An Experimental Approach to the Study of the Folding of Staphylococcal Nuclease*

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SUMMARY

Two inactive fragments of staphylococcal nuclease (a protein consisting of 149 residues, devoid of sulphydryl groups and disulfide bonds) have been prepared; one (nuclease-P\(_{1-126}\)) contains Residues 1 through 126, and the other (nuclease-P\(_{127-149}\)) contains Residues 127 through 149. Studies of nuclease-P\(_{1-126}\), employing circular dichroism, optical rotation, immunodiffusion, and solvent perturbation, reveal a loose and disorganized structure very different from that of nuclease. Nuclease-P\(_{127-149}\) is also structureless, as determined by measurements of circular dichroism and the fluorescence spectrum of the tryptophan residue.

It was shown previously that the cleavage of nuclease by trypsin in the presence of thymidine 3',5'-diphosphate and calcium ions yields nuclease-P\(_{1-49}\) (Residues 6 through 149), which has full enzymic activity and a conformation similar to that of nuclease. An enzymically active (8 to 10% of the native level) derivative, nuclease-T, composed of two fragments bonded noncovalently, nuclease-T-P\(_{49,126}\) (Residues 6 to 48) and nuclease-T-P\(_{49,149}\) (mixture of Residues 49 through 149 and 50 through 149), is subsequently formed. The association of separated nuclease-T-P\(_{49,126}\) and nuclease-T-P\(_{49,149}\) regenerates both enzymic activity (of nuclease-T) and a three-dimensional structure similar to that of nuclease.

When the fragments are mixed, noncovalent bonding of nuclease-P\(_{1-126}\) with nuclease-T-P\(_{49,149}\), but not with nuclease-P\(_{127-149}\), is indicated by generation of enzymic activity, increase in ellipticity (from 215 through 240 m\(^{-1}\)), positive reaction with antinuclease serum, and a shift in the emission maximum of fluorescence of the single tryptophan residue.

Trypsin digestion of noncovalently bonded nuclease-P\(_{1-126}\) and nuclease-T-P\(_{49,149}\) in the presence of thymidine 3',5'-diphosphate and calcium ions yields nuclease-T and small fragments derived from Residues 1 through 5 and 49 through 126. Thus, nuclease-T-P\(_{49,149}\) appears to bind only with the nuclease-T-P\(_{1-126}\) portion of nuclease-P\(_{127-149}\) (Residues 6 through 48) to form a conformation resembling that of nuclease-T, whereas the other portions of the polypeptide chain of nuclease-P\(_{1-126}\) protrude from the nuclease-T structure as essentially random coils.

We conclude that almost the entire amino acid sequence of nuclease is essential to determine unique folding and that the native conformation of nuclease cannot be formed during synthesis until the polypeptide chain has been extended beyond Residue 126. The results are not consistent with the concept of sequential folding from the NH\(_2\) terminus during assembly of the chain.

Although it is generally assumed that protein structure forms spontaneously on the basis of the genetic information in the primary amino acid sequence (2, 3), a suggestion has recently been made by Phillips (4) that folding occurs in a sequential manner as the chain is assembled from its NH\(_2\) terminus (5, 6). A test of this suggestion may be made by examining the structures of polypeptides of various lengths, derived from the NH\(_2\) terminus of protein chains, prepared either by isolation from the native proteins or by organic synthesis. Although RNase-S-peptide (Residues 1 to 20) of ribonuclease-S (7) shows a small negative circular dichroism with a peak at 225 to 227 m\(^{-1}\) (8, 9), this property is not influenced by changes of temperature or pH in a manner to be expected of \(\alpha\)-helical structure (9). Available data (8, 9, 12) suggest that RNase-S-peptide is essentially devoid of helix but assumes helical character when combined noncovalently with RNase-S-protein (Residues 21 through 124).

Furthermore, S-protein and S-peptide are involved in a mutual, cooperative interaction which introduces a conformational stability to the complex that is not present in either of the two components alone (13). A similar cooperativity exists with derivatives of staphylococcal nuclease (14, 15). A derivative lacking the first 5 residues of the structure (nuclease-P\(_{1,126}\)\(^{1}\)) has full activity and a structure very similar to the native enzyme (16-19). Limited cleavage of this derivative with trypsin yields two fragments (nuclease-T-P\(_{49,149}\), Residues 6 through 48, and nuclease-T-P\(_{1-126}\), Residues 49 through 149 and 50 through 149) of nuclease-T-P\(_{127-149}\).

1 Designations of nuclease fragments are changed from those published previously (14, 15). The residues comprising the fragments are indicated in parentheses. For example, nuclease-T-P\(_{49,126}\) and P\(_{49,126}\) are now designated nuclease-T-P\(_{1-126}\) and P\(_{1-126}\), respectively; P\(_{49,126}\) and P\(_{49,126}\) are P\(_{49,126}\) and P\(_{49,126}\), respectively; and nuclease-P\(_{127-149}\) is nuclease-P\(_{1-126}\). Other abbreviations used are: pdTp, deoxythymidine 3',5'-diphosphate; DNP-, dinitrophenyl-...
149), both of which are devoid of activity and appear to be structureless. The two fragments combine reversibly to regenerate approximately 8% of native enzymic activity and most of the characteristic helical, spectral, and chemical properties of nuclease, much as in the case of RNase-S.

The present communication describes the isolation and characterization of a large, essentially structureless fragment containing 85% of the polypeptide chain of nuclease and including the NH₂ terminus. The nuclease-T-P(1,6,149) portion of this fragment combines noncovalently with fragment nuclease-T-P(6,10,149) to form an active complex with the physical characteristics of nuclease-T. Digestion of this complex with trypsin in the presence of specific ligands produces nuclease-T with the removal of the superfluous or redundant portions of the sequence.

The results presented in this paper argue against the ordered folding of the polypeptide chain from the amino to the carboxyl terminus during synthesis. It should be emphasized that these studies do not permit further conclusions about the mechanism of the interaction between nuclease-T(1,126) and nuclease-T-P(6,10,149) or the pathway of the aggregation and folding processes. The results may be considered only in terms of the final equilibrium state, which we interpret to be the thermodynamically most stable conformation.

EXPERIMENTAL PROCEDURES

Purified staphylococcal nuclease (Foggi strain) (20, 21) was obtained as described previously (15, 22). The protein was trifluoroacetylated as described previously (18, 23). Nuclease-T and its components, nuclease-T-P(6,149) and nuclease-T-P(6,10,149) (actually a mixture of nuclease-T-P(6,149) and nuclease-T-P(6,10,149)) were prepared as reported earlier (14, 15).

