, 10 mM CaCl$_2$, containing 2.5 pmoles of pdTp, respectively. The samples were brought to pH 4 with acetic acid, dialyzed against 5 mM ammonium acetate, pH 4.8, and passed through a column of fine grade Sephadex G-25 equilibrated with the same buffer. About 0.1 pmole of each protein sample was digested with trypsin, subjected to peptide mapping, and examined by ninhydrin staining and autoradiography (Fig. 5). Three major and two minor radioactive peptides were detected in the unprotected sample which appeared to have been specifically labeled by the affinity reagent.

Table II

<table>
<thead>
<tr>
<th>Preparation</th>
<th>DNase activity$^b$</th>
<th>14C per mole of enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system (A)</td>
<td>610</td>
<td>0.7</td>
</tr>
<tr>
<td>Early peak</td>
<td>17</td>
<td>1.1</td>
</tr>
<tr>
<td>Late peak</td>
<td>2150</td>
<td>0.1</td>
</tr>
<tr>
<td>In absence of Ca++ (B)</td>
<td>2120</td>
<td>0.2</td>
</tr>
<tr>
<td>Early peak</td>
<td>20</td>
<td>0.9</td>
</tr>
<tr>
<td>Late peak</td>
<td>2510</td>
<td>0.2</td>
</tr>
<tr>
<td>Protected by pdTp (C)</td>
<td>2300</td>
<td>0.1</td>
</tr>
<tr>
<td>Early peak</td>
<td>5</td>
<td>0.9</td>
</tr>
<tr>
<td>Late peak</td>
<td>2100</td>
<td>0.08</td>
</tr>
</tbody>
</table>

* These data refer to the experiment described in Fig. 4.

$^b$ Specific activity; the change in optical density (260 nm) of DNA per min caused by 1 mg of protein (12).

Fig. 4. Affinity chromatography on nuclease-specific Sepharose column (19) of staphylococcal nuclease treated under various conditions with the affinity labeling Reagent I. Forty millimicro-moles of nuclease, in 45 ml of 0.05 M borate buffer, pH 9.4, were treated with 120 nmoles of 14C Reagent I (1.1 μCi per pmole) in (A) the presence of 10 mM CaCl$_2$, (B) the absence of Ca++, and (C) the presence of 0.4 mM pdTp and 10 mM CaCl$_2$. After 22 hours at room temperature the DNase activities were 26% (A), 88% (B), and 96% (C). The solutions were then brought to pH 5 with acetic acid, concentrated to 5 ml, dialyzed for 8 hours against 0.05 M ammonium acetate, pH 4.8, and passed through a column of fine grade Sephadex G-25 equilibrated with the same buffer to separate the protein from the unreacted reagent. The effluent protein was lyophilized and redissolved in 2 ml of 0.05 M borate buffer, pH 8.0, containing 10 mM CaCl$_2$. This sample was then applied on a column (0.5 X 7 cm) which contained Sepharose 4B conjugated with the inhibitor, pdTp-aminophenyl (1 amole per ml of Sepharose). The column was then washed with 0.05 M borate buffer, pH 8.0, and 10 mM CaCl$_2$. The bound enzyme was eluted with NH$_4$OH, pH 11 (arrow). The specific DNase activities, and the incorporation of label, of the various peaks from these columns are presented in Table II. Native nuclease, which adsorbs very strongly to such affinity columns, can be eluted with NH$_4$OH, pH 11.
Fig. 5. Peptide maps and radioautograms of tryptic digests of 0.1 µmole of staphylococcal nuclease treated with the affinity labeling Reagent I in the presence (A) and absence (B) of Ca\(^{++}\), as described in the text. The sample protected with pdTp, not shown here, resulted in patterns identical with those of B. The column (1.3 × 12 cm), as illustrated in Fig. 4. The first, slightly retarded peak emerging from this column contained 18.5 mg of protein (65% of the total protein recovered) with 0.4% of the native level of DNase activity and 1.15 moles of \(^{14}\)C per mole of protein. Ten milligrams of protein (35% of the total recovered) were eluted with NH\(_2\)OH, pH 11; this material had 86% of the DNase activity of the native enzyme and contained 0.3 mole of \(^{14}\)C per mole of enzyme. Radioautograms of peptide maps from tryptic digests of the material from the first peak were identical with that of Fig. 5AII. The radioautogram of the chromatogram containing the peptides derived from the second peak did not show any of the five specific radioactive spots; it was very similar to that shown in Fig. 5BII. It seemed clear that the radioactivity incorporated in the protein peak which emerged early during affinity chromatography was caused by specific tagging of binding site residues. On the other hand, the radioactive label found in the enzymatically active fraction which adsorbed strongly to the inhibitor Sepharose complex is distributed randomly over many residues throughout the protein.

