Topography of the Active Site of Staphylococcal Nuclease

AFFINITY LABELING WITH DIAZONIUM SUBSTRATE ANALOGUES

PEDRO CUATRECASAS

From the Laboratory of Chemical Biology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014

SUMMARY

Staphylococcal nuclease reacts stoichiometrically at tyrosine 115 with the diazonium derivative of deoxythymidine 3'-p-aminophenylphosphate 5'-phosphate. This reaction, which results in enzymatic inactivation, can be prevented by addition of the competitive inhibitor, deoxythymidine 3',5'-diphosphate, or by the omission of Ca++ from the reaction mixture.

Specific modification of the tryptophan residue at position 140 and of the histidine residue at position 46 occurs upon treating the enzyme with the diazonium derivative of deoxythymidine 3'-p-aminophenylphosphate. The diazonium derivative of deoxythymidine 5'-p-aminophenylphosphate reacts selectively with tyrosine 85.

The chemical exploration of the binding site of this enzyme with a variety of affinity labeling reagents permits certain stereochemical formulations that can be used to compare the solution and crystal structures of this protein.

Affinity labeling of the binding sites of antibodies and enzymes has been of value in the identification of amino acids located in specialized regions of these proteins (1). Studies have been reported which chemically probe the substrate binding site of staphylococcal nuclease by reacting the enzyme with 14C-bromoacetamidophenyl derivatives of various competitive inhibitors (2). This report extends further the chemical exploration of the active site of staphylococcal nuclease by presenting studies of the reactions of this enzyme with diazonium derivatives of various nucleotide inhibitors and substrates. In addition to illustrating and extending the principles of affinity labeling in general, these studies attempt to elucidate the stereochemical orientation of binding site residues of this protein in solution, and provide a basis for comparisons with the tertiary structure determined by x-ray crystallography (3).

The reagents used in these studies (Fig. 1) are obtained by reduction and diazotization of corresponding p-nitrophenylphosphorylthymidine derivatives (2, 4). Reagent I is derived from pdTp-nitrophenyl, which, because of the free 5'-phosphate group, is a potent competitive inhibitor, having a dissociation constant of 1.1 × 10⁻⁴ M (4, 5). Reagent II is derived from nitrophenyl-pdT, having a dissociation constant of 2.2 × 10⁻⁴ M, which is slowly cleaved by staphylococcal nuclease to yield p-nitrophenylphosphate and thymidine (4). Reagent III is a derivative of the inhibitor, dTp-nitrophenyl, which has a dissociation constant of 2.2 × 10⁻⁴ M, and Reagent IV is derived from p-aminophenylphosphate, a compound not susceptible to hydrolysis and not having appreciable affinity for the enzyme.

In these studies the specificity of the labeling reaction was determined by the stoichiometry of inactivation, the prevention of inactivation (as well as azotization of the specific residue) by the addition of a strong competitive inhibitor, pdTp (6, 7), the requirement of Ca++ for optimal binding and reaction (7, 8), the qualitative differences in the reactions of different inhibitors with identical functional groups (diazonium), and by identification of the uniquely labeled residues in the primary structure of the protein.

EXPERIMENTAL PROCEDURE

Materials

Staphylococcal (Foggi strain) nuclease was purchased from Worthington or prepared by a modification (9) of techniques described by Fuchs, Cuatrecasas, and Anfinsen (10). N-Acetyl-L-tyrosine, N-acetyl-L-histidine, and pdTp were purchased from Calbiochem; Sepharose 4B was from Pharmacia. Deoxythymidine 3',5'-di-p-nitrophenylphosphate was synthesized as previously described (4) or purchased from Ash Stevens, Inc., Detroit, Michigan. Chromatographically pure dTp-nitrophenyl was obtained from Raylo Chemical Ltd., Alberta, Canada, and nitrophenyl-pdT from Raylo and Calbiochem. pdTp-nitrophenyl was prepared by treating nitrophenyl-pdT-nitrophenyl with snake venom diesterase (4).

Methods

Ribonuclease and deoxyribonuclease assays were performed spectrophotometrically as described earlier (6). Assays with the synthetic, small molecular weight substrate, nitrophenyl-pdT-nitrophenyl, were performed as previously described (2, 4). dTp-nitrophenyl, p-nitrophenyl ester of deoxythymidine 3'-phosphate; pdTp, dThyminde 3',5'-diphosphate; nitrophenyl-pdT-nitrophenyl, p-thymidine 3'-p-aminophenylphosphate ester of thymidine; dTp-aminophenyl, deoxythymidine 3'-p-aminophenylphosphate; DNS, dimethylaminonaphthalenesulfonyl.
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 (11).

Amino acid analyses were performed according to the procedure of Spackman, Moore, and Stein (12) with the aid of a Spinco model 120 amino acid analyzer. Samples were hydrolyzed in constant boiling HCl in evacuated, sealed tubes at 112°C for 20 hours.

The reaction mixtures used for the affinity labeling experiments contained low concentrations of staphylococcal nuclease (10^{-4} M to 10^{-7} M). Protein concentration was determined spectrophotometrically (280 nm) using E_{1%26;280} of 9.30 (10). The molecular weight used in the calculations was 18,600 (13). The concentration of protein to which the affinity reagent had been covalently attached was determined by amino acid analysis. The concentration of the covalently bound azo nucleotide was estimated from ultraviolet and visible spectral measurements.