The quantitative determination of nuclease and its derivatives was made on the basis of amino acid analyses by the method of Spackman, Moore, and Stein (24) unless otherwise specified. Light absorption was measured with a Zeiss PMQII spectrophotometer. A Cary recording spectrophotometer, model 15, was used for the study of difference spectra according to the method of Hendrikvitz and Laskowski (25).

Fluorescence studies were performed in a 3-ml quartz cuvette with an Aminco-Bowman spectrophotofluorometer, made available through the kindness of Dr. R. F. Chen, National Institutes of Health. The machine was equipped with an RCA P28 photomultiplier tube and a grating blazed at 300 mÅ, giving an essentially linear response over the 280 to 400 mÅ range (26). The excitation beam was passed through a horizontally polarizing filter to reduce scatter (27) and the unfiltered emission was recorded by an American Instrument X-Y recorder. Both spectrophotometric and fluorometric studies were performed at 25°.

Optical rotatory dispersion and circular dichroism were measured with a Cary model 6001 recording spectropolarimeter equipped with a circular dichroism attachment. A jacketed cell holder designed by Dr. R. Resnik of the National Institutes of Health was used in combination with a Haake (type F) bath for the measurement of optical rotation as a function of temperature. The sample solution, in a 3-ml quartz cuvette of 1-cm light path placed in the cell holder, was equilibrated at a given temperature for 15 min before measurement. The temperature was measured with a thermometer placed in the Haake bath. Other details of measurement and calculation of mean residue rotation have been described previously (20).

The mean residue molecular weights of fragments were calculated on the basis of the amino acid sequences. The same cuvette was also used for the measurement of circular dichroism at 25°. The buffer used for the measurements of light absorption, fluorescence, circular dichroism, and optical rotation was 0.05 M Tris-HCl-0.1 M NaCl, pH 7.

Partially purified antinuclease serum, provided by Dr. Sara Fuchs (28), was used for double immunodiffusion by the method of Ouchterlony (29), with the use of Hyland immuno-plates (Pattern B). Nuclease activity was assayed by the method described by Cuatrecasas, Fuchs, and Anfinsen (30), with DNA as substrate. Other methods and materials have been described elsewhere (14, 15, 17, 18) unless otherwise specified.

Preparation of Nuclease-P(1,126) (Residues 1 through 196)—Approximately 10 µmoles of trifluoroacetylated nuclease were dissolved in 20 ml of 0.05 M NH₄HCO₃, and the pH of the solution was adjusted to 8 by the addition of 1 M NH₄OH. Forty microliters of a 1% trypsin solution (diisopropylfluorophosphate-treated, Worthington) were added. After incubation for 10 min at 25°, 400 µl of a solution of 1% soybean trypsin inhibitor (Worthington) were added to the mixture to terminate the digestion, and the mixture was lyophilized. The dried sample was dissolved in 20 ml of 1 m piperidine, and after incubation at 0° for 24 hours the solution was lyophilized. The dried sample was dissolved in 10 ml of 0.3 M ammonium acetate, pH 6, and applied to a phosphocellulose column (1 x 13 cm, Whatman, Chromedia, p 1) equilibrated with the same buffer. Gradient elution was performed at 4° with the aid of a Varigrad containing 300 ml of 0.3 M ammonium acetate, pH 6, and 1 M ammonium acetate, pH 8, in the first and second chambers, respectively, at a flow rate of approximately 50 ml per hour. The elution pattern is shown in Fig. 1.

The fractions comprising the first absorbance peak to emerge were enzymically inactive and contained nuclease-P(12,149), which was further treated as described below.

The enzymically active fractions comprising the second absorbance peak (effluent, 280 to 420 ml (Fig. 1)) were pooled and dialyzed against 0.05 M Tris-HCl, pH 8, and concentrated by lyophilization.

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**Fig. 1.** Chromatography on a phosphocellulose column of the deblocked mixture obtained from partial digestion of trifluoroacetylated nuclease with trypsin. Fractions were collected every 15 min and absorbance was determined at 280 mÅ (○), as well as pH (△) and conductivity (▲), as described previously (15). Enzymic activity (◇) was assayed with suitably diluted aliquots. The presence of two peaks is presumably caused by incomplete removal of trifluoroacetylated groups. Native nuclease treated similarly, but without trypsin digestion, showed a similar pattern (see the text).
lyophilized. The dried material was dissolved in 2 ml of 0.01 \text{M} acetic acid-0.1\% ammonium acetate buffer and applied on a Sephadex G-75 column (2 x 188 cm) equilibrated with the same buffer. Gel filtration was performed at a flow rate of 8 ml per hour at 25°C with the same buffer. The elution pattern is shown in Fig. 2. Two poorly separated fractions emerged. The first (effluent, 170 to 210 ml) possessed enzyme activity, and the second (effluent, 211 to 240 ml) was essentially inactive (Fig. 2). The fractions comprising each peak were pooled and lyophilized. The sample obtained from the second peak was purified further by a second gel filtration on Sephadex G-75 as described above. The resulting material constituted nuclease-P(1-126).

The sample obtained from the enzymically active fraction contained both deprotected nuclease and nuclease-P(1-126) as judged by polyacrylamide gel electrophoresis (pH 2.3, see below). Fractionation of the mixed sample by chromatography on a pdTp-Sepharose column was performed according to the method described by Cuatrecasas, Wilchek, and Anfinsen (31). The chromatographic pattern is shown in Fig. 3. The fractions comprising the first absorbance peak contained nuclease-P(1-126), which was enzymically inactive and unabsorbed. These fractions were pooled and lyophilized. The dried sample was dissolved in 1 ml of H$_2$O and dialyzed against 6 liters of H$_2$O with four changes for 4 hours at 25°C in order to remove the salt (see the legend to Fig. 3). The dialyzed solution of nuclease-P(1-126) was lyophilized. The total molar yield of nuclease-P(1-126) from the initial nuclease was approximately 20%.

\textbf{Preparation of Nuclease-P(127-149) (Residues 127 through 149)—}\n
The fractions containing nuclease-P(127-149) obtained from the chromatography on phosphocellulose (Fig. 1) were pooled and lyophilized. Examination of a two-dimensional peptide mapping (32) showed only one major ninhydrin-positive spot in addition to a few minor ones. The sample was subjected to gel filtration on a Sephadex G-50 column (Fig. 4). The fractions comprising the absorbance peak were pooled and lyophilized. The resulting nuclease-P(127-149) gave the same spot on a two-dimensional peptide map as that observed with the material before gel filtration, but no minor spots were observed.$^{5}$ Amino acid analysis and sequence determination of the sample identified the fragment as Residues 127 to 149 of nuclease as described previously (19).