The radioactive peptides shown in Fig. 5AIII, were purified by preparative paper chromatography and electrophoresis from tryptic digests of the protein obtained from the first peak of the affinity chromatography experiments described above. The relative amounts of these peptides, and the yields obtained by these purification procedures, are shown in Table III. Amino acid analysis of these peptides revealed that Peptides A1a and A1b, which accounted for nearly 80% of the total labeled material, represented the regions of the primary sequence (Fig. 6) consisting of Residues 49 to 53 and 46 to 49, respectively (Table...
Paper after elution. Complete, judged by the total radioactivity remaining on the paper with 50% aqueous pyridine was in all cases more than 90% under "Experimental Procedure." Elution of the peptides from preparative chromatography and electrophoresis as described treated with trypsin. The radioactive peptides were purified by the total radioactivity remaining on the paper after elution.

Radioactive peptides obtained from tryptic digests of staphylococcal nuclease treated with affinity labeling Reagent I

Protein, 0.8 μmole (on the basis of radioactivity), obtained from the first peak of the affinity chromatography of nuclease treated with Reagent I (experiment described in the text) was treated with trypsin. The radioactive peptides were purified by preparative chromatography and electrophoresis as described under "Experimental Procedure." Elution of the peptides from paper with 50% aqueous pyridine was in all cases more than 90% complete, judged by the total radioactivity remaining on the paper after elution.

Table III

Radioactive peptides obtained from tryptic digests of staphylococcal nuclease treated with affinity labeling Reagent I

<table>
<thead>
<tr>
<th>Radioactive peptide</th>
<th>μmol</th>
<th>14C recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>0.17</td>
<td>36</td>
</tr>
<tr>
<td>Alb</td>
<td>0.19</td>
<td>40</td>
</tr>
<tr>
<td>Alc</td>
<td>0.07</td>
<td>15</td>
</tr>
<tr>
<td>Ald</td>
<td>0.02</td>
<td>4</td>
</tr>
<tr>
<td>Ale</td>
<td>0.02</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>0.47</td>
<td>60%</td>
</tr>
</tbody>
</table>

* The position on the radioautogram is described in Fig. 5.
* Determined on the basis of radioactivity (1.10 μCi per μmole).
* Percentage of the starting radioactivity recovered by the purification procedures.

Table IV

Amino acid compositions of purified peptides derived from tryptic digests of nuclease treated with affinity Reagent I

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Ala</th>
<th>Alb</th>
<th>Alc</th>
<th>Ald</th>
<th>Ale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Found</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Calculated</td>
<td>2</td>
<td></td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Found</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Calculated</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Found</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Calculated</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* See Fig. 5 and Table III for peptide designations. Values are expressed as μmole ratios.
* No corrections have been made for losses on hydrolysis. Values less than 0.1 residue are described as 0. Values for Peptides Ala, Alb, and Alc are averages from triplicate determinations, those for Peptides Ald and Ale from single analysis.

...and more text...
FIG. 6. Amino acid sequence proposed for staphylococcal nuclease V8 (26). Residues 20 and 31 have been inverted and histidine has been substituted for leucine at position 124 in accordance with the amino acid sequence of nuclease from the Foggi strain determined by the DNS-chloride method (21), was valine. After one step of the Edman DNS-chloride degradation procedure, the new amino terminal residue was an anodally migrating amino acid which did not correspond to the usual DNS-amino acid standards, and was presumably the derivative of carboxymethyl-tyrosine. There was no apparent reason for the unusual cleavage by trypsin of the bond between asparagine 118 and asparagine 119 to produce peptide Ale.

The position in the primary structure of nuclease of the two minor peptides, AId and Ale, could not be established with certainty. Their amino acid contents, however, suggest that they may also represent alkylation of the 48 to 49 region (Table IV). Peptide AId resembles Peptide Ala in its amino acid composition. The small differences in electrophoretic mobility (Fig. 5) could possibly be the result of a difference in the affinity reagent portion of this peptide rather than of the peptide portion. For example, it is possible that a small amount of the phosphodiester bond of the reagent could have been chemically hydrolyzed during the purification procedures. The amino acid composition of Peptide Ale (Table IV) suggests that this peptide may represent the sequence 46 to 53, with alkylation of either lysine 48 or 49 (Fig. 6). Unfortunately, the quantities of these 2 peptides were so small (Table III) that no other studies could be done to further characterize them.

