A Solid Phase Synthetic Study of Structure-Function Relationships in the Amino-terminal Region of Staphylococcal Nuclease

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IRWIN M. CHAIKEN* AND CHRISTIAN B. ANFINSEN
From the Laboratory of Chemical Biology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014

SUMMARY

Synthetic peptides corresponding to residues (6 through 47), (9 through 47), (11 through 47), (12 through 47), (10 through 47), and (18 through 47) in staphylococcal nuclease were prepared by the Merrifield solid phase method. Whereas synthetic-(6-47) and synthetic-(9-47) are about equally effective in generating both RNase and DNase activity upon mixing with native nuclease tryptic fragment nuclease-T-(49-149), synthetic-(10-47) is only partially effective and synthetic-(11-47) through synthetic-(18-47) generate essentially no activity. Synthetic-(6-47) is also the most capable of forming a stable complex with nuclease-T-(49-149), as judged by the resistance of the DNase activity of this semisynthetic nuclease-T species to destruction by trypsin in the presence of deoxythymidine 3',5'-diphosphate and Ca++ as well as by the extent of the shift and intensification of the fluorescence emission spectrum of the tryptophanyl residue in nuclease-T-(49-149). By these latter criteria, synthetic-(9-47) and synthetic-(10-47) form progressively weaker nuclease-T complexes than does synthetic-(6-47). On the other hand, all of the synthetic peptides through synthetic-(18-47) are able to bind, at least slightly, with nuclease-T-(49-149) as judged by the ability to enhance the binding of [125I]-nuclease-T-(49-149) to antibody. The results for these peptides, considered together with structural features in the 2 A model of nuclease elucidated by Dr. F. A. Cotton and his associates, suggest important structural functions for residues lysine 9 and glutamic acid 10 in both nuclease-T and nuclease.

Selective tryptic digestion of the major extracellular nuclease of Staphylococcus aureus in the presence of calcium ions and an inhibitor, deoxythymidine 3',5'-diphosphate, has yielded a fragment, nuclease-(6-48),1 that differs from native nuclease in lacking the first 5 residues at the amino terminus (2). This derivative is completely active and has physical characteristics that are extremely similar to those of the native enzyme. Further tryptic digestion of nuclease or nuclease-(6-149) yields two smaller fragments, nuclease-T-(6-48) (residues 6 through 48) and nuclease-T-(49-149) (residues 49 or 50 through 149), which are, themselves, structureless and inactive but can combine non-covalently to form nuclease-T, a species having about 8 to 10% of the activity of nuclease and a structure that also appears to be very similar to that of the native enzyme (2, 3).2 Based on the properties of both nuclease-(6-149) and nuclease-T, at least the first 5 amino acid residues at the amino terminus of nuclease are unessential to enzymic structure and function.

Solid phase synthesis of a fragment corresponding to residues 6 through 47 has been accomplished (4) and applied to studies of the function of certain amino acids in nuclease-T-(6-48)3 in the formation of active nuclease-T (5-7). Crude synthetic-(6-47) can combine with nuclease-T-(49-149) to yield a partially active semisynthetic nuclease-T (4). Synthetic-(9-47) is about as effective as synthetic-(6-47) in forming the active complex (5), suggesting that residues 6 to 8 are unessential for the formation of active nuclease-T. On the other hand, synthetic-(18-47) does not form an active complex with nuclease-T-(49-149) (5).

We have now prepared and characterized several other solid phase synthetic truncated fragments of nuclease-T-(6-48) in order to elucidate further the structural requirements, at the amino terminus of nuclease, for enzymic activity and conformation.

MATERIALS AND METHODS

Synthetic peptides were prepared by the solid phase procedure of Merrifield (8). The initial attachment of N-α-t-butyloxycarbonyl-where the trivial name denotes the origin and X and Y denote the NH2-terminal and COOH-terminal amino acid residues, respectively.