\textbf{Preparation of Control Sample of Deblocked Nuclease—}\n
Approximately 0.7\% of trifluoroacetylated nuclease was deblocked and subjected to chromatography on a phosphocellulose column (1 x 8.5 cm) as described above. The volume of buffer in each chamber of the Varigrad was 150 ml. The elution pattern obtained was similar to that shown in Fig. 1 except that the first absorbance peak, due to nuclease-P(127-149) (Fig. 1), was lacking and the specific activity of the enzymically active fractions was approximately twice as high as presented in Fig. 1. The active fractions were pooled and lyophilized. The dried material was used as control material and is referred to as “deblocked nuclease,” unless otherwise specified. Trifluoroacetylated nuclease, itself, was inactive. The piperidine treatment employed to remove trifluoroacetyl groups restored 70 to 80\% of the original activity of nuclease. Deblocked nuclease was digested with trypsin in the presence of pdTp and Ca$^{2+}$ (14). A portion of the nuclease was degraded extensively, as shown by two-dimensional peptide maps of the digest (17). Nucleases-P(1-126) and -P(18, 19-149) were isolated from nuclease-T prepared from deblocked nuclease as described previously (14). The results of amino acid analysis and two-dimensional peptide maps of the tryptic digests of nuclease-T-P(1-126) and -P(18, 19-149) were essentially identical with those from native nuclease (14, 15).
RESULTS

Cleavage of Trifluoroacetylated Nuclease by Trypsin—Extensive digestion of trifluoroacetylated nuclease with trypsin resulted in the cleavage of the peptide bonds involving carboxyl groups of all arginine residues and some tyrosyl residues, as reported previously (18). The relative rates of cleavage of susceptible bonds were examined as follows. Approximately 0.25 μmol of trifluoroacetylated nuclease was digested in 0.7 ml of 0.07 M NH₄HCO₃, pH 8, with 10 μg of trypsin. Aliquots of 150 μl each were removed after 0, 15, 30, and 60 min of incubation, and 100 μg of soybean trypsin inhibitor were added to stop digestion. The mixtures were lyophilized. Each dried sample was dissolved in 0.2 ml of 1 M piperidine to remove trifluoroacetyl groups as described above. The deblocked samples were examined for enzymic activity, and quantitative determinations of NH₂-terminal groups by the dinitrofluorobenzene method were performed (17). The control aliquot, taken before addition of trypsin, yielded only DNP-alanine, and the quantity was approximately the same in the later aliquots (0.003 to 0.005 μmoles per 150-μl sample, uncorrected for losses during acid hydrolysis). The next end group to appear was bis-DNP-lysine, which reached a maximum level after 30 min of incubation (0.005, 0.008, and 0.007 μmoles after 15, 30, and 60 minutes, respectively). The other end groups observed were DNP-glutamic acid, -leucine, and -threonine, all of which increased at about the same rate up to 60 min of incubation (DNP-glutamic acid, 0.003, 0.011, and 0.015 μmole for the 15-, 30-, and 60-min aliquots; DNP-leucine, 0.005, 0.012, and 0.019 μmole for corresponding aliquots; DNP-threonine estimated, qualitatively, by visual inspection). The rate of decrease in enzymic activity paralleled the increase in bis-DNP-lysine. After 30 min, approximately 5% of the activity remained.

Leucine 36, threonine 82, glycine 88, glutamic acid 106, and lysine 127 are the residues following the 5 arginine residues of nuclease. None of the residues following trypsin-susceptible tyrosine residues are lysine (18). Therefore, the appearance of lysine as an NH₂ end group during the digestion indicated cleavage of the bond between residues 126 and 127. Extrapolation of the curves relating amounts of DNP-amino acids to incubation time indicated that, at 8 min of incubation, approximately 50% cleavage of the bond between residues 126 and 127 had occurred, while less than 10% of each of the other bonds had been cleaved. Accordingly, 8 to 10 min was chosen as the incubation time of trifluoroacetylated nuclease with trypsin, under the conditions described in "Experimental Procedures" for the preparation of nuclease-P(1-126).

Identification of Nuclease-P(1-126)—The purified nuclease-P(1-126) produced a single zone and had a slightly faster electrophoretic mobility than deblocked and native nucleases upon polyacrylamide gel electrophoresis at pH 2.3, as shown in Fig. 5. Nuclease-P(126) was subjected to gel filtration on Bio-Gel P-200 under conditions which separate the two components of nuclease-P (14). The sample, approximately 2 μmoles, was dissolved in 0.5 ml of 50% CH₃COOH and applied to a Bio-Gel column (1.2 × 210 cm) equilibrated with 50% CH₃COOH. The column was eluted with 50% CH₃COOH at a flow rate of 7 ml per hour, at 4°C. The fractions, collected every 30 min, were examined for absorbance at 280 μm. Aliquots (0.1 ml each) of the fractions were subjected to alkaline hydrolysis (34), and the amino acid contents of the hydrolysates were determined.

* DNP-glycine was not observed, presumably owing to destruction by acid. Glutamine 106 would be obtained as DNP-glutamic acid after acid hydrolysis.
by the ninhydrin color reaction (35). Only a single major peak was observed, at the position where nuclease would be expected (14, 15). The fractions comprising the peak were pooled and lyophilized. The dried sample was identical with the original material, as judged by amino acid analysis, polyacrylamide gel electrophoresis at pH 2.3, and a two-dimensional peptide map of the tryptic digest. The results are consistent with a single large fragment.

The NH₂ end group of nuclease-P₁₋₁₂₆ was exclusively alanine by dinitrophenylation (17). DNP-alanine, 0.006 μmole (uncorrected), was obtained from a 0.06-μmole sample. Carboxypeptidase B (having slight carboxypeptidase A activity) released arginine, histidine, and leucine from nuclease-P₁₋₁₂₆ in a yield of approximately 50% after 3 hours of incubation at 25°, indicating the carboxyl-terminal sequence, (His, Leu)Arg. Hydroxyanalysis of nuclease-P₁₋₁₂₆ yielded ornithine in a yield of approximately 15% (36). Carboxypeptidase A treatment for 3 hours did not release significant amounts of free amino acids from nuclease-P₁₋₁₂₆. Deblocokd nuclease yielded glutamine upon carboxypeptidase A treatment for 3 hours, and carboxypeptidase B treatment did not release free amino acids (17).