Tryptic digests of nuclease were obtained by incubating samples with trypsin (1% by weight) for 3 hours at 37°C in 0.05 M ammonium bicarbonate, pH 8.0. Peptide mapping was performed on tryptic digests from 0.1 µmole 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 (1:10:290), pH 3.6, for 60 to 70 min. Phenol red served as a reference standard in the chromatographic dimension. Peptides were located by dipping in 0.25% ninhydrin in ethyl alcohol.

In the preparative purification of the colored affinity-labeled peptides, about 0.05 µmole of sample from a tryptic digest was applied per cm of paper. After chromatography on Whatman No. 3MM paper for about 30 hours, in the above solvent, the colored 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. The central portions of the colored streaks were cut out, and the maps were stained with ninhydrin to evaluate the degree of purity achieved by these procedures. The paper strips containing the yellow peptides were 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. The peptides were then eluted with NH_{4}OH, pH 11.

Preparation of Monoazo-N-acetyltyrosyl and Monoazo-N-acetylhistidyl Derivatives of pdT-aminophenyl—These model compounds were prepared to determine the spectral and chemical properties necessary to properly modulate the reaction of the diazonium reagents with the protein. Diazotization of pdTp-aminophenyl was accomplished by dissolving 16 µmoles of this compound in 150 µl of cold 2 N HCl and adding 60 µl (0.7 mg) of sodium nitrite in water. After 7 min at 5°C, 1.8 ml of cold 0.05 M borate buffer, pH 8.8, were added and the entire mixture was added immediately to a solution containing 100 µmoles of N-acetyl-L-tyrosine or N-acetyl-L-histidine in 330 µl of 0.05 M borate buffer, pH 8.8. The pH was adjusted and maintained at 8.2 (histidine) or 9.8 (tyrosine). The reaction was allowed to proceed for 18 hours, and the compounds were purified by aqueous elution from preparative thin layer chromatograms developed in 2-propanol-NH_{4}OH-water (7:1:20). The ultraviolet and visible spectra of the purified derivatives are shown in Fig. 2. The molar extinction coefficients for the azohistidyl derivative were 4,600 (in acid, at 377 nm) and 3,700 (in base, at 433 nm); and for the azotyrosyl derivative 26,200 (in acid, at 335 nm) and 12,100 (in base, at 474 nm). Spectrophotometric titrations indicated that the pK of the azotyrosyl compound is 9.4, and that of the azohistidyl compound 11.1 (Fig. 3).

Preparation of Diazonium Reagents and Reactions with Enzyme—The diazonium reagents depicted in Fig. 1 were prepared from the corresponding aminophenyl derivatives by treating with nitrous acid. The aminophenyl compounds were prepared from the respective nitrophenyl derivatives by catalytic hydrogenation using palladium on charcoal, as previously described (4). Ten micromoles of the aminophenyl nucleotide were dissolved in 150 µl of 2 N HCl. The subsequent reactions were carried out at 4°C. Fifty microliters (containing 0.8 mg) of sodium nitrite were added over a 1-min period to the continuously stirred solution containing the nucleotide. After 7 min, 2.8 ml of cold 0.1 M borate buffer, pH 8.1, were added. Aliquots of the diazonium compound were used immediately in the modification reactions. The experimental conditions used to modify the enzyme differed with each reagent and are described in detail in the tables and figure legends.
FICL 3. Spectrophotometric titrations of the model aeo compounds prepared by reacting the diazonium Reagent I with N-acetyl-L-histidine and N-acetyl-L-tyrosine (Fig. 2) as described in the text. Titrations were performed in 5 mM Tris-HCl buffer containing 0.1 M NaCl. The pK of the histidyl compound is 11.1, and that of the tyrosyl compound is 9.4.

RESULTS

Affinity Labeling with Reagent I—Specific enzymatic inactivation was readily demonstrated with Reagent I (Table I). Marked inactivation occurred with a 1- or 2-fold molar excess of reagent. The stoichiometry of this inactivation was confirmed by analysis of the ultraviolet spectrum of the modified protein (Table I). Loss of enzymatic activity and the acquisition of color could be prevented by addition of the competitive inhibitor, pdTp. The reaction with Reagent I could also be prevented by omitting Ca++, from the reaction mixture. This supports further the specificity of the reaction, since Ca++ is required for binding of substrates and inhibitors to the active site of the enzyme (6-8).

TABLE I

Affinity labeling of staphylococcal nuclease with Reagent I

| Molar excess of reagent | Additions | DNase activity | Residues modified per mole of protein |
|-------------------------|-----------|----------------|--------------------------------------|
| 2                       | Ca++      | 13             | 0.9                                  |
| 2                       | None      | 83             | 0.1                                  |
| 2                       | Ca++, pdTp| 92             | 0                                    |
| 4                       | Ca++      | 10             | 1.0                                  |
| 4                       | None      | 78             | 0.1                                  |
| 4                       | Ca++, pdTp| 96             | 0.1                                  |
| 8                       | Ca++      | 5              |                                      |
| 8                       | None      | 67             |                                      |
| 8                       | Ca++, pdTp| 91             |                                      |
| 12                      | Ca++      | 3              | 1.7                                  |
| 12                      | None      | 51             | 0.8                                  |
| 12                      | Ca++, pdTp| 87             | 0.6                                  |

* Compared to a sample of nuclease maintained in the same conditions without any additions.

a Estimated by ultraviolet spectroscopy using the azotyrosyl derivative as standard; obtained after passing the protein through a column of Sephadex G-25 equilibrated with 0.1 M NH₄ acetate, pH 5.0.
Table II