1 Nuclease-T-(49-149) is usually obtained as an approximately equimolar mixture of fragments containing either residues 49 through 149 or 50 through 149 (2, 3). Both of these latter fragments have been isolated and shown to be equally competent in forming a nuclease-T complex with nuclease-T-(6-48) (2). The nuclease-T-(49-149) used here is such a mixture; the denotation used is for simplification.

2 Residue 48 (lysine) can be removed from nuclease-T-(6-48) by carboxypeptidase B without change in activity of nuclease-T (2).
Amino acid compositions of synthetic peptides

| Residue | 6-47 Found | 6-47 Theory | 9-47 Found | 9-47 Theory | 10-47 Found | 10-47 Theory | 11-47 Found | 11-47 Theory | 12-47 Found | 12-47 Theory | 16-47 Found | 16-47 Theory | 18-47 Found | 18-47 Theory |
|---------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
| Lys*    | 6.3        | 6          | 4.8        | 5          | 3.7        | 4          | 3.6        | 4          | 3.6        | 4          | 4.1        | 4          | 2.8        | 3          |
| His*    | 1.8        | 2          | 0.8        | 1          | 0.7        | 1          | 1.1        | 1          | 0.7        | 1          | 0.6        | 1          | 1.0        | 1          |
| Arg*    | 0.9        | 1          | 1.1        | 1          | 0.8        | 1          | 1.4        | 1          | 0.7        | 1          | 0.7        | 1          | 0.6        | 1          |
| Asp     | 3.3        | 3          | 3.1        | 3          | 3.1        | 3          | 3.0        | 3          | 3.4        | 3          | 3.4        | 3          | 3.5        | 3          |
| Thr*    | 4.7        | 5          | 4.2        | 5          | 4.7        | 5          | 4.6        | 5          | 4.9        | 5          | 4.1        | 4          | 4.1        | 4          |
| Glu     | 2.9        | 3          | 3.1        | 3          | 2.8        | 3          | 3.7        | 4          | 2.8        | 3          | 2.6        | 2          | 2.9        | 3          |
| Pro     | 4.0        | 4          | 4.4        | 4          | 3.0        | 4          | 3.7        | 4          | 2.8        | 3          | 2.6        | 2          | 2.9        | 3          |
| Gly     | 2.3        | 2          | 2.1        | 2          | 2.3        | 2          | 2.2        | 2          | 2.4        | 2          | 2.4        | 2          | 2.4        | 2          |
| Ala     | 2.3        | 2          | 2.5        | 2          | 2.4        | 2          | 2.3        | 2          | 2.0        | 2          | 1.5        | 1          | 1.0        | 0          |
| Val     | 2.1        | 2          | 2.1        | 2          | 2.0        | 2          | 2.0        | 2          | 2.0        | 2          | 2.2        | 2          | 2.1        | 2          |
| Met     | 1.0        | 2          | 1.6        | 2          | 1.9        | 2          | 1.2        | 2          | 1.8        | 2          | 1.9        | 2          | 1.5        | 2          |
| Ile     | 2.0        | 2          | 2.5        | 2          | 2.5        | 2          | 2.1        | 2          | 2.4        | 2          | 1.3        | 1          | 1.1        | 1          |
| Leu     | 5.9        | 6          | 5.1        | 5          | 5.6        | 5          | 5.9        | 5          | 5.9        | 5          | 4.4        | 4          | 4.3        | 4          |
| Tyr     | 0.8        | 1          | 0.5        | 1          | 0.6        | 1          | 0.6        | 1          | 0.6        | 1          | 0.6        | 1          | 0.6        | 1          |
| Phe     | 1.1        | 1          | 1.0        | 1          | 1.1        | 1          | 1.1        | 1          | 1.0        | 1          | 1.0        | 1          | 1.0        | 1          |
| e-Trifluoroacetyl lysine* | 0.6 | 0 | 0.3 | 0 | 0.2 | 0 | 0.2 | 0 | 0.5 | 0 | 0.2 | 0 | 0.4 | 0 |

* Total of free and e-trifluoroacetylated lysine.
* Corrected for 5% destruction during acid hydrolysis.