Since the sequence His-Leu-Arg corresponds to Residues 124 to 126, the above observations indicate that nuclease-P₁₋₁₂₆ is a single polypeptide chain containing Residues 1 to 126. This conclusion is supported by amino acid analyses (Table I) and by two-dimensional peptide maps of tryptic digests of nuclease-P₁₋₁₂₆ (17, 18). Three peptide spots, corresponding to Residues 127 to 133, 134 to 136, and 137 to 149, were absent in the peptide map of nuclease-P₁₋₁₂₆. All other expected major spots were observed on the map.

### Table I

**Amino acid composition of nuclease-P₁₋₁₂₆**

Duplicate samples (0.0072 μmole each) were hydrolyzed for 20, 48, and 72 hours. The values of threonine, serine, methionine, and tyrosine were obtained by extrapolation to zero time. Other values are averages of all analyses. The results are presented on the basis of 3 moles of phenylalanine per polypeptide molecule.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Theory (16, 21)</th>
<th>Found</th>
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<tbody>
<tr>
<td></td>
<td>Residues 1-126</td>
<td>Nuclease</td>
</tr>
<tr>
<td>Lysine</td>
<td>19</td>
<td>23</td>
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<tr>
<td>Histidine</td>
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<tr>
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</tr>
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<tr>
<td>Glutamic acid</td>
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<td>18</td>
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<td>Proline</td>
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<tr>
<td>Glycine</td>
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</tr>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
<td>Tryptophan</td>
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<td>1</td>
</tr>
</tbody>
</table>

* Seven tyrosines per molecule as determined spectrophotometrically (36).
* Determined by measurement of fluorescence (see the text).

Nuclease contains only one tryptophan, at Position 140 (18). Therefore, the presence or absence of tryptophan in nuclease-P₁₋₁₂₆ was examined by fluorescence spectroscopy. As shown in Fig. 6, deblocokd and native nucleases produced identical fluorescence with a peak at 333 μm, with a much higher intensity than tryptophan itself, when excited by light of 295 μm, the wave length selected to avoid fluorescence emission by tyrosine residues (37). However, nuclease-P₁₋₁₂₆ produced the same fluorescence spectrum as free tryptophan (37). The presence of a tryptophan residue in nuclease-P₁₋₁₂₆ was excluded by its fluorescence spectrum.

**Properties of Nuclease-P₁₋₁₂₆**—Purified nuclease-P₁₋₁₂₆ showed traces of enzymic activity (less than 1% of native nuclease). However, nuclease-P₁₋₁₂₆ did not bind pdTp in the presence of Ca²⁺, as examined by the method of Hummel and Dreyer (38) (Fig. 7), in contrast to native nuclease (39) (Fig. 7). Nuclease-P₁₋₁₂₆ was degraded completely by trypsin in the presence, as well as in the absence, of pdTp and Ca²⁺, as judged by the two-dimensional peptide map of the digest, again in contrast to native nuclease (14). The difference in ligand binding could be utilized to remove the traces of contaminating, intact nuclease (deblockked nuclease) from nuclease-P₁₋₁₂₆ preparation, as described in "Experimental Procedures." It can be concluded, therefore, that nuclease-P₁₋₁₂₆ is inactive. Addition of nuclease-P₁₋₁₂₆, which is itself completely inactive, to nuclease-P₁₋₁₂₆ did not generate enzymic activity (see below).

The results of measurements of circular dichroism (from 215 to 240 μm) of nuclease-P₁₋₁₂₆ and -P₁₋₁₂₆ and deblocokd nuclease...
are presented in Fig. 8. Debocked nuclease behaved in a manner similar to native nuclease (15). The ellipticity of nuclease-P(1-126) was low, resembling that of nuclease-T-P(49,50-149) (15). Nuclease-P(1-126) exhibited much lower ellipticity than deblocked nuclease, but higher than nuclease-T-P(648), nuclease-T-P(49-50), and nuclease-T-P(49,50-149) (15), and nuclease-P(12-149). A minimum at 220 mp was observed only with deblocked and native nucleases, as reported previously for nuclease-T (15).

The measurement of optical rotation at 233 mp as a function of temperature (25-70°) was performed with deblocked nuclease and nuclease-P(1-126). Fig. 9 shows the calculated mean residue rotations at 233 mp of deblocked nuclease, nuclease-P(1-126), and nuclease-T-P(648) and -P(49,50-149). The latter two are included as reference values (14). Debocked nuclease showed a change of the mean residue rotation as a function of temperature similar to that observed with native nuclease (14). The helicity of deblocked nuclease was calculated to be approximately 15%, according to the method of Simmons et al. (40). Nuclease-P(1-126) showed very little change of its mean residue rotation with temperature, as with nuclease-T-P(648) and -P(49,50-149) (14). The above results suggest that nuclease-P(1-126) has essentially no helical structure. Nuclease-P(12-149) is a covalent part of nuclease-T-P(648,10-149), which exhibited no helicity (14, 15) (Fig. 9), and the circular dichroism (215 to 240 mp) of nuclease-P(1-126) was similar to that of nuclease-T-P(648,10-149) (Fig. 8) (15). Therefore, nonhelicity is also indicated for nuclease-P(12-149). The addition of nuclease-P(12-149) to nuclease-P(1-126) did not increase the ellipticity at 215 to 240 mp over the sum of the values given by the two materials alone (see below).

The immunological properties of nuclease-P(1-126) and nuclease-P(12-149) were examined by immunodiffusion. As shown in Fig. 10, both nuclease-P(1-126) and nuclease-P(12-149) differed immunologically from native and deblocked nuclease, which were immunologically identical. A mixture of nuclease-P(1-126) and nuclease-P(12-149) did not react with the antiserum (Fig. 10).
addition of 8 M urea did not change, significantly, the state of the tyrosine residues of native nuclease but appeared to make the tryptophan residue perturbable. However, the perturbability of the tyrosine residues of nuclease T increased in the presence of 8 M urea. The tyrosine residues of nuclease-P (1,126) were already highly perturbable in the absence of 8 M urea, and no further change occurred upon addition of this reagent.

Preliminary experiments on the relative susceptibility to nitration of tyrosine residues by tetranitromethane (44, 45) in nuclease-P (1,126), nuclease-T, nuclease-T-P (49, 50, 10, 10), and deblocked nuclease, compared with native nuclease (46), have also supported the conclusion that native nuclease-P (1,126) does not possess the conformation of nuclease (or deblocked nuclease) but has a loose, motile structure.

**Foldng of Nuclease-P (1,126) upon Association with Nuclease P (49, 50, 10, 10)**—When nuclease-P (1,126) and nuclease-T-P (49, 50, 10, 10) were mixed in aqueous solution, a level of enzymatic activity comparable to that observed with nuclease-T (approximately 8% of the enzymatic activity of intact nuclease (14, 15)) (Table III) was generated. Immunological activity toward antinuclease serum (Fig. 11) was also induced. On the other hand, nuclease-P (1,126) did not generate enzymatic activity with nuclease-P (1,126) (Table III), and the enzymatic activity formed by mixing nuclease-T-P (49, 50, 10, 10) and nuclease-P (1,126) was not affected in the presence of an 8-fold molar excess of nuclease-P (1,126).