**Affinity Labeling with Reagents II, IV, and V**

Reaction of staphylococcal nuclease with Reagent II under conditions similar to those used with Reagent I caused a depression of DNase activity (Table V). Protection from catalytic inactivation in this case could also be affected by omitting Ca++, or by adding the competitive inhibitor, pdTp. The incorporation of radioactivity into protein was higher with this reagent (Table V) than with Reagent I (Table I), probably as the result of the larger quantities of reagent used, necessitated by the weaker affinity of Reagent II for the enzyme.

Radioautographs of peptide maps of tryptic digests of nuclease treated with Reagent II were performed essentially as described for the studies with Reagent I (Fig. 7). Two major radioactive peptides, RII1 and RII2, were found only when the reaction of radioactivity into protein was higher with this reagent (Table V) than with Reagent I (Table I), probably as the result of the larger quantities of reagent used, necessitated by the weaker affinity of Reagent II for the enzyme.

<table>
<thead>
<tr>
<th>Addition</th>
<th>DNase activity %</th>
<th>[3H] per molar excess</th>
<th>[3H] per molar excess</th>
<th>[3H] per molar excess</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca++</td>
<td>53</td>
<td>68</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>108</td>
<td>100</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Ca++, pdTp</td>
<td>116</td>
<td>75</td>
<td>1.7</td>
<td></td>
</tr>
</tbody>
</table>

*From the experiments conducted with a 10-fold molar excess of Reagent II. The values were determined after lyophilization and passage of the protein through a column of fine Sephadex G-25 (0.05 M ammonium acetate, pH 4.8).*
FIG. 7. Radioautograms of tryptic digests of 0.1 pmole of nuclease treated with a 10-fold excess of the affinity labeling Reagent II in the presence of Ca++ (A), and protected by addition of Ca++ and pdTp (B) as described in Table V. Before tryptic digestion, the samples were passed through Sephadex G-25 to remove excess reagent (see text). The radioautograms from the sample protected from inactivation by omitting Ca++ from the reaction mixture was identical with that of B. The major radioactive peptide with the slower migration in the chromatographic dimension (in A) is designated as Peptide RII1, the other as Peptide RII2. The peptide map, stained with ninhydrin, corresponding to A is shown for reference (C); Peptides RII1 and RII2 are circled.

For purification and identification of the Peptides RII1 and RII2 (Fig. 7), 1.7 pmoles of nuclease in 100 ml of 0.05 M borate buffer, 10 mM CaCl2, were treated with 12.3 pmoles of Reagent II. After 4 days the DNase activity had fallen to 21%. The protein was then dialyzed, lyophilized, and passed through Sephadex G-25, as described for Reagent I. This material contained 1.8 moles of 14C per mole of protein. Tryptic digestion and purification by preparative chromatography and electrophoresis were performed on 0.9,umole of this protein. The two labeled peptides were readily obtained in pure form; 0.20 pmole of Peptide RII1 and 0.22 pmole of Peptide RII2 were recovered.

The amino acid compositions of the two purified peptides demonstrated that they are overlapping peptides consisting of Residues 82 to 87 and 85 to 87 (Table VI and Fig. 6). The tyrosine residue at position 85 was the residue specifically alkylated.

Reagent II is the bromoacetyl derivative of aminophenyl-dT. The latter can be cleaved by staphylococcal nuclease into aminophenylphosphate and dT (15). It was therefore important to determine if hydrolysis of Reagent II occurred, and if alkylation occurred before, during, or after such hydrolysis. Table VII shows that the product of hydrolytic cleavage of Reagent II, bromoacetyl p-aminophenol (Reagent IV), is itself capable of inactivating staphylococcal nuclease. Inactivation is, however, less effective since larger amounts of this reagent must be used to achieve similar inactivation. As with the affinity labeling Reagents I and II, reaction of nuclease with Reagent IV is dependent on the presence of Ca++ and is prevented by the competitive inhibitor, pdTp.

