Structure-Function Relationships at the Active Site of Nuclease-T' *

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SUMMARY

Solid phase peptide synthesis has been used to study structural requirements at the active site of staphylococcal nuclease-T', the noncovalent complex of the polypeptides nuclease-T-(6-48) and nuclease-T-(49-149) (residues 6 through 48 and 49 through 149 of native nuclease, respectively). In light of the importance of glutamic acid 43 in nuclease-T' catalysis (CHAIKEN, I. M., AND SANCHEZ, G. R. (1972) J. Biol. Chem. 247, 6743–6747), analogues of nuclease-T-(6-48) with progressively longer deletions at the COOH terminus have been synthesized, and their binding to native nuclease-T-(49-149) to produce an active complex has been investigated. The results indicate that residues glutamic acid 43, threonine 44, lysine 45, histidine 46, proline 47, and lysine 48 are not necessary for formation of a nuclease-T'-like complex. On the other hand, threonine 44 is critical for normal enzymic activity. The effect of threonine 44 appears to be due to its contribution to the peptide bond with glutamic acid 43 and not to its specific side chain moiety.

The synthesis of an analogue of nuclease-T-(6-48) with alanine at position 19 in place of aspartic acid was also undertaken. No enzymic activity is generated by this peptide in the presence of nuclease-T-(49-149), although complex formation appears to occur. This finding is consistent with the view, based on the crystal structure of the nuclease-Ca++-diphosphate complex (COTTON, F. A., BIER, C. J., DAY, V. W., HAZEN, E. E., JR., AND LARSEN, S. (1972) Cold Spring Harbor Symp. Quant. Biol. 36, 243–249), that the side chain carboxyl group of aspartic acid 19, along with those of aspartic acid residues 21 and 40 and glutamic acid 43, participates in the binding of the essential calcium ion.

Native enzyme in its specificity toward substrates, in its requirement for calcium, and in over-all conformation (2-5). Recent work (6) has indicated that the crystal structure of nuclease-T' with Ca++ and the strong inhibitor deoxythymidine 3',5'-diphosphate bound is isomorphous with that of the native nuclease-Ca++-pdTp complex, the structure of which has been determined to approximately 2.5 A resolution (7). On this basis, the high resolution model of nuclease has been used in considerations of structure-function relationships in nuclease-T'. In native nuclease, according to the model, the NH2-terminal third of the polypeptide chain contributes a number of important residues to the interaction between the protein, Ca++, and pdTp. In particular, the side chain carboxyl groups of aspartyl residues 19, 21, and 40, and glutamyl residue 43 are located very close to the essential Ca++, while the guanidino group of arginine 35 appears to interact with the 5'-phosphate group of the inhibitor. Because of the close similarity between nuclease and nuclease-T', these interactions presumably play an important role in the latter complex as well. In fact, studies with synthetic analogues of nuclease-T-(6-48), the smaller polypeptide component of nuclease-T', have shown that aspartic acid 21 and 40, glutamic acid 43, and arginine 35 are critical for enzymic activity (9, 10). Furthermore, the results have suggested participation of glutamic acid 43 in the formation of the active site (11).

In an attempt to investigate further the structural requirements at the active site of nuclease-T' and the role of glutamic acid 43 at this locus, we have undertaken the synthesis and the role of glutamic acid 43 in this crystal structure of nuclease-T' (6-48) with progressively larger deletions at the COOH terminus and studied the ability of these peptides to bind to nuclease-T-(49-149) and to generate nuclease-T' activity. In addition, we have investigated the effect of substituting alanine for aspartic acid 19, the side chain of which may be involved in ligand binding and enzymic activity based on its proximity to the essential Ca++. The results have suggested participation of glutamic acid 43 in the formation of the active site (11).

EXPERIMENTAL PROCEDURES

Nuclease, nuclease-T-(6-48), and nuclease-T-(49-149) were prepared according to published procedures (2, 3). For this...
work, nuclease-T-(49-149) was freed from nuclease-T-(50-149) by the method of Taniuchi and Anfinsen (3). All experimental work was performed using nuclease-T-(49-149). Synthetic peptides were prepared by the Merrifield method (15, 16) of solid phase peptide synthesis, using chloromethylated Bio-beads S-X-1 (1% cross-linked, 200 to 400 mesh; 0.75 meq of chlorine per g) as the solid support. Amino acid blocking groups, coupling procedures, cleavage of the peptide from the resin with hydrogen fluoride, and removal of t-trifluoroacetyl groups of lysine residues with piperidine were as previously described (9, 17). Removal of t-butyloxycarbonyl blocking groups was carried out with 50% trifluoroacetic acid in methylene chloride (18). The deblocked peptide mixture was passed through Sephadex G-25 (fine), and the peak fraction was rechromatographed on the same column. This material was used in the experimental work.

The following peptides (shown schematically in Fig. 1) were prepared: synthetic-(6-47), [Ala49]synthetic-(6-49), synthetic-(6-44), synthetic-(6-43), synthetic-(6-42), [Ala39]synthetic-(6-47), [Asp38]synthetic-(6-43), synthetic-(9-44), and [Ala46]synthetic-(9-44). Analogues were always synthesized in parallel with synthetic-(6-47), the peptide corresponding to the native sequence (omitting residue lysine 48, which has been shown not to be necessary for nuclease-T' activity (3, 4)), in order to control the synthesis.