The circular dichroism (215 to 240 m\(\mu\)) of the mixture of

**Table II**

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<th>Nuclease residues</th>
<th>Tryptophan residues</th>
<th>Difference absorption</th>
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<td>Urea (+)</td>
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</tr>
<tr>
<td></td>
<td>(\Delta \alpha)</td>
<td>(\Delta \alpha)</td>
</tr>
<tr>
<td>Nuclease-P (1,126)</td>
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<tr>
<td>Nuclease-P (1,126)</td>
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<td>1</td>
</tr>
<tr>
<td>Nuclease-P (1,126)</td>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

* Zero absorption is assumed at 295 m\(\mu\) instead of 310 m\(\mu\).
nuclease-P(1-126) and nuclease-T-P(49,50-149) increased over the
sum of the values given by the two components alone (Fig. 12).
On the other hand, the circular dichroism observed with a mix-
ture of nuclease-P(1-126) and nuclease-T-P(127-148) was not
greater than the sum of the individual values (Fig. 12). Therefore,
generation of secondary structure was indicated upon interaction
of nuclease-P(1-126) and nuclease-T-P(49,50-149), as in the case of
nuclease-T-P(6-48) and nuclease-T-P(49,50-149) (14, 15).

The tryptophan fluorescence of nuclease (Fig. 6) indicated
that tryptophan 140 resides in a nonpolar environment (37).
Thus, the position of the maximum emission band of nuclease,
with 295 nm excitation, was 333 nm, while the maxima of nuc-
lease-P(127-148), nuclease-T-P(49,50-149), and tryptophan itself
were at 348 nm, with much lower intensity (Figs. 6 and 13). The
wave length of the maximum emission (343 nm) and the intensity
of tryptophan fluorescence of nuclease-T fell between those of

<table>
<thead>
<tr>
<th>TABLE III</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Generation of enzymic activity upon mixing nuclease-P(1-126) and nuclease-T-P(49,149)</strong></td>
</tr>
</tbody>
</table>

A 1% aqueous solution of one sample was mixed with an equal
volume of a 1% aqueous solution of the other. Suitable diluted
aliquots of the mixture were used for the assay. (Two volumes
of a 1% solution of nuclease-P(1-126) were mixed with 1 volume of a
1% solution of nuclease-T-P(49,149) in one experiment.) Maximum
enzymic activity was generated under these conditions
within 1 min.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Addition</th>
<th>Molar ratio of the first sample to the added sample</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>to the first sample</td>
<td>Toward DNA</td>
</tr>
<tr>
<td>Nuclease-T-P(49,149)</td>
<td>None</td>
<td></td>
<td>0.035*</td>
</tr>
<tr>
<td>Nuclease-T-P(49,149)</td>
<td>None</td>
<td></td>
<td>0.016*</td>
</tr>
<tr>
<td>Nuclease-T-P(49,149)</td>
<td>Nuclease-T-P(49,149)</td>
<td>2.2</td>
<td>0.616</td>
</tr>
<tr>
<td>Nuclease-T-P(49,149)</td>
<td>Nuclease-T-P(49,149)</td>
<td>0.81</td>
<td>0.816</td>
</tr>
<tr>
<td>Nuclease-T-P(49,149)</td>
<td>Nuclease-T-P(49,149)</td>
<td>1.6</td>
<td>0.992</td>
</tr>
<tr>
<td>Nuclease-T-P(49,149)</td>
<td>Nuclease-T-P(49,149)</td>
<td>2.8</td>
<td>0.064</td>
</tr>
</tbody>
</table>

*Activities found with mixtures have been corrected for the low
values shown by the samples of nuclease-T-P(49,149), P(1-126),
and nuclease-P(1-126). Neither nuclease-T-P(49,149) nor nuclease-T-
P(49,10-149) added in 15- to 36-fold molar excess, affected the
activities of nuclease or nuclease-P(1-126) (15). The relatively high
enzymic activities observed with the nuclease-P(1-126) sample is
presumably due to contaminating intact nuclease (see the text).

<table>
<thead>
<tr>
<th>TABLE IV</th>
</tr>
</thead>
</table>
| **Change in enzymic activities (against DNA) of mixtures of nuclease-
P(1-126) and nuclease-T-P(49,149) incubated with trypsin in
presence and absence of pdT-p and Ca** |

<table>
<thead>
<tr>
<th>Sample</th>
<th>Addition</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 min  10 min  20 min  30 min  60 min  120 min</td>
</tr>
<tr>
<td>Nuclease-P(1-126) and nuclease-T-P(49,149)</td>
<td>pdT-p, Ca**</td>
<td>100 96 69 49 47</td>
</tr>
<tr>
<td>Nuclease-P(1-126) and nuclease-T-P(49,149)</td>
<td>None</td>
<td>100 8 3 0 0 0</td>
</tr>
</tbody>
</table>

Fig. 12. Circular dichroisms of the mixture of nuclease-P(1-126) and
-T-P(127-148) (left), and of nuclease-P(1-126) and nuclease-T-
P(49,50-149) (right). Nuclease-P(1-126) (0.05 mmole) and nuclease-
P(127-148) (0.070 mmole) were mixed in 70 μl of water and diluted with
25 ml of 0.05 M Tris-HCl, 0.1 M NaCl pH 7. Nuclease-P(1-126) (0.021 mmole) and nuclease-T-P(49,50-149) (0.020 mmole) were mixed
in 45 μl of water and diluted with 3 ml of the same buffer.

On the other hand, the circular dichroism observed with a mix-
ture of nuclease-T-P(49,50-149) and nuclease-T-P(127-148) shifted to
337 nm with increased intensity (Fig. 13). A similar shift in the maximum emission (to 338 nm)
and increase in intensity were observed with tryptophan fluorescence
of nuclease-T-P(49,50-149) upon mixing with nuclease-P(1-126) (Fig. 13). However, the fluorescence properties of nuclease-
P(1-126) did not change when mixed with nuclease-P(1-126). The
observations indicate that the environment of tryptophan residue of
nuclease-T is more polar than in nuclease, but less polar than in nuclease-T-P(49,50-149) or nuclease-P(1-126). The binding of nuclease-T-P(49,50-149) with either nuclease-
T-P(49,149) or nuclease-P(1-126) forms an environment for the trypto-
phan residue which is similar to that with nuclease-T. 