**TABLE I**

Synthetic peptides were prepared which consisted of residues (6 through 47), (9 through 47), (10 through 47), (11 through 47), (12 through 47), (16 through 47), and (18 through 47) of nuclease.

The amino acid compositions for these peptides, shown in Table I, correspond closely to the expected values based on the structure of nuclease-T-(6-48), as presented in Fig. 1. The synthetic peptide preparations were further examined by correlation of tryptic peptide maps with the map for nuclease-T-(6-48) (2), as shown for synthetic-(9-47), -(11-47), and -(18-47) in Fig. 2. All three peptide maps contain ninhydrin- and Pauly-positive Components I and II (cross-hatched), shown previously to correspond to the tryptic peptides 36 to 47 and 25 to 28, respectively (2, 7). As expected, however, only the map for synthetic-(9-47) contains the component (III) corresponding to tryptic Peptide 10 to 16. The tryptic Peptide 11 to 16, which should be present for synthetic-(11-47), has not been identified but appears to be migrating in the abnormally large dense area in which Component II is located. In addition, synthetic-(9-47) and synthetic-(11-47) show a component (IV) corresponding to tryptic...
FIG. 2. Peptide maps of synthetic-(9-47), -(11-47), and -(18-47) after tryptic digestion. Each peptide (0.5 mg) was treated with 0.01 mg of trypsin in 0.5 M ammonium bicarbonate for 2 hour at room temperature. Termination of digestion by lyophilization was followed immediately by electrophoresis-chromatography as described under “Materials and Methods.” All maps are ninhydrin-stained (18), with the major and minor density components circled in solid and dashed lines, respectively. The cross-hatched areas are components positive to subsequent treatment with Pauly reagent (19). Phenol red (PR) was run as a marker in the chromatographic dimension. The numbered components are explained in the text. The component labeled Y in each map stains yellow with ninhydrin.

Peptide 17 to 24. Synthetic-(18-47), which should not contain this component, contains a component (V), with similar electrophoretic and chromatographic mobilities, which may represent Peptide 18 to 24.

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The DNase and RNase activities generated by all of the synthetic peptides upon mixing with nuclease-T-(49-149) were measured. For DNase activity, 0.01 μmole of each synthetic peptide was incubated with 0.01 μmole of nuclease-T-(49-149) in 600 μl of 0.05 M Tris buffer, pH 8, for 1 hour at room temperature before activity assays were carried out. A similar incubation, except in a total of 60 μl, was carried out prior to RNase activity assays. As shown in Table II, synthetic-(6-47) and synthetic-(10-47) generate the highest levels of activity, while synthetic-(10-47) is much less effective. All other synthetic peptides effect no significant activity. In addition, the relative amount of enzymic activity generated for each peptide, as compared with that effected for synthetic-(6-47), is similar for both DNA and RNA substrates. Clearly, an abrupt transition in ability to promote enzymic activity upon addition to nuclease-T-(49-149) occurs upon loss of residues 9 and 10. As found previously (4, 5, 7), the DNase activity generated for crude synthetic-(6-47) is about 5% that effected with nuclease-T-(6-48).

Since it is apparent that synthetic-(6-47), -(9-47), and -(10-47) can effect enzymically active complexes with nuclease-T-(49-149) (Table II), the stability of these complexes to tryptic digestion was studied and compared with that found for native nuclease-T [nuclease-T-(6-48) mixed with nuclease-T-(49-149)] (3). Nuclease-T-(49-149) (2.3 × 10⁻³ μmole) was incubated with 2.3 × 10⁻³ μmole of each synthetic peptide, or nuclease-T-(6-48), in 0.1 ml of 0.05 M Tris buffer, pH 8, with or without 0.01 M CaCl₂ and 0.0001 M deoxythymidine 3',5'-diphosphate. After incubation at room temperature for 1 hour, 1 μl of 1% trypsin was added to each sample and incubation continued. The DNase activities of the trypsin-treated peptide mixtures, as well as of the untreated controls, were measured at various times after trypsin addition. In all cases, mixtures of synthetic peptides, or nuclease-T-(6-48), with nuclease-T-(49-149) treated in the absence of deoxythymidine 3',5'-diphosphate and Ca²⁺, lost essentially all enzymic activity within 1 hour, in agreement with previous results for both native (3) and semisynthetic (4, 5, 7) nuclease-T. On the other hand, as shown in Table III, the activities generated by all synthetic peptides upon incubation with nuclease-T-(49-149) are protected, to differing extents, against proteolytic destruction by inhibitor and calcium ions. Such protection corresponds to the effect found for the mixture of nuclease-T-(6-48) with nuclease-T-(49-149).
Tryptic stability of activity of (synthetic peptide)-(nuclease-T-(49-149)) mixtures in presence of deoxythymidine 3',5'-diphosphate and Ca++