Time course of inactivation of staphylococcal nuclease by affinity Reagent I

The indicated molar excess of Reagent I was added to a solution of 5.3 × 10^-3 m nuclease in 0.05 m borate buffer, pH 8.4, containing 10 mM CaCl₂. At varying times an aliquot of the reaction mixture was diluted 20-fold with 0.1% albumin and assayed for DNase activity (6).

| Time of reaction (min) | Molar excess of reagent | DNase activity (in %) |
|------------------------|-------------------------|-----------------------|
| 2                      | 2                       | 29                    |
| 4                      | 4                       | 16                    |
| 8                      | 12                      |                       |
| 2                      | 2                       | 23                    |
| 4                      | 4                       | 10                    |
| 8                      | 8                       |                       |
| 2                      | 2                       | 11                    |
| 4                      | 4                       | 4                     |
| 8                      | 4                       |                       |

Even with enzyme concentrations as low as 10^-7 M the rate of inactivation was so rapid that it was difficult to obtain reliable kinetic constants (Table II). Inactivation increased with increasing pH in the range 7 to 10 (Fig. 4). Since binding of the inhibitor is maximal at pH 7 (7), this dependence on H⁺ concentration suggests azotisation of a functional group of an amino acid having a relatively high pK (i.e. tyrosine or lysine). The protection from inactivation afforded by the competitive inhibitor, pdTp, is less effective at higher pH values. This is presumably due to the higher reactivity of the modified residue and to the weaker binding of pdTp to the enzyme observed at higher pH values (7, 14).

The ultraviolet spectrum of the protein modified by reaction with Reagent I suggested the presence of an azotyrosyl moiety (Fig. 5). This was supported by finding that the changes in the visible region of the spectrum occurred with a pK of 9.2 (Fig. 5, inset). The affinity-labeled protein described in Fig. 5 was chromatographed on a column of Sepharose to which the competitive inhibitor, pdTp-aminophenyl, had been conjugated (11) (Fig. 6). These experiments demonstrated that the residual enzymatic activity of the modified protein resulted from a small amount of native nuclease which adsorbed strongly to the column. The major protein peak, which was enzymatically inactive and which contained virtually all the yellow color applied to the column, emerged unretarded through the column. Samples of nuclease treated with Reagent I under the same conditions described in Fig. 5, except for the omission of Ca⁺⁺ or the addition of pdTp to the incubation mixture, were virtually devoid of color and adsorbed completely to the affinity column. These studies demonstrated that catalytic inactivation resulting from reaction of nuclease with Reagent I was complete, specific, and stoichiometric.
The monoazo-substituted nuclease derivative purified by affinity chromatography (Fig. 6) was incapable of hydrolyzing DNA, RNA, and the synthetic substrate, nitrophenyl-pdTp-nitrophenyl.

Peptide maps of tryptic digests of this modified nuclease revealed one major (N1a) and one minor (N1b) peptide (Fig. 7). These two peptides were purified from a tryptic digest of a large sample of modified protein (Table III); Peptide N1a represented 90% of the total material purified. The ultraviolet spectrum of the purified Peptide N1a (Fig. 5) was identical with that of the model compound prepared by reaction of Reagent I with N-acetyltyrosine (Fig. 2). Amino acid analyses of the N1a peptide revealed that it represents Residues 114 to 127 in the primary structure of the protein (Table IV). The tyrosine at position 115 was the residue specifically modified in the azotization reaction. The amino acid composition of the minor peptide, N1b, which also has an azotyrosyl spectrum, is very similar to that of N1a except for a higher content of serine, glycine, and proline. If these amino acids represent contaminants, the reason for the unusual electrophoretic mobility of this peptide compared to that of N1a remains unexplained. Unfortunately, the amount of this peptide available for study was insufficient to permit its further characterization.

Affinity Labeling with Reagent II—Enzymatic inactivation of staphylococcal nuclease occurred after treatment with the diazonium Reagent II (Table V). This reagent, however, was less effective than Reagent I, as indicated by the requirement of larger amounts of reagent, longer incubation periods, and higher enzyme concentrations to achieve similar degrees of inactivation. Furthermore, unlike the reaction with Reagent I, additional Ca++ was not needed to cause inactivation with Reagent II. Nevertheless, azotization of an active site residue was likely since the...
TABLE IV
Amino acid composition of purified peptides derived from tryptic digests of nuclease treated with affinity Reagent I

| Amino acid | Peptide | Calculated<sup>a</sup> |
|------------|---------|-----------------------|
|            | N1a     | N1b                   |                           |
| Lysine     | 1.9     | 2.2                   | 2                         |
| Histidine  | 2.0     | 2.1                   | 2                         |
| Arginine   | 1.0     | 0.9                   | 1                         |
| Aspartic acid | 2.4    | 2.7                   | 2                         |
| Threonine  | 1.1     | 1.0                   | 1                         |
| Serine     | 0.1     | 1.0                   | 0                         |
| Glutamic acid | 2.3    | 2.1                   | 2                         |
| Proline    | 0.9     | 2.1                   | 1                         |
| Glycine    | 0.1     | 1.5                   | 0                         |
| Alanine    | 0.1     | 0.6                   | 0                         |
| Valine     | 1.0     | 0.9                   | 1                         |
| Methionine | 0       | 0                     | 0                         |
| Isoleucine | 0       | 0.3                   | 0                         |
| Leucine    | 1.0<sup>c</sup> | 1.0<sup>d</sup>   | 1                         |
| Tyrosine   | 0<sup>d</sup> | 0.2<sup>d</sup>   | 1                         |
| Phenylalanine | 0    | 0.1                   | 0                         |

<sup>a</sup> Specifically labeled peptide, as designated in Fig. 7 and Table III. No corrections have been made for losses on hydrolysis. Values less than 0.1 residue are described as 0. Values for Peptide N1a are averages of five separate determinations, those for Peptide N1b are from a single analysis.