Formation of Nuclease-T-P(6-48) from Nuclease-P(1-126)—If nuc-
lease-T-P(49,50-149) binds with the nuclease-T-P(6-48) portion
of nuclease-P(1-126) to form a three-dimensional structure similar to
that of nuclease-T, the rest of the polypeptide chain (Residues
1 through 5 and Residues 49 through 126) of nuclease-P(1-126)
would not participate in the formation of nuclease-T conformation,
but should extend from the nuclease-T structure, as illustrated
schematically in Fig. 14. Since nuclease-T is protected from digestion by trypsin in the presence of pdT-p and Ca++
(14), the nuclease-T moiety of the nuclease-P(1-126)-nuclease-
T-P(49,50-149) complex should be similarly protected from trypsin
cleavage by the ligands. The residual portions (Residues 1
through 5 and Residues 49 through 126) of the complex, if flex-
ible, should be digested completely by trypsin. This hypoth-
esis was confirmed experimentally as follows. Nuclease-P(1-126)
(0.57 μmole) and nuclease-T-P(49,50-149) (0.62 μmole) were dis-
solved in 2 ml of 0.05 M NH4HCO3, pH 8, with 4 mg of pdT-p and
0.01 m Ca++. Trypsin (0.2 mg) was added to the mixture.
Aliquots of 5 μl were removed at incubation times of 0, 10, 30,
60, and 120 min. Soybean trypsin inhibitor (20 μg) was added to each aliquot to terminate digestion. Enzymic activity was assayed in each aliquot after suitable dilution. After 2 hours of incubation at 25°, soybean trypsin inhibitor (1 mg) was added to the mixture, which was then lyophilized. A similar incubation of nuclease-P (0.02 μmole) and nuclease-T-P (69, 81-149) (0.02 μmole) without pdTTP and Ca++ was carried out as a control. Parallel aliquots were assayed for enzymic activity.

As presented in Table IV, approximately 50% of the enzymic activity of the mixture of nuclease-P (69, 126) and nuclease-T-P (69, 81-149) remained after 2 hours of incubation with trypsin in the presence of pdTTP and Ca++. However, the mixture without pdTTP and Ca++ lost enzymic activity in 30 min.

A part of the lyophilized sample (1 mg) was examined by two-dimensional peptide mapping. Major ninhydrin-positive spots found on the map correspond to the tryptic peptides derived from Residues 1 to 5 and 49 to 126 as judged by their positions (17, 18). A streak, indicating an undigested, large fragment,

![Fluorescence emission spectra of nuclease-T](image1)

**Fig. 13.** Fluorescence emission spectra of nuclease-T (●), nuclease-T-P (69, 126) (△), the mixture of nuclease-P (69, 126) and nuclease-T (69, 126) (□), the mixture of nuclease-T-P (69, 126) and -P (69, 81-149) (□), and the mixture of nuclease-P (69, 126) and nuclease-T (69, 81-149) (□), with 300 μM excitation. The ordinate is fluorescence intensity in arbitrary units. Observed values of fluorescence intensity with nuclease (0.5 × 10^{-4} M), nuclease-T (1.4 × 10^{-4} M), nuclease-T-P (69, 126) (0.2 × 10^{-4} M), nuclease-T (69, 81-149) (2.2 × 10^{-4} M), nuclease-P (69, 126) (3.1 × 10^{-4} M) and nuclease-T (69, 149) (3.3 × 10^{-4} M) were used to calculate the values presented in the figure, on the basis of equimolar concentrations of each sample. Nuclease-P (69, 126) (0.0002 μmole) and nuclease-P (69, 126) (0.015 μmole) were mixed in 7 μl of water and diluted with 3 ml of 0.01 M Tris-HCl-0.1 M NaCl, pH 7. Nuclease-P (69, 126) (0.0092 μmole) and nuclease-T (69, 126) (0.0066 μmole) were mixed in 10 μl and diluted with 3 ml of the same buffer. The fluorescence intensities of the mixtures were calculated on the basis of the concentrations of nuclease-T (69, 126) and nuclease-P (69, 126). Other details are given in the legend to Fig. 6 and under “Experimental Procedures.” The emission spectra of nuclease-T-P (69, 126) and nuclease, being identical with those of tryptophan and debranned nuclease, respectively, serve as standards to compare with those shown in Fig. 6.

![Schematic diagram of the formation of nuclease-T'](image2)

**Fig. 14.** Schematic diagram of the formation of nuclease-T' from nuclease-P (69, 126) and nuclease-T-P (69, 81-149) (presented as -P (69, 149) (15)). Nuclease-T'-P (69, 149) and the nuclease-T-P (69, 149) portion of nuclease-P (69, 126), are postulated to form the conversion of nuclease-T'. The rest of nuclease-P (69, 126) is digested with trypsin in the presence of pdTTP and Ca++, leaving nuclease-T' (surrounded by line) intact.

![Polyacrylamide gel electrophoresis of nuclease-T'](image3)

**Fig. 15.** Polyacrylamide gel electrophoresis of nuclease-T' and nuclease-T, and a mixture of the two. Approximately 40 μg of each sample alone and a mixture of 20 μg of each, were run at 3 mA per tube for 4 hours at 4° on 2.3 gel with reversed polarity. The lower zone corresponds to nuclease-T- (69, 149) and the upper zone to nuclease-T (69, 126) (14). The two components of nuclease-T dissociate on 2.3 gel, but do not on standard gel (14, 15).

was also observed (14). The rest of the sample was dissolved in 0.5 ml of 0.01 n acetic acid-0.1% ammonium acetate and applied on a Sephadex G-50 column (medium, 1 × 25 cm), equilibrated with the same buffer. Large fragments were well separated from the small peptides, as described previously for the separation of nuclease-T from nuclease-T (69, 126) (Residues 1 through 5) (14). The fractions containing large fragments had enzymic activity equivalent to that of nuclease-T' (approximately 8% of the

