Ligand-induced Resistance of Staphylococcal Nuclease and Nuclease-T to Proteolysis by Subtilisin, α-Chymotrypsin, and Thermolysin

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SUMMARY

Staphylococcal nuclease, consisting of a polypeptide chain of 149 amino acid residues devoid of cysteine and disulfide bonds, is highly susceptible to digestion by a variety of proteases. In the presence of deoxythymidine 3',5'-diphosphate and calcium ions, marked stability is acquired to digestion by trypsin, subtilisin, α-chymotrypsin, and thermolysin. Nuclease-T, the enzymically active product of trypsin digestion in the presence of ligands, which consists of two noncovalently associated chains (residues 6 to 48 and 49 and 50 to 149), is also resistant to digestion by proteases when ligands are present. These observations indicate the very close similarity of the tertiary structures of nuclease and nuclease-T.

† Staphylococcal nuclease, Foggi strain (5, 12), obtained from Worthington or prepared on a large scale from culture medium (13), was purified by phosphocellulose chromatography (14). Nuclease-T and nuclease-P(6-149) were prepared as described previously (8, 9). Diisopropyl fluorophosphate-treated trypsin and α-chymotrypsin were obtained from Worthington and subtilisin (NAGASE) by Enzyme Development Corporation (64 Wall Street, New York). Thermolysin (15, 16) was kindly donated by Dr. T. H. Jukes and Dr. H. Matsuoka.

* Edman Degradation—The procedures described by Edman and Begg (11) and Blomback et al. (18) were used as follows. All reagents used were analytical grade. Pyridine (Fisher, infrared spectroanalyzed) was redistilled after refluxing with KOH. Dimethylallylamine (K and K Laboratories, Inc.) (19), trifluoroacetic acid (Eastman) (17), phenylisothiocyanate (Eastman) (20), and propionic acid (Fisher) (20) were purified by redistillation after pretreatment to remove traces of aldehyde according to the methods described by Cope and Towe (19), Edman and Begg (17), and Blomback (20). Water used to make the pyridine buffer (18) and 1 N HCl (17) was glass distilled. Ethylene chloride (Fisher), formamide (Eastman), and n-butylacetate (Matheson Coleman and Bell) were used without further purification.

†† Approximately 0.05 μmole of each dried sample was dissolved in 0.2 ml of pyridine-dimethylallylamine-trifluoroacetic acid buffer, pH 9 (18), in a conical 8-ml glass tube with a standard taper glass cap described previously (2). Phenyl isothiocyanate (5 μl) was added to the solution. The tube was flushed with N2 and stoppered. The solution was incubated at 40° for 1 hour and -P1 and -P1b, respectively (9). Nuclease-P(6-149) refers to the derivative previously called nuclease-P(6-149) (9).

1 The abbreviations used are: pdTp, deoxythymidine 3',5'-diphosphate; PTH, 3-phenyl-2-thiohydantoin; PTC, phenylthiocarbamyl; DPN+, 2,4-dinitrophenyl-. Fragments of nuclease are given names that indicate the residues in the structures. For example, nuclease-T-P1P and -P1(8, 9) are now termed nuclease-T-P1P(6-49) and -P1(49-50-149). The latter contains a mixture of nuclease-T-P1P(6-49) and -P1(49-149), formerly termed nuclease-T-P1P, tibility of ribonuclease A in the presence of ligands has recently been reported by Markus et al. (11). In this communication the ligand-induced resistance of both nuclease and nuclease-T to proteolysis by subtilisin, α-chymotrypsin, and thermolysin is described, and the results are considered in relation to the tertiary structures of these macromolecules.

EXPERIMENTAL PROCEDURES

Staphylococcal nuclease, Foggi strain (5, 12), obtained from Worthington or prepared on a large scale from culture medium (13), was purified by phosphocellulose chromatography (14). Nuclease-T and nuclease-P(6-149) were prepared as described previously (8, 9). Diisopropyl fluorophosphate-treated trypsin and α-chymotrypsin were obtained from Worthington and subtilisin (NAGASE) by Enzyme Development Corporation (64 Wall Street, New York). Thermolysin (15, 16) was kindly donated by Dr. T. H. Jukes and Dr. H. Matsuoka.