Removal of the phosphate group from Reagent IV results in the total loss of inactivating capacity of the reagent, as shown by the lack of enzymatic inactivation with Reagent V, bromoacetyl p-aminophenol (Table VII). Reagent IV, then, in spite of the absence of the nucleotide moiety, behaves like an affinity labeling reagent. Phosphodiester compounds devoid of a nucleoside constituent are not cleaved by the enzyme (15). Affinity of Reagent IV to the binding site might be favored because of the phosphate moiety, since the phosphate groups of substrates and inhibitors are major contributors to the strength of attachment (13, 15, 16). However, no inhibition of hydrolysis of synthetic substrates could be shown with p-nitrophenylphosphate under conditions which would detect inhibition if the dissociation constant had been smaller than 5 mM. The calcium-dependent inactivation caused by Reagent IV cannot be linked by specific orientation at the active site, as with true substrate analogues. The observed alkylation of nuclease may be caused by a calcium-dependent association of the phosphate moiety of this reagent with anionic groups in the protein, perhaps assisted by unusual reactivity of the alkylated group. Thus, demonstration that the potential product of hydrolytic cleavage of Reagent II, bromoacetyl p-aminophenolphosphate (Reagent VI), is itself capable of inactivating staphylococcal nuclease, does not constitute proof that this is the inactivating reaction that actually occurs when the protein is treated with Reagent II. The fact that Reagent IV is a much less effective reagent (Table VIII), and that its affinity for nuclease must be very poor, suggest that this may not be the case.

The following studies were performed to determine the compo-
Values less than 0.1 residue are designated as 0. Figures are molar ratios.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>RI1</th>
<th>RI2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Found*</td>
<td>Calculated*</td>
<td>Found*</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.1</td>
<td>1</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.9</td>
<td>0</td>
</tr>
<tr>
<td>Serine</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>Proline</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.0</td>
<td>1</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>Valine</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>Leucine</td>
<td>1/</td>
<td>0</td>
</tr>
<tr>
<td>Carboxymethyllysine</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Carboxymethyltyrosine*</td>
<td>1.1/</td>
<td>0.9/</td>
</tr>
</tbody>
</table>

* Peptides RI1 and RI2 are the two which react specifically with the affinity reagent (see text and Fig. 7). Values are expressed as molar ratios.

** No corrections have been made for decomposition on hydrolysis. Values less than 0.1 residue are designated as 0. Figures are averages from triplicate determinations.

* Calculated from the region of the sequence (Fig. 6) containing Residues 82 to 87.

a Calculated from the region of the sequence (Fig. 6) containing Residues 85 to 87.

b Arbitrarily selected as 1 residue for reference.

c Residue involved in alkylation.

d Calculated using tyrosine as standard.

Comparison of effects of affinity labeling Reagents II, IV, and V on DNase activity of staphylococcal nuclease

The indicated amount of affinity labeling reagent was added to a solution containing 1.3 × 10⁻⁶ m nuclease in 0.05 m borate buffer, pH 9.4. Calcium, when present, was 10 mM, and pdTp was 8.2 × 10⁻⁴ m. The data presented is the percentage of native enzyme DNase activity remaining after incubation for 48 hours at room temperature.

<table>
<thead>
<tr>
<th>Reagent II</th>
<th>DNase activity in %</th>
<th>DNase activity in %</th>
<th>DNase activity in %</th>
<th>DNase activity in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>+Ca⁺⁺⁺</td>
<td>36</td>
<td>8</td>
<td>76</td>
<td>95</td>
</tr>
<tr>
<td>+Ca⁺⁺⁺, pdTp</td>
<td>95</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>109</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Reagent IV</td>
<td>51</td>
<td>22</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>+Ca⁺⁺⁺</td>
<td>98</td>
<td>88</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>+Ca⁺⁺⁺, pdTp</td>
<td>106</td>
<td>89</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>109</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reagent V</td>
<td>103</td>
<td>88</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td>+Ca⁺⁺⁺</td>
<td>98</td>
<td>106</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Ca⁺⁺⁺, pdTp</td>
<td>106</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>112</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Amino acid compositions of purified peptides derived from tryptic digests of nuclease treated with affinity Reagent II

 Tyrosine, Leucine, Methionine, Valine, Alanine, Glycine, Proline, Serine, Threonine, Glutamic acid, Aspartic acid, Arginine, Histidine, Lysine, 0-Carboxymethyltyrosine was produced upon acid hydrolysis. Enzymes prevented subsequent hydrolysis by the other enzyme. 0-Carboxymethyltyrosine was produced upon acid hydrolysis.