Nuclease-T' refers to the product formed by recombination of fragments nuclease-T-P(69, 126) and nuclease-T-P(69, 81-149), the two nonequivalently bonded polypeptides of nuclease-T (14, 15). Nuclease-T' refers to the nuclease-T formed by trypsin digestion of the P (69, 126)-P (69, 15) complex in the presence of pdTTP and Ca++.
enzymic activity of native nuclease (14, 15)) on the basis of absorbance at 280 mp (14, 15) (see also Table IV). The fractions containing the peptides gave no enzymic activity, and two-dimensional peptide maps of the pooled fractions, after lyophilization, accounted for all the peptide spots found with the original mixture, with no evidence of the larger, streaking materials. For more positive identification, the peptide fraction was subjected to ion exchange chromatography as described previously (17). The resulting samples were examined by amino acid analysis and two-dimensional peptide mapping. The results, together with the observations on staining two-dimensional peptide maps of the original sample before chromatography by ninhydrin, Sakaguchi, and Pauli reagents (47), identified, qualitatively, peptides containing residues 1 to 5, 49 to 53, 50 to 63, 54 to 61, 54 to 63, 65 to 70, 71 to 81, 82 to 87, 85 to 87, 88 to 97, 98 to 105, and 114 to 126. Polyacrylamide gel electrophoresis of the lyophilized fractions containing the large fragment (nuclease-T"P(6-49)) showed two zones, indistinguishable from those given by nuclease-T, as shown in Fig. 15. The table shows that the amino acid composition of nuclease-T" was like that of nuclease-T (Table V). The fluorescence spectrum of the tryptophan of nuclease-T" was also similar to that of nuclease-T (see Fig. 13). The yield of nuclease-T" (0.14 umole) was approximately 30%. The above results indicated that nuclease-T" remained intact during digestion of the mixture of nuclease-P(1,126) and nuclease-T-P(6-48) with trypsin in the presence of pdTp and Ca++. That is, the nuclease-T-P(49) portion of nuclease-P(1,126) remained undigested and the "tails" (Residues 1 through 5 and 49 through 126) were completely digested.

To obtain further evidence for the formation of nuclease-T-P(6-48) (nuclease-T"-P(6-48)) from nuclease-P(1,126), 1.7 umoles of nuclease-P(1,126) and 1.5 umoles of nuclease-T-P(49,10-126) were mixed in 4 ml of 0.05 M NH4HCO3, pH 8, and digested with trypsin in the presence of pdTp and Ca++ for 100 min at 25°C as described above. Nuclease-T" was isolated, by gel filtration on a Sephadex G-50 column, from the digestion mixture. The sample was dissolved in 0.5 ml of 50% CH3COOH and subjected to gel filtration on a Bio-Gel P-20 column (2 × 196 cm) to separate nuclease-T-P(49,50-149) and nuclease-T"-P(6-49) (14).

The separated nuclease-T-P(49,50-149) and nuclease-T"-P(6-48) were identified by amino acid analysis (Table V), NH2-terminal sequence analysis (Table V), two-dimensional peptide maps of the tryptic digests (18), and polyacrylamide gel electrophoresis at pH 2.3 (12). Nuclease-T"-P(49,50-149), upon mixing with nuclease-T-P(49,40-149), generated the enzymic activities against DNA and RNA of nuclease-T (approximately 8% of the enzymic activity of native nuclease) (14, 15). Approximately 0.5 umole each of the separated nuclease-T"-P(49,50-149) and nuclease-T"-P(6-48) was obtained. Taking into account the loss during separation (14), the yield of nuclease-T" from nuclease-T-P(1,126) and nuclease-T-P(49,50-149) was approximately 50%.

**DISCUSSION**

It has been shown in previous studies that Staphylococcal nuclease possesses a compact, globular structure (20, 48). Measurements of the fluorescence properties of the single tryptophan residue, in this paper, and of solvent perturbation of tyrosine and tryptophan fluorescence (40) and light absorption (40) suggest, further, that the distribution of polar and nonpolar side chains between the surface and the interior of the molecule is probably very similar to that already demonstrated for other proteins (12, 50-54).

An enzymically fully active derivative, nuclease-P(6-48), which lacks the NH2-terminal 5 residues of nuclease (Fig. 16), seems to have essentially the same structure as that of nuclease (22, 24). The enzymic activity of nuclease-P(6-48) and that of nuclease are recovered completely after heat denaturation.4

Nuclease-T, an enzymically less active derivative produced by trypsin digestion of nuclease in the presence of pdTp and calcium ions, possesses the same amino acid sequence as that of nuclease-P(6-48) with the exception of the cleavage of the bond between Residues 48 and 49 (Fig. 16). The structure of nuclease-T is composed of nuclease-T-P(4-49) and -P(49, 50-149).7

---

**Table V**

Amino acid compositions of nuclease-T", nuclease-T"-P(49-149), and nuclease-T"-P(49,50-149).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Nuclease-T&quot;</th>
<th>Nuclease-T&quot;-P(49-149)</th>
<th>Nuclease-T&quot;-P(49,50-149)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>22.6 (21-22)</td>
<td>7.0 (7)</td>
<td>13.5 (14-15)</td>
</tr>
<tr>
<td>Histidine</td>
<td>4.2 (4)</td>
<td>2.2 (2)</td>
<td>2.5 (2)</td>
</tr>
<tr>
<td>Arginine</td>
<td>5.3 (5)</td>
<td>1.2 (1)</td>
<td>4.7 (4)</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>14.4 (14)</td>
<td>3.2 (3)</td>
<td>10.8 (11)</td>
</tr>
<tr>
<td>Threonine</td>
<td>7.5 (8)</td>
<td>4.3 (6)</td>
<td>3.8 (7)</td>
</tr>
<tr>
<td>Serine</td>
<td>4.6 (4)</td>
<td>0.2 (0)</td>
<td>3.2 (4)</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>18.4 (18)</td>
<td>3.5 (4)</td>
<td>15.8 (15)</td>
</tr>
<tr>
<td>Proline</td>
<td>6.1 (6)</td>
<td>3.5 (4)</td>
<td>16.2 (12)</td>
</tr>
<tr>
<td>Glycine</td>
<td>9.9 (10)</td>
<td>2.2 (2)</td>
<td>6.6 (8)</td>
</tr>
<tr>
<td>Alanine</td>
<td>11.6 (13)</td>
<td>2.2 (2)</td>
<td>9.1 (11)</td>
</tr>
<tr>
<td>Valine</td>
<td>8.7 (9)</td>
<td>2.3 (2)</td>
<td>6.5 (7)</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.7 (4)</td>
<td>1.9 (2)</td>
<td>2.5 (2)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5.4 (5)</td>
<td>1.9 (2)</td>
<td>2.8 (3)</td>
</tr>
<tr>
<td>Leucine</td>
<td>10.7 (11)</td>
<td>5.6 (7)</td>
<td>5.5 (5)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>5.3 (7)</td>
<td>2.3 (2)</td>
<td>5.6 (6)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.0 (5)</td>
<td>1.0 (1)</td>
<td>2.5 (2)</td>
</tr>
<tr>
<td>NH2-terminal residues</td>
<td></td>
<td>Lys-Leu</td>
<td>Lys-Gly and Gly-Val</td>
</tr>
</tbody>
</table>