<sup>b</sup> Calculated from the region of the sequence containing Residues 114 to 127. The tyrosine at position 115 is the residue specifically modified.

<sup>c</sup> Arbitrarily selected as 1 residue for reference.

<sup>d</sup> Residue involved in azo linkage with the diazonium reagent (see spectra of Fig. 5).

TABLE V
Effect of affinity labeling with Reagent II on DNase activity of staphylococcal nuclease

| Addition | 1.7 molar excess of reagent | 6.8 molar excess of reagent | 13.6 molar excess of reagent |
|----------|----------------------------|----------------------------|----------------------------|
|          | %                          | %                          | %                          |
| Ca++     | 10 min                     | 85                         | 75                         | 68                         |
|          | 6 hrs                      | 68                         | 52                         | 36                         |
|          | 24 hrs                     | 64                         | 27                         | 14                         |
| None     | 10 min                     | 78                         | 51                         | 46                         |
|          | 6 hrs                      | 67                         | 25                         | 31                         |
|          | 24 hrs                     | 61                         | 25                         | 12                         |
| Ca++, pDTp | 10 min                   | 100                        | 92                         | 94                         |
|          | 6 hrs                      | 92                         | 84                         | 82                         |
|          | 24 hrs                     | 90                         | 79                         | 64                         |

* Compared to a sample of nuclease maintained in the same conditions without any additions.

Fig. 8. Affinity chromatography on columns (0.7 X 7 cm) of Sepharose coupled with pDTp-aminophenyl of staphylococcal nuclease treated with affinity labeling Reagents II, III, and IV. Samples of nuclease (1.1 X 10⁻⁴ M) in 0.05 M borate buffer, pH 8.4, 10 mM CaCl₂, were treated with a 10-fold molar excess of Reagent II or III, or with a 14 fold excess of Reagent IV. After reaction for 5 hours at room temperature, the mixtures had lost 83% (Reagent II), 87% (Reagent III), and 56% (Reagent IV) of the DNase activity. Parallel samples protected by the inhibitor, pDTp, had lost 18% (Reagent II), 38% (Reagent III), and 46% (Reagent IV) of the enzymatic activity. The samples were titrated to pH 5 with acetic acid, dialyzed for 24 hours against distilled water, and lyophilized. Approximately 3 mg of modified protein, dissolved in 1.5 ml of 0.05 M borate buffer, pH 8.0, 10 mM CaCl₂, were applied to the column which was developed with the same buffer. Elution of material adsorbed to the column was achieved with NH₄OH, pH 11.1 (arrow). The specific DNase activities of the protein present in the early, unretarded peaks were 1%, 3%, and 0.5% for Reagents II, III, and IV, respectively. The specific activity of the strongly bound protein was the same as that of native nuclease.

Presence of the competitive inhibitor, pDTp, prevented inactivation.

Evaluation of the degree and stoichiometry of the reaction was difficult with this reagent because the ultraviolet spectra of the modified native enzyme, as well as that of the protected enzyme, indicated reaction of both tyrosine and histidine residues. The absorbance in the visible region of the spectrum of the protected protein, however, was less intense than that of the native modified enzyme, suggesting that there was a difference in the extent of reaction in the two samples.

Affinity chromatographic purification of the protein treated with Reagent II revealed that the major portion of the protein emerged as an early, enzymatically inactive peak (Fig. 8). Peptide maps of a tryptic digest of this protein contained only two major yellow spots (Fig. 9). A peptide map of a tryptic digest of the protein which adsorbed to the nuclease-specific
TABLE VI
Amino acid compositions of purified peptides derived from tryptic digests of nuclease treated with affinity Reagent II

| Amino acid      | Peptide\(^b\) | N\(_{IIa}\) | Calculated\(^a\) | N\(_{IIb}\) | Calculated\(^a\) |
|-----------------|--------------|------------|----------------|------------|----------------|  
| Lysine          | 1.1 1        | 0          | 0.1 1          | 0          | 0              |  
| Histidine       | 0            | 0          | 0.1 1          | 0          | 0              |  
| Arginine        | 0.9 1        | 0.1 0.1 1  | 0.1 1          | 0          | 0              |  
| Aspartic acid   | 1.6* 1       | 0.9 0.1 1  | 0.1 1          | 0          | 0              |  
| Threonine       | 0.9 1        | 0.2 0      | 0.1 1          | 0          | 0              |  
| Serine          | 0.1 0        | 0          | 0.1 1          | 0          | 0              |  
| Glutamic acid   | 0.1 0        | 0          | 0.1 1          | 0          | 0              |  
| Proline         | 0            | 0          | 0.1 1          | 0          | 0              |  
| Glycine         | 1.0 1        | 1.0 0      | 1.0 1          | 0          | 0              |  
| Alanine         | 0.2 1        | 0.2 0      | 0.2 0          | 0          | 0              |  
| Valine          | 0            | 0          | 0              | 0          | 0              |  
| Methionine      | 0            | 0          | 0              | 0          | 0              |  
| Isoleucine      | 0            | 0          | 0              | 0          | 0              |  
| Leucine         | 0            | 0          | 0              | 0          | 0              |  
| Tyrosine        | 0* 1         | 0* 0       | 0* 0           | 0          | 0              |  
| Phenylalanine   | 0            | 0          | 0              | 0          | 0              |  