There is considerable evidence indicating that inactivation of nuclease by Reagent II did not proceed by reaction with bromoacetyl p-aminophenylphosphate, present in solution as a result of enzymatic cleavage. Nuclease modified by reaction with Reagent II behaved quite differently from that modified by 0-Carboxymethyltyrosine with either venom or staphylococcal diesterase.

Amino acid-water, 4 : 1 : 5) a single reaction product (RF 0.3) was readily separated from Reagent II (RF 0.6) and from K-acetyl tyrosinamide (RF 0.8). It was purified by preparative thin layer chromatography (1-butanol-acetic acid-water, 4 : 1 : 5) a single reaction product (RF 0.3) was readily separated from Reagent II (RF 0.6) and from K-acetyl tyrosinamide (RF 0.8).

It was not possible from these experiments to specify whether, during the reaction of nuclease with Reagent II, the latter was first cleaved, releasing bromoacetyl p-aminophenylphosphate, which subsequently reacted with a residue in the active site of the enzyme. Alkylation could have occurred simultaneously with hydrolysis, or was perhaps uniquely coupled with hydrolysis in a concerted process. As a third possibility, alkylation could have occurred first, followed by enzymatic hydrolysis.

Scheme I

```
CH₃CONHCH₂
```

CH₂-C-HN-O-CH₂

O

O

OH

O

O

CH₂-CONHCH₂

```
Fig. 8. Comparison of ultraviolet spectrum of Peptide RII (Fig. 7 and Table VI) obtained from the reaction of Reagent II with staphylococcal nuclease at tyrosine 85 (—, \(7 \times 10^{-4}\) M) with the spectrum of (a) the model compound made by reacting N-acetyl tyrosineamide with Reagent II, as described in the text (—O—O, \(4 \times 10^{-4}\) M), and (b) the spectrum of this model compound after removal of thymidine by hydrolysis with staphylococcal nuclease and purification by thin layer chromatography (—, \(1 \times 10^{-4}\) M). Spectra were obtained in acetic acid, pH 3.4. Very minor changes in the spectra were observed by raising the pH to 11, indicating that in all cases alkylation had occurred with the hydroxyl group of tyrosine.

Reagent IV on nuclease-specific Sepharose columns (Fig. 9). The former was not appreciably retarded in its passage through such columns, despite the fact that considerable catalytic inactivation had apparently occurred. The enzyme treated with Reagent IV, on the other hand, passed unretarded through these affinity columns. The proportion of protein found in this early peak (78%) reflected the amount of protein which had been catalytically inactivated (80%). Amino acid analysis of the protein from the first peak (Fig. 9, Reagent IV) showed 0.9 residue of carboxymethyllysine, and no carboxymethyltyrosine. This is in contrast to the effects of Reagent II, which alkylates tyrosine 85 (Table VI). The second peak of protein which had been treated with Reagent IV (Fig. 9) had 0.1 residue of carboxymethyllysine and 0.1 residue of carboxymethyltyrosine. These results provided strong evidence that nuclease had been alkylated differently by these two affinity reagents.

The principal protein fraction derived from reaction with Reagent II retained the capacity to bind to substrate analogue inhibitors, as shown by its adsorption to the nuclease-specific Sepharose column (Fig. 9). Since the specific DNase activity

Fig. 9. Affinity chromatography on columns (0.5 X 5 cm) of Sepharose coupled with pdTp-aminophenyl of staphylococcal nuclease treated with affinity Reagents II (upper) and IV (lower). In the former experiment, 2 mg of nuclease treated for 26 hours with a 10-fold molar excess of Reagent II in the presence of Ca\(^{+2}\) were applied on the column; the protein had been passed through Sephadex G-25 to remove excess reagent. The first peak contained 0.3 mg of protein having 0.2% DNase activity and 3.1 moles of ^{14}C per mole of protein. The second major peak contained 1.6 mg of protein having 32% DNase activity and 2.2 moles of ^{14}C per mole of protein. Radioautograms of peptide maps from tryp tic digests of this peak showed prominent Peptides RIII and RII. In the experiment with Reagent IV (lower), the sample applied on the column was 3.4 mg of protein which had been reacted for 48 hours with a 15-fold excess of reagent in the presence of Ca\(^{+2}\), and then passed through Sephadex G-25. The protein had 22% of the DNase activity of the native enzyme. The early, unretarded protein peak contained 2.3 mg of protein, with 14% DNase activity. The second peak had 0.7 mg of protein having DNase activity equal to that of the native enzyme. After rechromatography of the first peak, the protein again emerged unretarded; its DNase activity was 0.4% of the native enzyme.
of this protein was 30% of that of the native enzyme, it was important to determine if this derivative, substituted at tyrosine 85, was contaminated with native enzyme, or if it retained inherent catalytic function.