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extracted five times with approximately 1 ml of benzene. The aqueous phase was lyophilized in the tube. The dried material was washed three times with approximately 1 ml of ethyl acetate and dried again under reduced pressure. To the dried sample in the tube approximately 100 μl of trifluoroacetic acid was added, and the solution was incubated at 80° for 10 min under N₂. The volume of the solution was then reduced to approximately 50 μl by streaming N₂ through the tube. Approximately 1 ml of 1-chlorobutane was added to the solution. After centrifugation in a clinical centrifuge, the supernatant solution was removed. The residue was washed once with approximately 1 ml of 1-chlorobutane. The residue was then cooled in Dry Ice-alcohol and dried over P₂O₅ and KOH under reduced pressure overnight to be used for the next cycle. The supernatant solution and the 1-chlorobutane wash were combined in another 8-ml conical tube and evaporated to dryness under a stream of N₂, and 0.2 ml of 1 M HCl was added. The solution was incubated at 80° for 10 min under N₂. After cooling, the solution was extracted three times with approximately 0.5 ml of ethyl acetate. The extracts were combined and evaporated to dryness under a stream of N₂. The dried sample was dissolved in a small volume of ethylene chloride (approximately 20 μl) and applied on a silica gel plate containing an ultraviolet fluorescent indicator (Brinkman, thin layer chromatography plate Silica Gel F-254). Thin layer chromatography was performed at 25° with the use of Solvents D and E described by Edman and Sjöquist (20) with 3-phenyl-2-thiohydantoin amino acids obtained from Sigma as standards. Spots were located both by the extinction of fluorescence (16, 18) and by the iodine-azide reagent described by Sjöquist (21).

Quantitative Determinations—The amounts and concentrations of nuclease and its derivatives were determined by amino acid analysis by the method of Spackman, Moore, and Stein (22), as described previously (2). The enzymic activity of nuclease was assayed by the method of Cuatrecasas, Fuchs, and Anfinsen (23). All other materials and methods have been described elsewhere (2, 3, 8, 9), unless otherwise specified.

RESULTS

Preparation of Nuclease-S—As reported previously (8), the enzymatic activities of nuclease in digestion mixtures containing pdTp and Ca²⁺ remain at approximately 80% of the original value after 2 hours of incubation with subtilisin or a-chymotrypsin, whereas only a small percentage of the original activity remains in the absence of the ligands 3 Two-dimensional peptide maps of the digestion mixtures indicated the presence of large fragments (8). The characterization of the large fragments is described below.

Approximately 35 mg of nuclease were dissolved in 3.5 ml of 0.05 M NH₄HCO₃, pH 8, containing 0.01 M CaCl₂ and 3.5 mg of pdTp. To the solution, 35 μl of a freshly prepared 1% solution of subtilisin were added, and incubation was performed for 34 hours at 25°. Digestion was stopped by lyophilization. The dried sample was subjected to chromatography on a phosphocellulose column, as described previously (8, 9). The elution pattern is shown in Fig. 1. An absorbance peak having enzymic activity against both RNA and DNA appeared at the position expected for native nuclease (8, 9). The yield of enzymically active material was approximately 57% on the basis of absorbance at 280 μm. The fractions were pooled and lyophilized. The material thus obtained is designated “nuclease-S.”

Properties of Nuclease-S—Nuclease-S yielded a single zone upon electrophoresis on both “standard” and “pH 2.3” polyacrylamide gels and had the same mobilities as native nuclease (8, 9). End group analysis by the dinitorfluorobenzene method (2) yielded only bis-DNP-lysine in a yield of 35% (uncorrected) on the basis of a molecular weight of 16,300 (see below), as previously observed for nuclease-P₁₄₉ (9). The first step of Edman degradation of nuclease-S also yielded ε-phenylthiocarbamyl-lysine-PTH and a trace of alanine-PTH. The two-dimensional peptide map of a tryptic digest of nuclease-S contained all major spots accounting for fragments produced from Residues 6 through 149 (2, 3, 5), as observed with nuclease-P₁₄₉ (9), including Component P₁₂₅ (9, 3), which consists of Residues 137 to 149. Hydrazinolysis (24) of nuclease-S gave negative results, consistent with glutamine (Residue 149) as the COOH-terminal residue.