* Peptides N\(_{IIa}\) and N\(_{IIb}\) are the two that react specifically with the affinity reagent (see text and Fig. 9).
* No corrections have been made for decomposition on hydrolysis. Values less than 0.1 residue are designated as 0. Figures are averages of triplicate (N\(_{IIa}\)) or duplicate (N\(_{IIb}\)) determinations.
* Calculated from the region of the sequence containing Residues 82 to 87.
* Calculated from the region of the sequence containing Residues 85 to 87. In both peptides, tyrosine 85 is the residue specifically azotized.
* Arbitrarily selected as 1 residue for reference.
* Residue involved in azo linkage with labeling reagent.

FIG. 9. Peptide maps of tryptic digests of 0.1 mole samples of nuclease treated with affinity labeling Reagents II (left) and III (right). The modified enzyme samples were from the early, unretarded peaks obtained by the affinity chromatography experiments described in Fig. 8. The yellow, specifically modified peptides indicated in the figure were identified by their absence in peptide maps of corresponding enzyme samples which had been modified with the labeling reagents in the presence of the competitive inhibitor, pdTp. Column contained no visible yellow spots. Also, peptide maps of protein derivatives prepared by reaction with Reagent II in the presence of pdTp, which also were yellow, contained no yellow peptide spots. This indicates random reaction of residues in the latter case, as well as in the case of the yellow active protein which adsorbs to the affinity column of Fig. 8. These results indicated that the enzymatic inactivation was indeed specific and related to selective reaction of 1 or 2 critical residues in the enzymatic binding region of the protein.

The two yellow peptides present in the tryptic digests of nuclease treated with Reagent II (Fig. 9) were purified by preparative chromatography and electrophoresis at pH 3.6 and 6.5 as described under “Methods.” The amino acid composition of these purified peptides demonstrated that they are overlapping peptides consisting of Residues 82 to 87 and 85 to 87 (Table VI). The tyrosine residue at position 85 was the residue specifically azotized.

Reagent II is a derivative of aminophenyl-pdT, a compound that can be slowly cleaved by staphylococcal nuclease into aminophenyl phosphate and dT (4). Unlike the observations made with the bromoacetamidophenyl derivative of this nucleotide (2), hydrolysis of the diazotized derivative did not occur during the labeling reaction. This was demonstrated by the release of dT upon incubation of purified Peptide N\(_{IIa}\) with staphylococcal nuclease, and by the release of pdTp upon incubation with snake venom diesterase.

Affinity Labeling with Reagents III and IV—Reagent III, which is a derivative of the low affinity competitive inhibitor, dTp-aminophenyl, is somewhat more effective in inactivating staphylococcal nuclease than is Reagent II (Table VII). Maximal enzymatic inactivation requires addition of Ca\(^{++}\), as observed with the synthetic substrate DNA.

Similar random reaction of the ε-amino groups of lysine of staphylococcal nuclease, not accompanied by enzymatic inactivation, occurs on reaction of this protein with \(^{14}C\)-bromocacetamidophenyl affinity labeling reagents (2) and DNS-chloride (15). It is not surprising that such reactions of the exposed lysines are catalytically benign, since alkylation of as many as 9 lysine residues did not cause enzymatic inactivation (16).
The experimental conditions were the same as those described in Table V. Enzyme activity was determined at 24 hours.

| Sample | DNase activity in following molar excess of reagent |
|--------|-----------------------------------------------|
|        | 1.7 | 3.3 | 6.0 | 13.4 | 26 |
| Reagent III |     |     |     |      |    |
| ÷ Ca²⁺ | 62  | 14  | 7   |      |    |
| + Ca²⁺, pdTp | 92  | 88  | 76  |      |    |
| None   | 58  | 32  | 8   |      |    |
| Reagent IV |     |     |     |      |    |
| ÷ Ca²⁺ | 88  | 42  | 20  |      |    |
| + Ca²⁺, pdTp | 96  | 82  | 76  |      |    |
| None   | 97  | 77  | 53  |      |    |

The specific tryptic peptides described in Fig. 9 were purified as described in the text from a sample of 0.9 μ mole of modified protein.

| Peptide | Amountc | Residue azotizedb |
|---------|---------|------------------|
| NIIIa   | 0.04    | Histidine        |
| NIIIb   | 0.07    | Histidine        |
| NIIIc   | 0.09    | Not histidine or tyrosinec |

* Determined by amino acid analysis.

b Determined from ultraviolet spectra by comparison to the spectra described in Fig. 2 and by amino acid composition (Table IX).

The spectrum of Peptide NIIIc is shown in Fig. 10.

served with Reagent I, and the inactivation can be prevented with pdTp.