See text for procedures for peptide incubations, trypsin treatment, and activity assays.

| Peptide              | DNase activity remaining after incubation with trypsin for times designated |
|----------------------|----------------------------------------------------------------------------|
|                      | 1 hr | 1½ hrs | 2 hrs |
| Nuclease-T-(6-48)    | 92   | 91     |       |
| Synthetic-(9-47)     | 68   | 72     | 67    |
| Synthetic-(9-47)     | 55   | 38     | 37    |
| Synthetic-(10-47)    | 45   | 28     | 90    |

It has been shown previously (20) that nuclease-T-(6-48), when added to nuclease-T-(49-149), causes a distinct blue shift and intensification of the fluorescence emission spectrum of the tryptophan residue in nuclease-T-(49-149), this effect being interpreted as due to the burying of the tryptophanyl side chain in a hydrophobic environment during the folding of the native fragments to form nuclease-T. This effect was studied for the series of analogues from synthetic-(6-47) through synthetic-(18-47). Nuclease-T-(49-149) (9 x 10^-3 amole) was incubated with each of the crude synthetic peptides (9 x 10^-5 amole, or a 10-fold molar excess over nuclease-T-(49-149)) in 0.4 ml 0.05 M Tris buffer, pH 8, containing 0.01 M CaCl2 and 0.0005 M deoxythymidine 3',5'-diphosphate. After incubation for 1 hour at room temperature, the fluorescence emission spectrum of each sample, as well as of nuclease-T-(49-149) alone, was recorded. The spectrum for nuclease-T-(49-149) alone was corrected for the slight emission found for the buffer; the spectrum for each (synthetic peptide)-nuclease-T-(49-149) mixture was corrected for the spectrum shown for the synthetic peptide alone in buffer. The corrected spectra are shown in Fig. 3. Clearly, the intensification and shift of the spectral maximum from that of nuclease-T-(49-149) alone were greatest with synthetic-(6-47) and progressively less for synthetic-(9-47) through synthetic-(12-47). Synthetic-(16-47) and synthetic-(18-47) produced no significant effect on the fluorescence of nuclease-T-(49-149). The relative ability of each fragment to effect the shift and intensification of fluorescence corresponds quite closely to the relative ability of each peptide to produce enzymic activity (Table II). These correlations are discussed further below. The emission spectra for synthetic-(6-47), -(9-47), and -(18-47) are all referred to those found previously (4, 5, 7).