To establish that nuclease-S consists of a single polypeptide chain, a lyophilized digest of nuclease (10 mg) with subtilisin in the presence of pdTp and Ca²⁺, prepared as described above, was dissolved in 0.3 ml of 50% CH₃COOH and subjected to gel filtration on a Bio-Gel column by the method used for the separation of the two components of nuclease-T (8). The elution pattern is shown in Fig. 2. Only one enzymically active absorbance peak (at 280 μm) was observed in the fractions in which large polypeptide chains such as nuclease and nuclease-P₁₂₅ are resolved.

3 Incubation for 2 hours after further addition of subtilisin and a-chymotrypsin to the ligand-containing incubation mixtures caused only a slight (approximately 5%) further decrease in enzymic activity.

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Fig. 1. Chromatography of nuclease digested with subtilisin in the presence of pdTp and Ca²⁺ on a phosphocellulose column. The lyophilized sample (see the text) was dissolved in approximately 3 ml of 0.3 M ammonium acetate, pH 6, and applied on a phosphocellulose (Whatman, chromedia, Pl) (2) column (1 X 7 em) equilibrated with the same buffer. Gradient elution was performed at a flow rate of 50 ml per hour at 4°, using a Varigrad containing 150 ml each of 0.3 M, pH 6, and 1 M, pH 8, ammonium acetate buffers in the first and second chambers, respectively. The absorbance at 280 μm of fractions collected every 6 min were determined ( ), and enzyme activity was assayed with the use of both DNA (A) and RNA ( ) as substrates (3). The conductivity (C) and pH (D) were also determined as described previously (9).
are expected to appear (9) (Fig. 2). The material obtained from the pool of fractions gave bis-DNP-lysine by end group analysis. Since both residues 5 and 6 of nuclease are lysine (3), the above results indicated that nuclease-S was a fragment containing residues 5 to 149 or 6 to 149. Amino acid analyses were consistent with both assignments (Table I).

Two cycles of Edman degradation of nuclease-S were performed, with the use of nuclease and nuclease-P(41-149) as controls. The products of the first cycle were, for nuclease-S, e-PTC-lysine PTH; nuclease, alanine PTH; nuclease-P(41-149), e-PTC-lysine-PTH. Those of the second cycle were, for nuclease-S, leucine-PTH and e-PTC-lysine-PTH; nuclease, threonine-PTH; nuclease-P(41-149), leucine-PTH. The results with the control samples were consistent with the assigned sequences (3, 8, 9). The presence of both lysine and leucine as 2nd residues of nuclease-S indicated that nuclease-S is a mixture of two fragments postulated above.

Further support for the absence of cleavage between Residues 6 and 149 of nuclease-S was obtained as follows. Approximately 10 mg of nuclease-S were digested with 0.1 mg of trypsin in the presence of pdTp (2 mg) and Ca++ (0.01 M) in 1 ml of 0.05 M NH₄HCO₃, pH 8, for 2 hours at 25° (8, 9). Soy bean trypsin inhibitor (0.4 mg) was then added to the mixture, followed by lyophilization. The dried sample was dissolved in 0.6 ml of 50% CH₃COOH and subjected to gel filtration on a Bio-Gel P-20 column (1.2 x 200 cm) equilibrated with 50% CH₃COOH at 4°. The elution was performed at a flow rate of 7 ml per hour with 50% CH₃COOH at 4°. The absorbances at 280 μm of fractions collected every 30 min were determined. The elution pattern obtained was similar to that obtained for the separation of the samples of nuclease-S (0.0090 μmole) and nuclease-C (0.0011 μmole) were hydrolyzed for 20, 48, and 72 hours to permit correction for destruction of threonine and serine as described previously (2). The results shown for nuclease-S-P(6-48), nuclease-S-P(48, 50-149), nuclease-C, nuclease-C-P(41-149), and nuclease-C-P(46, 50-149) were obtained after 20 hours of hydrolysis. The values presented for each amino acid are molar ratios relative to phenylalanine. The assumed contents of phenylalanine are based on the amino acid compositions of nuclease-S, nuclease-S-P(6-48), nuclease-S-P(48, 50-149), nuclease-C, nuclease-C-P(41-149), and nuclease-C-P(46, 50-149).