Affinity chromatography of nuclease modified by reaction with Reagent III demonstrated the presence of two equally yellow protein fractions, the major one appearing as an early inactive peak, the other as a strongly adsorbing and enzymatically active peak (Fig. 8). Peptide maps of tryptic digests of the protein in the major, early, peak contained three yellow spots which were not present in peptide maps of the material in the strongly adsorbed protein peak (Fig. 9). These three yellow peptides were not seen in peptide maps prepared from samples which had been protected with pdTp. It appeared probable, therefore, that in spite of the poor affinity of Reagent III for the enzyme, it had reacted with a specific residue in the binding site of the protein in such a way as to affect the catalytic properties of the enzyme. No uniquely labeled residues could have been present in the protected protein since no distinct yellow spots were seen in the peptide maps derived from this protein; the color of this azoprotein must have resulted from partial reaction of several residues.

The three specifically azotized peptides (Fig. 9) were purified as described under "Methods." The yields obtained by these purification procedures, and the identification of the azo residue in each peptide by ultraviolet spectroscopy, are described in Table VIII. The spectra of Peptides NIIIa and NIIIb indicated the presence of azohistidyl derivatives. The amino acid compositions indicated that these two peptides represented overlapping peptides comprising Residues 36 to 48; azotization occurs at histidine 46.

From the region of the sequence containing Residues 137 to 149, reaction appears to be with tryptophan 140.

The spectrum of Peptide NIIIc (Table VIII) did not resemble that of the model azo compounds prepared from tyrosine or histidine (Fig. 10). This peptide, furthermore, did not show the pH-dependent spectral shifts typical of the azotyrolyl and azohistidyl derivatives. The amino acid composition of Peptide NIIIc demonstrated that this peptide represented Residues 137 to 149 in the primary structure of the protein (Table IX). This region of the sequence contains no tyrosine or histidine. The only residue which could conceivably have reacted with the diazonium reagent to produce a colored product is the tryptophan at position 140. Unfortunately, further characterization of this peptide was not possible because of the small quantities available for study. Preliminary studies with model compounds indicate that tryptophan can indeed react with diazonium reagents to yield colored derivatives.
Fig. 10. Ultraviolet and visible spectra of the Peptide N-XIIIc (3.0 × 10⁻³ m), the tryptic peptide derived from staphylococcal nuclease modified with affinity labeling Reagent III (Table IX and Fig. 9). The tryptophanyl residue at position 140 is presumed to have reacted with the diazonium reagent. The spectrum was obtained at pH 12.2; no significant spectral changes were observed at lower pH values.

Fig. 11. Schematic representation of the residues of staphylococcal nuclease which were found to react with the various affinity labeling diazonium reagents described in Fig. 1. Reagent I reacts entirely with tyrosyl residue at position 115. Reagent II reacts with tyrosine 85. Reagent III reacts specifically with histidine 46 (55%) and with tryptophan 140 (45%). Dashed parallel lines indicate the site of hydrolysis by the enzyme, provided that a phosphodiester bond is present (4). Strong competitive inhibition results if the nucleotide has a free 5'-phosphate.

Reagent IV, although quite ineffective as an affinity labeling reagent, did cause enzymatic inactivation which could be partially prevented by pdTp (Table VII). The major portion of the modified protein passed unretarded through the nucleasespecific affinity column (Fig. 8). Peptide maps derived from tryptic digest of this material, however, contained many very light yellow spots, and the patterns observed in these maps could not be distinguished unequivocally from the patterns of peptide maps prepared from protein reacted with Reagent IV in the presence of pdTp. Therefore, no uniquely labeled active site residues could be identified in the reaction of Reagent IV with staphylococcal nuclease.

DISCUSSION

Localization of the amino acid residues of staphylococcal nuclease to which the diazonium affinity labeling reagents attach, summarized in Fig. 11, contributes toward the elucidation of the functional organization of residues in or near the active site region of the enzyme. These studies, together with those reported for the 14C-bromoacetamidophenyl nucleotide derivatives (2), and other investigations of the effects of chemical modifications of nucleases (17, 18), provide a description of certain stereoechemical properties of this protein in solution which serve as a basis for comparison with the structure as determined by x-ray crystallography.

The tyrosyl residue at position 115, which can be specifically modified with tetraniotromethane in the presence of pdTp and Ca⁺⁺ (17), is labeled selectively with the diazonium derivative of the strong competitive inhibitor, pdTp-aminophenyl. The bromoacetamidophenyl derivative of this same nucleotide inhibitor reacted specifically with the same residue (tyrosine 115) in 15% yield and equally with lysines 48 and 49 in 80% yield (2). The diazonium derivative of dTp-aminophenyl reacts in about 50% yield with histidine 46. These results suggest that there may be some flexibility in the 46 to 49 region of the protein, and that this region as well as that of tyrosine 115 must be near the binding site. The selective cleavage of the 48 to 49 region by trypsin in the presence of pdTp and Ca⁺⁺ (19, 20) is also consistent with these observations. The x-ray crystallographic analysis of staphylococcal nuclease at 2 A resolution supports these interpretations. Models reconstructed from the 2 A data obtained on pdTp-nuclease crystals indicate that the three-dimensional structure of the active site permits precise attachment of the alkylating and diazonium reagents of pdTp-aminophenyl to the hydroxyl and ortho carbon positions, respectively, of tyrosine 115. Furthermore, the crystallographic data demonstrate that a peptide loop containing residues 46 through 49 lies near the binding site. This loop is not rigidly fixed, and attachment of the bromoacetamidophenyl derivative of pdTp-aminophenyl to lysines 48 and 49 is sterically permitted if the labeling derivative is rotated about the 5'-phosphate to the side opposite that which contains tyrosine 115.