---

1) Nuclease-T-P(49,149-149) is a mixture of approximately equal amounts of nuclease-T-P(49,149) (18 lysine residues) and -P(6-48) (14 lysine residues) (15).
2) Low values presumably due to degradation during hydrolysis.
3) Two steps of Edman degradation were performed successively.
4) Identification of glycine-phenylthiohydantoin was tentative.
5) The assignment of two NH2-terminal sequences for the two types of nuclease-T-P(49,149-149), P(49-149), and -P(49,149), was made on the basis of the peptide map of a tryptic digest of nuclease-T"-P(49,50-149) (15).
6) Complete recovery of enzymic activity was observed with nuclease-P(6-48), and nuclease-T after heating at 68°C for 15 min (14, 15). (The enzymic activity of nuclease-T was, of course, approximately 8% of that of nuclease.) The helical content of the renatured nuclease was also the same as that of native nuclease as measured by the change of the optical rotation at 232 mp as a function of temperature (14, 15) (see the legends to Figs. 8 and 9).
Folding of Staphylococcal Nuclease

Fig. 16. Relation of the amino acid sequences of nuclease, nuclease-P(1-126), nuclease-T-P(49-149) (see Footnote 6), nuclease-T-P(48-64), nuclease-P(61-126), and nuclease-P(127-149), showing the location of the tryptophan residue when present.

NUCLEASE

H₂N ALA LYS LYS LEU

NUCLEASE-P(6-149)

H₂N LYS LEU

NUCLEASE-T-P(6-48)

H₂N LYS LEU PRO LYS COOH

NUCLEASE-T-P(49-149)

H₂N LYS LEU

NUCLEASE-P(1-126)

H₂N ALA LYS LYS LEU PRO LYS LYS PRO LEU COOH

NUCLEASE-P(127-149)

H₂N LYS LEU PRO LYS PRO LEU COOH

NUCLEASE-T appears to be qualitatively like those of native nuclease and nuclease-P(6-126). Neither of the two noncovalently interacting fragments of nuclease-T, nuclease-T-P(6-48) and nuclease-T-P(49,50-149), shows ordered structure in solution when examined alone (14, 15, 55), but combination of the two results in changes in tryptophan fluorescence consistent with the movement of this residue from a polar to a nonpolar environment (37). The helicity, sedimentation velocity, and immunological properties of the P(6-48)-P(49,50-149) complex (nuclease-T'), as well as affinities for both DNA and RNA, are also closely similar to those of native nuclease (14, 15). The finding that the binding of pdTp and calcium ions to nuclease-T induces resistance to proteolysis by trypsin, subtilisin, a-chymotrypsin, and thermolysin, in the same manner as that observed with native nuclease and nuclease-P(61-126), also supports the structural similarity of nuclease-T and nuclease-P(1-126), and nuclease-P(127-149), showing the location of the tryptophan residue when present.

The present studies indicate that nuclease-P(1-126) (Fig. 16) is essentially unstructured in solution under the conditions used. The combination of nuclease-P(1-126) with nuclease-T-P(6-126) (Fig. 14) with physical properties resembling those of nuclease-T'. Since the structureless 23-residue COOH-terminal fragment, nuclease-P(127-149) (Fig. 16), does not bind to nuclease-P(1-126), it seems quite likely that specific association of nuclease-T-P(48,50-149) and nuclease-P(1-126) involves the "P(6-48) portion" of nuclease-P(1-126) (i.e., Residues 6 through 48). This is supported by the fact that trypsin digests away, in the presence of pdTp and calcium ions, those portions of the sequence not included in Residues 6 to 48 with the production of a residual material indistinguishable from nuclease-T. One must conclude that these "superfluous" parts of the sequence, in spite of being rich in clusters of hydrophobic amino acid side chains and including much of the amino acid sequence of the NH₂-terminal portion of nuclease-T-P(48,50-149) (77% of its total residues; see Figs. 14 and 16), do not interfere with the P(6-48)-P(49,50-149) interaction. The formation of the ordered structure of the P(6-48)-P(49,50-149) complex must, therefore, be thermodynamically favorable and attainable without the penetration of a significant energy barrier. It is known that polypeptide chain biosynthesis takes place by the stepwise addition of monomers from the NH₂ terminus (5, 6). The nuclease fragment, nuclease-P(1-126), appears to possess a loose structure, and it is certainly devoid of many of the physical properties that characterize the native, parent protein. These observations indicate that the structure characteristic of native nuclease could not form as the polypeptide chain is being assembled since the information required to determine the native structure becomes available only at some point* in the synthesis after the addition of Residue 126.

Fairly high concentrations (0.5%) of nuclease-P(1-126) and nuclease-T-P(48,50-149) are required, in recombination experiments, for generation of maximum enzyme activity (approximately 8% of the enzymic activity of native nuclease) within a reasonably short time (approximately 1 min). The information for the correct structure is available, but the bimolecular nature of the interaction makes the process slow. Renaturation of native nuclease, with an uninterrupted polypeptide chain, is extremely rapid even at high dilution.

The reconstitution of biologically active material from inactive peptide fragments observed in the nuclease-T and P(1-126)-P(48, 50-149) systems, as well as with RNase-S-peptide and S-protein, is in marked contrast to the situation with insulin and chymotrypsin (57). These multichained "derived" proteins are not capable of spontaneous recombination after chain separation, and the required information for folding is present only in the

* The COOH-terminal glutamine residue is not necessary for the enzymic activity of nuclease (17).
precursor molecules, proinsulin (58-62) and chymotrypsinogen (57).

Note Added in Proof—Further evidence for the absence of ordered structure in nuclease-Pd(126) was obtained by measurements of viscosity. These were carried out at 20°C ± 0.01°C in 0.05 M Tris-HCl, pH 8—0.1 M NaCl and intrinsic viscosity values were calculated as described previously (20). The intrinsic viscosity of nuclease-Pd(126) was 0.008 ± 0.01 dl per g. The values for deblocked nuclease and native nuclease were 0.021 ± 0.002 and 0.019 ± 0.002 dl per g, respectively. The values obtained are comparable to those for performic acid-oxidized ribonuclease A and native ribonuclease A (63). The intrinsic viscosity of native nuclease in 6 M guanidine HCl-0.05 M Tris-HCl, pH 8, was found to be 0.170 ± 0.017 dl per g as expected from the formulation of Tanford (64).

Acknowledgments—We thank Dr. Ladislav Morávek and Mr. James L. Cone for providing partially purified nuclease from which purified protein was prepared for part of this work, and Dr. Alan Schechter for his help in the measurement of fluorescence.

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