### Table I

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nuclease-S</td>
</tr>
<tr>
<td>Lysine</td>
<td>10.0 (10)</td>
</tr>
<tr>
<td>Histidine</td>
<td>4.7 (4)</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.8 (5)</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>13.2 (14)</td>
</tr>
<tr>
<td>Threonine</td>
<td>7.8 (8)</td>
</tr>
<tr>
<td>Serine</td>
<td>3.8 (4)</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>18.7 (18)</td>
</tr>
<tr>
<td>Proline</td>
<td>4.8 (6)</td>
</tr>
<tr>
<td>Glycine</td>
<td>10.0 (10)</td>
</tr>
<tr>
<td>Alanine</td>
<td>13.7 (13)</td>
</tr>
<tr>
<td>Valine</td>
<td>9.2 (9)</td>
</tr>
<tr>
<td>Methionine</td>
<td>3.6 (4)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5.4 (5)</td>
</tr>
<tr>
<td>Leucine</td>
<td>11.9 (11)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>7.2 (7)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.0 (3)</td>
</tr>
<tr>
<td>Tryptophan*</td>
<td>1 (1)</td>
</tr>
</tbody>
</table>

* See the text.
| Nuclease-S-P(6-48) and C-P(41-149) were mixtures of residues 49 to 149 and 50 to 149, as in the case of nuclease-T-P(41, 50-149) (judged by peptide maps of the trypsin digests) (9).
| Low value presumably due to destruction during acid hydrolysis.
| Qualitative determination (see above).
nuclease-T-P(6-149) and -P(60, 50-149) (8, 9). Two fractions corresponding to nuclease-T-P(6-48) and -P(60, 50-149) were pooled and lyophilized. These are referred to below as nuclease-S-P(6-48) and -P(60, 50-149), respectively. The results of amino acid analysis (Table I), peptide mapping of tryptic digests (8, 9), and polyacrylamide gel electrophoresis at pH 2.3 (8, 9) indicated that these fragments are identical with nuclease-T-P(6-48) and -P(60, 50-149) (0), respectively. The yields of nuclease-S-P(6-48) and -P(60, 50-149) from nuclease-S were approximately 60%. The first cycle of Edman degradation of nuclease-S-P(6-48) yielded \( \epsilon \)-PTC-lysine-PTH, and the second cycle produced leucine-PTH, consistent with the results with nuclease-S described above. The specific activities of nuclease-S with both DNA and RNA as substrates were the same as those of intact nuclease (Table II), as found earlier (9) with nuclease-P(6-149).

Preparation and Characterization of Nuclease-C—Approximately 20 mg of nuclease were digested in 2 ml of 0.05 M \( \text{NH}_4 \text{HCO}_3 \), pH 8, with 0.5 mg of \( \alpha \)-chymotrypsin in the presence of 2 ml of pdTp and 0.01 M \( \text{Ca}^{2+} \) for 3 hours at 25°, followed by lyophilization. The dried sample, fractionated by chromatography on a phosphocellulose column (8, 9), yielded an elution pattern exactly as shown in Fig. 1. The enzymically highly active fractions were pooled and lyophilized. The material is referred to as nuclease-C.

This sample of nuclease-C was examined by polyacrylamide gel electrophoresis on "standard" and "pH 2.3" gels (8, 9), and NH₂-terminal groups were determined by the dinitrofluorobenzene method. Two-dimensional peptide mapping of a tryptic digest and amino acid analysis (Table I) were also performed, as described above. The results were the same as those obtained with nuclease-S, indicating essential identity of nuclease-C with nuclease-S. The presence of Peptide F25 (Residues 137 to 149) (2, 3) on the peptide map of the tryptic digest of nuclease-C indicated again that the COOH-terminal region of nuclease in nuclease-C is intact. Hydrazinolysis of nuclease-C (1 mg) also gave negative results, consistent with COOH-terminal glutamine.