Examination of the kinetic properties of the modified enzyme showed that this derivative was catalytically active, but that its ability to bind substrates was profoundly affected. It was apparent from studies using the substrate, DNA, that the modified enzyme was not significantly contaminated with native enzyme (Fig. 10). The low specific activity was the result of a severely depressed affinity for DNA, approximately 300-fold lower than that of the native enzyme; the maximal catalytic rate appeared to be only slightly, if at all, affected. Studies with the synthetic substrate, nitrophenyl-pdTp-nitrophenyl, confirmed this view. The apparent \( K_M \) of the modified enzyme was 5 mM, compared with 40 PM for the native enzyme. The \( k_{cat} \) was the same for the native and modified derivatives, about 10 min\(^{-1} \). In contrast to the protein modified with Reagent II, treatment with Reagent IV yielded material that failed to regain the capacity to hydrolyze DNA or synthetic substrates upon systematic variation of a number of assay conditions, such as substrate concentration, pH, and Ca\(^{2+} \) concentration.

**Affinity Labeling with Reagent III**

This reagent is the bromomethyl derivative of dTp-aminophenol, a nucleotide with much weaker affinity for staphylococcal nuclease than the comparable derivatives of Reagents I and II (15). Accordingly, it is a much less effective affinity labeling reagent (Table VIII). Much larger amounts of Reagent III must be used to depress enzymatic activity, and the amount of enzymatic activity which is lost is small in relation to the total number of \(^{14}C \) residues incorporated into the protein. Nevertheless, the observed inactivation is protected by the competitive inhibitor, pdTp, and by the absence of Ca\(^{2+} \). Protection against reaction, however, was not detected by comparing the amount of label incorporated into the native and protected samples of protein. This did not exclude the possibility of specific inactivation by stoichiometric reaction with a critical residue, since only a 10% difference in radioactivity would have been expected to accompany protection of the 30% of enzymatic inactivation which had been observed (Table VIII).

It seemed probable that extensive random alkylation of lysine (and possibly other) residues, not affecting enzymatic activity, had occurred in the native and protected samples as a result of the large excess of reagent and the long incubation periods which were used. Peptide maps of tryptic digests supported this view, since many radioactive spots were seen in all the samples. The radioactive peptide in the radioautograms did not coincide with the ninhydrin stained areas of the corresponding peptide maps, indicating the random nature of the reaction. However, the radioautograms corresponding to the peptide maps of the native enzyme, which had been 30% inactivated, showed one major radioactive spot which was represented by a weak ninhydrin stained region in the peptide map. This spot was not seen in the radioautograms prepared from samples which had been 8

\[^{14}C \text{Reagent III} (0.44 \mu \text{Ci per \( \mu \text{mole} \)) was added to a solution containing 2.2 \times 10^{-5} \text{ M nuclease in 0.05 \text{ M borate buffer, pH 9.4. Calcium, when present, was 10 mM, and pdTp was 7.6 \times 10^{-3} \text{ M. The data presented is the percentage of native enzyme DNase activity remaining after incubation for 36 hours at room temperature.}

**Table VIII**

<table>
<thead>
<tr>
<th>Addition</th>
<th>DNase activity in %</th>
<th>(^{14}C ) per mole of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-fold molar excess</td>
<td>30-fold molar excess</td>
<td></td>
</tr>
<tr>
<td>Ca(^{2+} )</td>
<td>71</td>
<td>58</td>
</tr>
<tr>
<td>None</td>
<td>64</td>
<td>104</td>
</tr>
<tr>
<td>Ca(^{2+} ), pdTp</td>
<td>121</td>
<td>88</td>
</tr>
</tbody>
</table>

*From the experiments conducted with a 17-fold excess of Reagent III. The values were determined after lyophilization and passage of the protein through a column of fine Sephadex G-25 (0.05 M ammonium acetate, pH 4.8).*

protected by pdTp or by omission of Ca\(^{2+} \). It appeared probable, then, that in spite of the unfavorable nature (poor affinity) of Reagent III as an affinity labeling reagent, it had reacted with a specific residue in the binding site of the enzyme in such a way as to affect the catalytic properties of the enzyme.

Purification of this specifically alkylated peptide from tryptic
digests was much more difficult than in the studies described for
Reagents I and II, because of the large number of other radio-
active peptides present and because the total amount of this
peptide was small.

The amino acid composition of the peptide isolated by prepara-