The regions of Residues 46 to 49 and of tyrosine 115 must be located in the section of the binding site which interacts with that part of the substrates or inhibitors lying to the 3'-COOH side of the basic structural element required for substrate recognition, R-dpT (4). This region must be very important in the binding function of the enzyme, since the nature of the corresponding parts of substrates or inhibitors determine directly their dissociation constants, but not the catalytic rate constants of substrates (4). The portions of substrates and inhibitors which extend to the "right" of the 5'-phosphate must interact with the region of the enzyme described by the reactions with the affinity labeling reagents derived from pdTp-aminophenyl (Reagent I) and dTp-aminophenyl (Reagent III).

The loss of catalytic activity of the protein derivatives modified by reaction with Reagents I and III cannot be ascribed to the modification of a singularly essential residue. An inhibitor molecule, once irreversibly bound to the active site, may block the access of substrate to this region and thus cause enzymatic inactivation. This is in fact the most likely explanation for the catalytic inerti of these protein derivatives since the functional groups of these affinity labeling Reagents I and III are attached to a portion of the inhibitor which, although directly

A. Arnone, C. J. Bier, F. A. Cotton, E. E. Hazen, D. C. Richardson, and J. S. Richardson, the manuscript describing the high resolution x-ray structure of the nuclease-inhibitor complex is in preparation. A model of this protein has been constructed at the National Institutes of Health by D. C. Richardson and J. S. Richardson; the crystallographic correlations and descriptions presented in the present paper emerged from extensive discussions with them.
The reaction of tryptophan 140 in 50% yield with the diazonium Reagent III suggests that this residue may be in the same general region of the protein described above, and that it must be at least partially accessible to the inhibitor. By a variety of chemical and physical probes, the single tryptophanyl residue of staphylocoecal nuclease was judged as being relatively buried and inaccessible to the aqueous environment (15, 17, 7).

The x-ray crystallographic analysis of this protein places this residue near the lower region of the binding site, and the indole ring appears to be in a partially inaccessible and hydrophobic region. If the diazonium derivative of dTp-aminophenyl is bound rigidly to the protein in the same position as pdTp, or the reagents derived from pdTp-aminophenyl, attachment to the tryptophan is virtually impossible, since a distance of at least 8 A exists between the diazonium group and the indole ring. It is possible, however, that dTp-aminophenyl, which is a very poor inhibitor, is accommodated preferentially in a binding “subsite” adjacent to that occupied by pdTp. This is in accordance with the belief that there are three substrate binding “subsites” on the protein which together can accommodate the ideal inhibitor, pdTp-dTpdT (8). It is not yet known how such a trinucleotide sequence fits into the active site of the protein. However, extending down from the pdTp binding site is a region on the protein structure which could conceivably accommodate the remainder of this 5’-phosphoryl oligonucleotide. If dTp-aminophenyl is placed in this region, the diazonium derivative of this inhibitor could probably reach the tryptophan residue at position 140. Although carbon 2 appears to be the position of the ring most accessible sterically to approach by the reagent, some movement of the tryptophan could possibly permit reaction at other positions in the ring.

Comparisons of the studies with the bromoaecomidophenyl derivatives (2) with the crystallographic analyses (3) also indicate that the dTp-aminophenyl reagents are positioned differently than pdTp in the active site. The bromoaecomyl reagent of dTp-aminophenyl reacted selectively with lysine 24, or possibly with methionine 26 (2). The x-ray crystal structure of the enzyme indicates that neither reaction is sterically possible if the reagent is positioned in the first subsite, that occupied by pdTp. In a slightly lower position, however, the reagent could reach lysine 24. The diazonium derivative of dTp-aminophenyl can also reach histidine 46 from this position.

It is instructive that tryptophan is capable of reaction with the diazonium Reagent III to produce a yellow derivative, since this amino acid is generally neglected in studies of protein modification by diazonium reagents such as diazonium-1H-tetrazole (21, 22). It has been demonstrated previously that indole (23) and 3-indolyacetic acid (24) can react with diazonium compounds to produce yellow derivatives. The reaction products, however, have not been characterized, and the reaction of tryptophan in proteins has not heretofore been described. Preliminary studies indicate that N-acetyltrypotphan and Gly-Gly-Trp readily react in neutral aqueous media with diazonium Reagent III, and with diazonium-1H-tetrazole, yielding derivatives with ultraviolet spectra similar to that described in Fig. 10. It is very possible that the reaction of the tryptophanyl residue of nuclease with a properly aligned active site affinity labeling reagent is assisted by the unique environment of this residue. Reaction of the tryptophan with 2-OH-4-nitrobenzylbromide, or with tetranitromethane (17), does not occur unless the protein is denatured, and physical studies suggest that this residue is relatively inaccessible to the solvent (7, 15, 17). Furthermore, significant reaction does not occur with the diazonium Reagent IV.

It is well known that diazonium compounds can react with lysine residues to form colorless derivatives. With the possible exception of the studies with Reagent IV, where no uniquely reacting colored derivative could be established, it is unlikely that this reaction was of significance in the studies described here. This is supported by the stoichiometry of the reactions, the identification of uniquely labeledazo derivatives, and the observations that lysyl residues react randomly without affecting enzymatic activity with DNS-Cl (15), dinitrodifluorobenzene (4), and acetyl anhydride (16).