Since \( \alpha \)-chymotrypsin has been shown to catalyze the unusual cleavage of the bond between Residue 5 (lysine) and 6 (lysine) of nuclease (3), the NH₂-terminal lysine residue found in nuclease-C was assumed to be Residue 6 (see below).

The absence of bond cleavage between Residues 6 to 149 of nuclease-C was also confirmed by gel filtration on a Bio-Gel column in 50% \( \text{CH}_3 \text{COOH} \) (see Fig. 2) and by the separation of nuclease-C-P(6-149) and -P(60, 50-149) from the trypsin digest of nuclease-C in the presence of pdTp and \( \text{Ca}^{2+} \), as described above for nuclease-S. The identity of nuclease-C-P(6-149) and -P(60, 50-149) with nuclease-T-P(6-149) and -P(60, 50-149) was proved by polyacrylamide gel electrophoresis at pH 2.3, amino acid analysis (Table I), and peptide mapping of tryptic digests. The first step of Edman degradation of nuclease-C-P(6-149) gave \( \epsilon \)-PTC-lysine-PTH. However, the second step also yielded \( \epsilon \)-PTC-lysine-PTH, as well as leucine-PTH, suggesting the fractional presence of lysine Residue 5 in nuclease-C as in the case of nuclease-S. Thus nuclease-C appears to be a mixture of two derivatives: Residues 5 to 149 and 6 to 149. Although cleavage of the bond between Residue 4 (threonine) and 5 (lysine) by \( \alpha \)-chymotrypsin is very unusual, such instances have been reported (26, 27).

The specific activity of nuclease-C was the same against DNA and RNA as the activities of nuclease and nuclease-S (Table II).

Digestion of Nuclease with Thermolysin—In preliminary experiments, approximately 70% of the original nuclease activity remained after 3 hours of incubation with thermolysin in the presence of pdTp and \( \text{Ca}^{2+} \), whereas almost all of the original activity disappeared in the absence of ligands.

To characterize the digestion product, 10 mg of nuclease were digested in 2 ml of 0.05 M \( \text{NH}_4 \text{HCO}_3 \), pH 8, with 1 mg of thermolysin in the presence of 1 mg of pdTp and 0.01 M \( \text{Ca}^{2+} \) for 4 hours at 25°. The digestion was stopped by the addition of 100 \( \mu l \) of 5 N \( \text{CH}_3 \text{COOH} \) to the mixture, followed by lyophilization. The dried sample was dissolved in 1 ml of 50% \( \text{CH}_3 \text{COOH} \) and subjected to gel filtration on a Bio-Gel column (2 x 200 cm) in 50% \( \text{CH}_3 \text{COOH} \), as described above. Fractions eluted at a flow rate of 4 ml per hour at 4° and collected every 30 min were read with absorbance at 280 nm. Fractions forming the 2 absorbance peaks that emerged (Fractions 48 to 53 and 159 to 198) were lyophilized. The first peak was of high molecular weight, comparable to nuclease (8, 9), whereas the second peak corresponded to relatively small fragments. Enzymic activity was found only in the material from the first peak, and the specific activity was the same as that of nuclease. End group analysis gave, exclusively, DNP-alanine. The material was also digested with trypsin in the presence of pdTp and \( \text{Ca}^{2+} \), as described previously (8). After 2 hours of incubation, soybean trypsin inhibitor was added to stop the digestion (8) and the mixture was lyophilized. A peptide map of the digest was the same as that obtained with native nuclease (8) and included the component previously identified as Residues 1 to 5 (8, 9). The enzymatically active material was therefore unchanged nuclease, and it may be concluded that all peptide bonds of nuclease are resistant, in the presence of pdTp and \( \text{Ca}^{2+} \), to the action of thermolysin.