tive paper chromatography and electrophoresis, as described
earlier, suggested that this peptide represented Residues 17 to
28, with alkylation of lysine 24. Concomitant alkylation of
methionine 26 could not be excluded.

**DISCUSSION**

Identification of the amino acid residues of staphylococcal
nuclease to which the affinity labeling reagents attach, sum-
marized in Fig. 11, is a step toward the elucidation of the stereo-
chemistry and functional organization of residues in or near the
active site region of the enzyme. Reagent I, the most specific
of the reagents, attaches equally to lysines 49 and 48 in at least
80% yield, and to tyrosine 115 in about 15% yield. This
indicates that these residues must be located in the region of the
binding site which interacts with that part of substrates or
inhibitors lying to the right of the basic structural element
required for substrate recognition, RpdT (15). This region must
be very important in the binding function, since the nature of
the corresponding parts of substrates (15) or inhibitors (16)
directly determine their dissociation constants, but not the
catalytic rate constants of substrates (15). The binding site
optimally recognizes a trinucleotide sequence, (pdT)₂ (18).
The sequence which extends to the right of the 5′-phosphate end
must interact with the region of the enzyme described by the
reactions of Reagent I.

It is instructive in the studies of staphylococcal nuclease, as
well as for affinity labeling procedures in general, that the very
specific Reagent I can react selectively with three different
residues of the protein. Only one attachment, however, can
occur per protein molecule, since the reactions are clearly
stoichiometric. Reaction at one site excludes reaction at
other active site residues, which further confirms the specificity
of the processes involved in these affinity labeling studies. An
inhibitor molecule, once irreversibly bound to the active site,
blocks the access to this region of another similar inhibitor
molecule. For this reason it can not be said that the amino acid
residues which react with the affinity labeling reagent are them-
selves moieties essential for binding or catalysis. These may
serve, rather, simply as attachment sites which hold the reagent
firmly localized, increasing, in a sense, its local concentration,
and allowing other parts of the reagent to interact so strongly
with the protein that its displacement by reversible substrates
and inhibitors is not possible. This is probably the ease for
the protein modified by attachment of Reagent I, which is cataly-
tically inactive. Alkylation occurs from a portion of the inhibitor
structure which corresponds to a structural portion of substrates
which, although directly affecting strength of binding, is not
essential for binding or hydrolysis. The free 5′-phosphate
attached to the nucleoside, which is the essential feature of the
inhibitor, must be lodged very tightly in the active site, thus
preventing the approach of a substrate. It has been postulated
that it is the bond between the phosphate and C-5 of the sugar
(Fig. 11) which would be hydrolyzed if the bond structure were
to be a diester bond (15).

Considerable latitude must be permissible in the sphere of
reaction of Reagent I, despite its very strong affinity for the
protein, since reaction can occur specifically with at least three
different residues of the enzyme (Fig. 11). The patterns of
reaction are probably determined by the relative proximity and
reactivity of the amino acid side chains which can interact and
chemically react with the functional group of the reagent.
The reaction of this reagent with residues in two distant parts
of the primary structure of the protein is useful information, since
it indicates that in the tertiary structure the region of lysine 48-
lysine 49 is probably near the region of tyrosine 115. Both
regions probably bear a relationship to the binding site of the
enzyme, in the manner discussed above.

The proximity of tyrosine 115 to the substrate binding site
of the enzyme, and to tyrosine 85, was predicted from studies of
tyrosine nitration with tetranirotromethane (30), and from studies
of intramolecular cross-linking of aminotyrosyl residues (31).
Although tyrosine 115 was not nitratable in the native enzyme,
it became unusually reactive to tetranirotromethane in the presence
of pdTp (30). The properties of the mononitrotyrosyl 115
nuclease derivative indicated that this residue may be exposed
in the aqueous solvent on addition of pdTp.

The region of the protein near lysine 24, or methionine 26, may
also be located in this general portion of the tertiary structure of
the protein, as suggested by the reactions of Reagent III (Fig.
11). This reagent is much less firmly bound than Reagent I