As a further means of characterizing the binding of nuclease-T-(49-149) with each of the synthetic peptides, advantage was taken of the ability of nuclease-T-(6-48) to enhance the binding of 125I-[nuclease-T-(49-149)] to antinuclease antibody (15), probably as a result of formation of antigenic sites specified by conformational features of nuclease-T. Previous results (15) have shown that synthetic-(6-47), -(9-47), and -(18-47) are all effective in enhancing 125I-[nuclease-T-(49-149)] binding. With the use of a procedure similar to that described previously (15), 2 μmoles of nuclease-T-(6-48) or synthetic peptide were incubated at 4°C with 47 μl of 3 mg per ml of antinuclease in 0.53 ml of 0.9% NaCl solution, buffered at pH 7.5 with 0.05 M Tris-HCl. After 24 hours, 212 μg of 125I-[nuclease-T-(49-149)] (0.2 μmole) were added to each sample and incubation continued at 4°C for an additional 24 hours. Then, 50 μl of sheep antirabbit immunoglobulin G antiserum were mixed with each sample. After 48-hour incubation at 4°C, the precipitate in each sample was centrifuged, washed with cold NaCl buffer solution, dissolved in 1 ml of 0.1 n NaOH, and tested for radioactivity content. For controls, normal rabbit immunoglobulin G was used instead of antinuclease antibody, in an amount necessary to produce a similar amount of precipitate as judged by the A280 of solutions of the final, dissolved precipitates. Each trial with antinuclease was corrected for the radioactivity nonspecifically bound to precipitate in the appropriate control (about 5 to 7% of the total radioactivity bound to antinuclease). The results are summarized in Table IV. As expected from previous studies on synthetic-(6-47), synthetic-(9-47), and synthetic-(18-47) (15), all of the synthetic peptides are able to effect an enhancement of 125I-[nuclease-T-(49-149)] binding to antinuclease. These data are discussed further below.

**DISCUSSION**

Synthetic analogues, differing from synthetic-(6-47) by deletions of residues at the amino-terminal end, have been tested for the extent to which they interact with nuclease-T-(49-149). Based on the ability to generate nuclease activity with the latter...
fragment, synthetic-(6-47) and synthetic-(9-47) are the most effective in forming a specifically folded semisynthetic enzyme complex corresponding to native nuclease-T. On the other hand, deletion of residue 9 (lysine) to yield synthetic-(10-47) results in a marked decrease in the ability to effect activity. Further deletion of residue 10 (glutamic acid) to give synthetic-(11-47) results in essentially complete loss in this property. As expected, peptides lacking further residues at the amino terminus, namely synthetic-(12-47), -(16-47), and -(18-47), are also unable to generate activity with nuclease-T-(49-149). The activity generated by each of synthetic-(6-47), -(9-47), and -(10-47) appears to be due to the specific formation of a semisynthetic nuclease-T complex as indicated by the resistance of this activity to tryptic inactivation in the presence of inhibitor and Ca^{2+} (Table III). On the other hand, the tightness of the conformational nuclease-T complexes formed probably decreases with decreasing chain length from synthetic-(6-47) to synthetic-(10-47), based on the differences in tryptic stability shown.

The ability of the peptide fragments to form a complex with nuclease-T-(49-149) having a conformational essential for activity is generally paralleled by a shift and intensification of the fluorescence emission spectrum of the tryptophanyl residue of nuclease-T-(49-149). Thus, the extent of shift and intensification shown in Fig. 3 is greatest for synthetic-(6-47), which effects the greatest activity, and insignificant for two of those peptides, synthetic-(16-47) and synthetic-(18-47), which effect no activity. As with activity, the extent of the fluorescence effect decreases most dramatically with the loss of residue 9 (from synthetic-(9-47) to synthetic-(10-47)) and decreases still further with the loss of residue 10 (from synthetic-(10-47) to synthetic-(11-47)). A significant decrease in fluorescence intensity and spectral shift is effected with the loss of residues 6 through 8 (from synthetic-(6-47) to synthetic-(9-47)) without a corresponding change in enzymic activity (Table II). This spectral difference suggests that synthetic-(9-47) forms a somewhat looser but essentially equally active complex with nuclease-T-(49-149), a conclusion also suggested by the decreased tryptic stability of the semisynthetic nuclease-T complex formed by synthetic-(9-47) (Table III).