Digestions of Nuclease-T with Subtilisin, \( \alpha \)-Chymotrypsin, and Thermolysin in Presence of pdTp and \( \text{Ca}^{2+} \)—Previous observations on the immunological properties, the content of helical structure, and the relative affinities of nuclease-T and nuclease for DNA and RNA (8, 9) have suggested that the tertiary structures of the two macromolecules are closely related. The rates of decrease in the enzymic activities of nuclease and nuclease-T during trypsin digestion in the absence of the ligands were qualitatively similar (Table III). A further investigation of this presumed similarity has been carried out by study of the relative susceptibilities of nuclease and nuclease-T to subtilisin, \( \alpha \)-chymotrypsin, and thermolysin in the presence and absence of ligands.

To avoid possible contamination with traces of nuclease-P(6-149) in nuclease-T (8, 9), which might influence the interpretation of the results of activity assays, nuclease-T' was reconstituted from nuclease-P(6-149) and nuclease-C, respectively.
essentially inactive nuclease-T-P(4, 50-146) and nuclease-T-P(49, 50-146) (8, 9). This material was used in the kinetic study of proteolytic digestion of nuclease T, as described in Table IV. The enzymic activity of nuclease-T remained unchanged, in the presence of pdTp and Ca++, after 100 min of digestion with either subtilisin, α-chymotrypsin, or thermolysin (Table IV). In the absence of

**Table III**

Comparison of rates of decrease in enzymic activities of nuclease and nuclease-T during trypsin digestion in absence of ligands

A digestion mixture containing, in 0.1 ml of 0.05 M NH₄HCO₃, pH 8, 500 μg of nuclease or nuclease-T and 10 μg of trypsin was incubated at 25°. Aliquots (5 μl each) were taken at times indicated, and soy bean trypsin inhibitor (20 μg each) was added to terminate the digestion. The enzymatic activity was assayed with suitably diluted aliquots. The values are presented as the percentage of the activity present at initial time.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Initial activity toward DNA at 5 min</th>
<th>Initial activity toward DNA at 10 min</th>
<th>Initial activity toward DNA at 15 min</th>
<th>Initial activity toward DNA at 20 min</th>
<th>Initial activity toward DNA at 30 min</th>
<th>Initial activity toward DNA at 60 min</th>
<th>Initial activity toward DNA at 120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease</td>
<td>62</td>
<td>33</td>
<td>19</td>
<td>13</td>
<td>3</td>
<td>0.4</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Nuclease-T</td>
<td>84</td>
<td>57</td>
<td>33</td>
<td>17</td>
<td>12</td>
<td>8</td>
<td>2</td>
</tr>
</tbody>
</table>

* The changes in activity toward RNA paralleled those toward DNA.

**Table V**

Amino acid compositions of samples of reisolated nuclease-T after incubation with subtilisin or α-chymotrypsin, in presence of pdTp and Ca++ (nuclease-T-S and nuclease T C, respectively) and samples of nuclease-T-P(4, 50) and nuclease-T-P(49, 50-146) separated from reisolated nuclease-T after incubation with thermolysin in presence of pdTp and Ca++ (nuclease-T-Th-P(4, 50) and nuclease-T-Th-P(49, 50-146), respectively)

Samples of 0.007 to 0.028 μmole were hydrolyzed for 20 hours (2). The values presented are molar ratios relative to phenylalanine, the residue numbers of which were selected as described in Table I. Theoretical values are presented in parentheses. Tryptophan was not determined (see the text). The method of the separation of nuclease-T-Th P(4, 50) and P(49, 50-146) was the same as that described earlier (8) (see also the text).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Nuclease-T-S</th>
<th>Nuclease-T-C</th>
<th>Nuclease-T-Th-P(4, 50)</th>
<th>Nuclease-T-Th-P(49, 50-146)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>21.8 (21-22)*</td>
<td>19.5 (21-22)*</td>
<td>6.6 (7)</td>
<td>16.7 (14-15)*</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.6 (4)</td>
<td>3.1 (4)</td>
<td>1.6 (2)</td>
<td>2.4 (2)</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.9 (5)</td>
<td>4.7 (5)</td>
<td>1.0 (1)</td>
<td>4.1 (4)</td>
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<td>6.5 (8)</td>
<td>3.8 (5)</td>
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<tr>
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<td>3.8 (4)</td>
<td>trace (0)</td>
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<tr>
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<td>15.3 (15)</td>
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<tr>
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<td>2.2 (2)</td>
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* See Footnotes 1 and 5 to the text.
the ligands, a rapid decrease in the enzymatic activity was observed (Table IV). The peptide map of nuclease-T after digestion with the proteolytic enzymes in the presence of pdTp and Ca++, for 2 hours at 25°, indicated the presence of large fragments, and only very small amounts of extensively digested material. Characterization of the large fragments was performed as follows.