to the enzyme, and its specific reaction with an active site residue
is a relatively minor part of its total reaction, in random fashion,
with other amino acid side chains. Independent evidence that
indicates that the enzyme region which reacts selectively with
Reagent III is near the substrate binding site is the observation
that tyrosine 27 is protected by pdTp from reaction with tetra-
nirotromethane (30).

It has been postulated that the substrate binding region of
nuclease can accommodate a trinucleotide sequence (16). Since
such a compound would probably cover an area of the protein
larger or longer than that covered by Reagent I, it would not be
surprising if the enzyme modified by attachment of this reagent
retained some weak residual capacity to bind nucleotides. This

![Fig. 11. Schematic representation of the residues of staphylo-
ococcal nuclease which were found to react with the various affinity
labeling reagents described in Fig. 1. At least 80% of the reaction
of Reagent I occurs at lysines 48 and 49 (equally distributed), 15%}
![Fig. 11. Schematic representation of the residues of staphylo-
ococcal nuclease which were found to react with the various affinity
labeling reagents described in Fig. 1. At least 80% of the reaction
of Reagent I occurs at lysines 48 and 49 (equally distributed), 15% occurs at tyrosine 115. Reagent II reacts with tyrosine 85. Re-
agent III probably reacts with lysine 24, possibly with methionine
26. Dashed parallel lines indicate the site of hydrolysis by the
enzyme, provided that a phosphodiester bond is present (15). Strong
competitive inhibition results if the nucleotide has a free
5′-phosphate.](http://www.jbc.org/content/244/16/4328.full.pdf)
was actually suspected from the fact that this modified protein was slightly retarded in its passage through columns of Sepharose to which the potent inhibitor, pOdP-aminophenyl, had been bound (Fig. 1).

The studies with the affinity labeling Reagent II suggest that tyrosine 85 may occupy a position in the tertiary structure of the protein which corresponds to the left side of the basic substrate structure, RpdT (Fig. 11). Reagent II, which is a weak substrate for staphylococcal nuclease, must have reacted with tyrosine 85 before or during hydrolysis of its phosphodiester bond. In either case hydrolysis of the pseudosubstrate must have occurred, and alkylation did not proceed by reaction with the product of the hydrolysis, bromoacetyl p-aminophenylphosphate. Preliminary data have suggested that hydrolysis of 5'-p-nitrophosphorylphosphate esters of thymidine may proceed via a phosphorylated enzyme step before release of p-nitrophosphorylphosphate. Provided that the rate of phosphorylation is faster than the rate of reaction of the alkylating group, it would be possible that the reaction of Reagent II could proceed first by fixation of the reagent in a favorable position by covalent linkage by way of a phosphorylated intermediate. Alkylation of a properly oriented and perhaps unusually reactive residue (tyrosine 85) could then occur. This type of reaction, involving a quasissubstrate containing a reactive functional group and proceeding first by the formation of an enzyme-substrate intermediate, has been invoked to explain the reaction of chymotrypsin with p-nitrophosphoryl bromoacetyl p-aminophenylphosphate (32). Acylation of the unique seryl residue was thought to immobilize the reagent, with subsequent alkylation of the methionyl residue 3 amino acids away in the linear structure. Another example of this type of affinity labeling appears to be the reaction of p-nitrophosphoryl diazoacetate with chymotrypsin (33). In the case of the reaction of staphylococcal nuclease with Reagent II, it is not possible from the available data to establish that such a mechanism has occurred.

It is of interest that nuclease alkylated at tyrosine 85 was catalytically active, although its affinity for synthetic substrates and for DNA was severely compromised. These observations emphasize that in affinity labeling studies the principle of catalytic inactivation is not an essential criterion for assigning a given residue to a location in the active site of the enzyme.

Studies on the reaction of staphylococcal nuclease with tetraniitromethane had indicated that cysteine 85 was an essential residue in the active site of staphylococcal nuclease (30). This residue was selectively nitrated in the native enzyme, with resultant loss of enzymatic activity. In the presence of pODP and Ca++ tyrosine 85 was not nitrated, and catalytic integrity was maintained. The present studies support the view that tyrosine 85 is located in the active site of the enzyme. Furthermore, the results presented indicate that this residue is not an essential participant in the hydrolytic processes.

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