The pattern of complex-forming ability of synthetic peptides with nuclease-T-(49-149) as studied by the enhancement of $32\mathrm{P}$-[nuclease-T-(49-149)] binding to antinuclease (Table IV) is quite different from that shown by the fluorescence spectra. All of the shortened synthetic peptides are effective in enhancing $32\mathrm{P}$-[nuclease-T-(49-149)] binding, with enhancement about equal for all of those peptides that generate activity, namely synthetic-(6-47), synthetic-(9-47), and synthetic-(10-47). The data suggest a small difference in enhancement between these peptides and the analogues synthetic-(11-47), -(12-47), -(16-47), and -(18-47). It seems reasonable to assume that the enhancement of $32\mathrm{P}$-[nuclease-T-(49-149)] binding can occur if the synthetic peptide can at least partially join in complex with nuclease-T-(49-149) to generate some, if not all, of the conformation-dependent antigenic sites of nuclease-T. In addition, as has been suggested from studies on the immunological properties of fragments of myoglobin (21), antibody against a specific antigenic site may combine with the fragment only when it assumes its native conformational state. The antibody may thus "trap" a particular conformational variant of the system in question, in this instance, semisynthetic nuclease-T. In this light, it may be suggested that synthetic-(11-47), -(12-47), -(16-47), and -(18-47), although they generate no active complex with nuclease-T-(49-149), can bind with this fragment well enough to effect, at least partially, the formation of a semisynthetic complex that is conformationally related to nuclease-T. That synthetic-(11-47) and synthetic-(12-47) can bind loosely to nuclease-T-(49-149) is, in fact, also suggested by the small fluorescence shift and intensification effected by these peptides (Fig. 3). The data for synthetic-(18-47) are in general agreement not only with previous immunological data (15), but with the previous demonstration that synthetic-(18-47) can bind to Sepharose to which nuclease-T-(49-149) has been covalently bound (5).

Based on the properties shown above for the shortened peptides synthetic-(6-47) through synthetic-(18-47), it may be concluded that residues 9 and 10 appear to be critical in allowing synthetic-(6-47) to form an active semisynthetic complex with nuclease-T-(49-149). Loss of residue 10 causes essentially complete loss of the ability to effect enzymic activity. On the other hand, synthetic-(11-47) through synthetic-(18-47), although they generate no activity, appear to be partially effective in forming a complex with nuclease-T-(49-149) with at least some of the conformational features of nuclease-T.4

The importance of residues 9 and 10 in maintaining nuclease structure is suggested by the localization of these residues in the three-dimensional structure of nuclease. Although neither lysine nor glutamic acid is close to the catalytic or substrate binding regions, both residues appear to be involved in interactions which could stabilize the nuclease structure by linking a significant part of the large pleated sheet structure (residues 13 to 35) to other parts of the folded nuclease molecule. Further investigations of the crystal structure of nuclease, now in progress at the Massachusetts Institute of Technology, as well as the synthesis of other peptide analogues in the nuclease-T-(6-48) region, will allow a more detailed description of the participation of residues 9 and 10 in nuclease structure and function.

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4 Synthetic-(1-47) also has been prepared and shown to be able to form an active semisynthetic complex with nuclease-T-(49-149). This complex has properties similar to those for the complex with synthetic-(1-47) but appears to be somewhat less stable. Based on the earlier observation that nuclease-(6-149) is as active as nuclease itself (2), it may be suggested that, although the NH$_2$-terminal 5 residues of nuclease serve no essential function, the existence of these residues in synthetic-(1-47) partially destabilizes the normal folding with nuclease-T-(49-149). The mechanism for interference has not been investigated.

5 Previous investigations of the crystal structure of nuclease and nuclease in a complex with deoxythymidine 3',5'-diphosphate and Ca$^{2+}$ have led to 4 A electron density maps for both protein forms (22), as well as to the more recently derived 2 A map of the nuclease complex with deoxythymidine 3',5'-diphosphate and Ca$^{2+}$ (A. Arnone, C. J. Bier, F. A. Cotton, E. E. Hazen, D. C. Richardson, J. S. Richardson, V. W. Day, and A. Tonath, manuscript in preparation). Based on the 2 A map, an atomic model of the nuclease-ligand complex has been built in our laboratory by D. C. Richardson and J. S. Richardson and used, along with the map, for discussions relevant to the work presented here.
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