Solutions containing 1 ml of 0.05 M NaH2PO4, pH 8, 8 mg of nuclease-T, 2 mg of pdTp, and 0.01 M CaCl2 were incubated with 0.35 mg of subtilisin, α-chymotrypsin, or thermolysin for 2 hours at 25° and then lyophilized. Each dried sample was fractionated on a phosphocellulose column (1 × 2.5 cm) by gradient elution as described in the legend to Fig. 1. Small amounts of extensively digested fragments were found in the initial fraction (see Fig. 1) as judged by peptide mapping. The fractions composing the second absorbance peak (see Fig. 1) were pooled and lyophilized. The recovery of enzymically active material was approximately 60% with each digest on the basis of absorbancy at 280 nM. Polyacrylamide gel electrophoresis at pH 2.3 indicated the same zones as those observed with nuclease-T (8).

End group analyses, by the dinitrofluorobenzene method, of the samples obtained from the proteolytic digests yielded only bis-DNP-lysine. The peptide maps of the tryptic digests of the samples showed the same spots as those observed with the tryptic digest of nuclease-T. The amino acid composition of the samples was also consistent with that of nuclease-T (Table V). The above observations indicate that most of the original nuclease-T remained intact after 2 hours of digestion in the presence of pdTp and Ca++.

DISCUSSION

The protection of nuclease against proteolytic cleavage by the binding of ligands can be explained in two ways (9). Sensitive bonds in the binding site may be masked by the ligands, or a decreased flexibility (28) or “motility” (29) may be induced throughout the structure of the macromolecule. The structural rigidification implied by the latter alternative may not be shown by hydrodynamic or spectroscopic techniques, which measure the average conformational state in a population. However, the measurement of the exchangeability of hydrogen atoms has clearly indicated that motility is markedly decreased when ligands are bound to a number of proteins in solution (10, 30, 31).

The limited attack of ligand-protected nuclease by trypsin, subtilisin, and α-chymotrypsin and the complete protection against thermolysin, which has specificity for peptide bonds involving hydrophobic amino acids (32), strongly support the view that generalized proteolytic attack of nonprotected protein takes place only on that small fraction of the population which is partially unfolded at any moment (33, 34) or on limited portions of the amino acid sequence in the three-dimensional structure which are sufficiently flexible to form “enzyme-substrate complexes” (35) with proteolytic enzymes. In the former case, such a minor fraction would not be detected by the usual physical measurements and is only made evident by such “historical” methods as hydrogen exchange or study of susceptibility to proteolysis, which can “record” infrequent deviations from a normally compact conformation.

The similarity of the effects of ligands on proteolytic susceptibility of nuclease and nuclease-T suggests that those two substances possess closely similar three-dimensional structures in solution. The results support earlier data on helical content, immunological reaction, sedimentation velocity, and affinity toward DNA and RNA (8, 9). Since all peptide bonds in nuclease-T were resistant to cleavage by trypsin, subtilisin, α-chymotrypsin, and thermolysin in the presence of pdTp and Ca++, the two ends of the discontinuous region of the polypeptide chain of nuclease-T must not be widely separated. The NH₂ terminus of nuclease (Residues 1 to 5) is susceptible to proteolysis by subtilisin and α-chymotrypsin as well as by trypsin (8, 9) in the presence of pdTp and Ca++, indicating ligand-insensitive flexibility in this portion of the nuclease structure.

REFERENCES

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Ligand-induced Resistance of Staphylococcal Nuclease and Nuclease-T to Proteolysis by Subtilisin, α-Chymotrypsin, and Thermolysin
Hiroshi Taniuchi, Ladislav Morávek and Christian B. Anfinsen

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