The essential nature of tyrosine 85, and its proximity to the substrate binding site and to tyrosine 115, were predicted from studies of modification with tetranitromethane (17), and from studies of intramolecular cross-linking of aminoxyryl residues (16). The bromoaecomidophenyl affinity labeling reagent derived from aminophenyl-pdT reacted exclusively with tyrosine 85 (2). The diazonium compound of the same nucleotide also reacted selectively with tyrosine 85. This residue, which demonstrates unusual reactivity toward tetranitromethane, appears to be very susceptible to chemical reaction and to be well situated stereochemically to interact with the 5’-phosphate portion of aminophenyl pdT. The stereochemistry of the binding site of this protein, as revealed by x-ray crystallographic studies (3), demonstrates that the distances and positions in this region are nearly perfect for the reaction of the bromoaecomyl and diazonium derivatives with the hydroxyl and ortho carbon, respectively, of tyrosine 85.

The bromoaecomidophenyl labeling reagent prepared from aminophenyl-pdT was cleaved by the enzyme during the labeling reaction, perhaps in a concerted fashion through the formation of an activated enzyme-substrate intermediate (2). No such cleavage was observed with the diazonium compound, probably because of the much more facile nature of the diazotization compared to the alkalation reaction. Once the aminophenyl 85 bond is formed, the enzyme is incapable of cleaving phosphodiester bonds. This parallels the lack of catalytic activity of the monoaecomyltyrosyl 85 nucleotide derivative (17).

The affinity labeling studies with diazonium and bromoaecomyl (2) compounds suggest that tyrosine 85 may occupy a position in the tertiary structure of the protein which corresponds to the 5’ carbon side of the basic substrate structure, R-pdT (Fig. 11). This position in a substrate or inhibitor molecule has been thought to correspond to the hydrolytic portion of the active site, the nature of R determining directly the catalytic rate constants but not the binding constants of the compounds (4).

Acknowledgment—Gratitude is expressed to Mrs. Juanita Eldridge for technical assistance.

REFERENCES
1. SINGER, S. J., Advan. Protein Chem., 23, 1 (1968).
2. CUATRECASAS, P., WILCHEK, M., AND ANFINSEN, C. B., J. Biol. Chem., 244, 4316 (1969).
3. ARDSON, A., BIER, C. J., COTTON, F. A., HAazen, E. E., RICHARDSON, D. C., AND RICHARDSON, J. S., Proc. Nat. Acad. Sci. U. S. A., 64, 430 (1969).
4. Unpublished observations.
4. Cuatrecasas, P., Wilchek, M., and Anfinsen, C. B., Biochemistry, 8, 2277 (1969).
5. Cuatrecasas, P., Wilchek, M., and Anfinsen, C. B., Science, 163, 1401 (1968).
6. Cuatrecasas, P., Fuchs, S., and Anfinsen, C. B., J. Biol. Chem., 242, 1541 (1967).
7. Cuatrecasas, P., Taniuchi, H., and Anfinsen, C. B., Brookhaven Symp. Biol., 21, 172 (1969).
8. Cuatrecasas, P., Fuchs, S., and Anfinsen, C. B., J. Biol. Chem., 242, 3063 (1967).
9. Moravek, L., Anfinsen, C. B., Cone, J., and Taniuchi, H., J. Biol. Chem., 244, 497 (1969).
10. Fuchs, S., Cuatrecasas, P., and Anfinsen, C. B., J. Biol. Chem., 242, 4768 (1967).
11. Cuatrecasas, P., Wilchek, M., and Anfinsen, C. B., Proc. Nat. Acad. Sci. U. S. A., 61, 696 (1968).
12. Spackman, D. H., Moore, S., and Stein, W. H., Anal. Chem., 30, 1190 (1958).
13. Taniuchi, H., Anfinsen, C. B., and Sodja, A., J. Biol. Chem., 242, 4752 (1967).
14. Cuatrecasas, P., Fuchs, S., and Anfinsen, C. B., J. Biol. Chem., 242, 4759 (1967).
15. Cuatrecasas, P., Eidelhoch, H., and Anfinsen, C. B., Proc. Nat. Acad. Sci. U. S. A., 58, 2043 (1967).
16. Cuatrecasas, P., Fuchs, S., and Anfinsen, C. B., Biochim. Biophys. Acta, 169, 417 (1968).
17. Cuatrecasas, P., Fuchs, S., and Anfinsen, C. B., J. Biol. Chem., 243, 4787 (1968).
18. Cuatrecasas, P., Fuchs, S., and Anfinsen, C. B., J. Biol. Chem., 244, 406 (1969).
19. Taniuchi, H., Anfinsen, C. B., and Sodja, A., Proc. Nat. Acad. Sci. U. S. A., 58, 1235 (1967).
20. Taniuchi, H., and Anfinsen, C. B., J. Biol. Chem., 243, 4778 (1968).
21. Hominishi, H., Iachimori, Y., Kurihara, K., and Shibata, K., Biochim. Biophys. Acta, 86, 477 (1964).
22. Sokolovsky, M., and Vallee, B., Biochemistry, 5, 3574 (1966).
23. Eagle, H., and Vickers, P., J. Biol. Chem., 114, 193 (1936).
24. Howard, A. N., and Wild, F., Biochem. J., 66, 651 (1957).
Topography of the Active Site of Staphylococcal Nuclease: AFFINITY LABELING WITH DIAZONIUM SUBSTRATE ANALOGUES
Pedro Cuatrecasas

J. Biol. Chem. 1970, 245:574-584.

Access the most updated version of this article at http://www.jbc.org/content/245/3/574

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/245/3/574.full.html#ref-list-1