DNase and RNase activities were measured using the assay system of Cuatrecasas et al. (21). Synthetic peptide (0.01 µmole) was incubated for 1 hour with nuclease-T-(49-149) (0.01 µmole) in 0.025 M Tris-Cl, pH 8.0, in a total volume of 100 µl. Aliquots of the incubation mixture were assayed against DNA and RNA assay solutions prepared as previously described (21). When the effect of calcium ion (as CaCl2) and pdTp was studied, these ligands were included in the incubation mixture at concentrations of 10 mM and 0.10 ±M, respectively.

For the determination of the fluorescence emission characteristics of semisynthetic peptide mixtures, synthetic peptide (0.05 µmole) was incubated with nuclease-T-(49-149) (0.0025 µmole) in 0.025 M Tris-Cl, pH 8.0, in the presence of 10 mM CaCl2 and 0.10 mM pdTp in a total volume of 200 µl. The samples were incubated for 1 hour and the fluorescence spectrum, upon excitation at 290 nm (22), was determined with an Aminco-Bowman spectrophotofluorometer (23). Appropriate blanks were run to correct for small contributions of the synthetic peptide, buffer, and ligands.

The resistance to trypsin of the activity of mixtures of synthetic peptides and nuclease-T-(49-149) was tested by addition of 5 µl of 0.2% trypsin (Worthington; diisopropylfluorophosphate-treated) to a solution containing synthetic peptide (0.025 µmole) and nuclease T (10 mg) in 0.0012 µmole in 0.025 M Tris-Cl, pH 8.0, in the presence and in the absence of CaCl2 (10 mM) and pdTp (0.10 mM) in a total volume of 100 µl. Aliquots were taken at time intervals and assayed for DNase activity as described above.

The resistance to trypsin of the fluorescence of mixtures of synthetic peptides and nuclease-T-(49-149) was tested by addition of 5 µl of 0.1% trypsin to a solution containing synthetic peptide (0.05 µmole) and nuclease-T-(49-149) (0.0025 µmole) in 0.025 M Tris-Cl, pH 8.0, in the presence and absence of CaCl2 (10 mM) and pdTp (0.10 mM) in a total volume of 200 µl (11). The fluorescence spectra of samples were determined as a function of time and corrected for the contribution of synthetic peptide, trypsin, ligands, and buffer.

Semisynthetic complexes of synthetic-(6-44), -(6-43), and -(6-42) with native nuclease-T-(49-149) were purified by a functional purification method developed previously (4, 11). In this method, a mixture of synthetic peptide and native nuclease-T-(49-149) is treated with trypsin in the presence of Ca++ and pdTp and then fractionated by phosphocellulose chromatography. Synthetic-(6-44) (150 mg) and native nuclease-T-(49-149) (15 mg) were dissolved in 15 ml of 0.1% NH4HCO3 containing 0.1 mM pdTp and 0.01 mM CaCl2 at pH 8.0. After incubation at room temperature for 15 min, 2 mg of trypsin (diisopropylfluorophosphate-treated) were added. The solution was allowed to incubate for 150 min, during which time the DNase activity of the mixture fell to 84% of the initial value. At this time, 9 mg of soybean trypsin inhibitor were added. After 10 min, the solution was lyophilized. Synthetic-(6-43) (55 mg) and synthetic-(6-42) (86 mg) were incubated as above with native nuclease-T-(49-149) (5.5 and 8.0 µg, respectively). After 15 min, trypsin (0.75 mg and 1.2 mg, respectively) was added and incubation was continued for 1 hour. Soybean trypsin inhibitor (3.3 mg and 5.2 mg, respectively) was then added. After 15 min, the solutions were lyophilized. For the synthetic-(6-42) ex-

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**Fig. 1.** Scheme showing the amino acid sequence of native nuclease-T-(6-48) (19) and of the synthetic peptides prepared in this work.
Table I
Enzymic activity effected by nuclease-T-(6-48) and synthetic analogues after incubation with nuclease-T-(49-149) in the presence and absence of Ca ++ and pdTp

| Peptide species | DNase | RNase |
|----------------|-------|-------|
|                | ΔOD 600/mg complex | ΔOD 600/mg complex |
| Synthetic-(6-43) | 154.5 | 223.7 |
| Synthetic-(6-44) | 12.1 | 18.2 |
| Synthetic-(6-45) | 0.1 | 0.1 |
| Synthetic-(6-46) | 0.0 | 0.2 |
| Synthetic-(6-47) | 0.1 | 0.2 |
| Synthetic-(6-48) | 0.1 | 0.2 |
| Synthetic-(6-49) | 0.1 | 0.2 |

* Values in parentheses represent DNase activity levels when synthetic peptides were incubated at a 20:1 molar ratio of peptide to nuclease-T-(49-149) in the presence of 0.1 M CaCl 2 and 0.1 mM pdTp.

* Activity level less than 0.1% that of native nuclease-T RNase activity.

A white powder was obtained by lyophilization of the resulting material used in the purification step. The lyophilized mixtures were dissolved in small volumes of 0.3 M ammonium acetate buffer, pH 5.8 (starting buffer), and applied to phosphocelulose columns equilibrated with the same buffer (column sizes: 22 x 1.0 cm for synthetic-(6-44) mixture, 7 x 1.0 cm for synthetic-(6-46) and -(6-42) mixtures). Elution was carried out first with starting buffer (until essentially complete elution of the initial peak occurred) and then with a gradient consisting of 200 cc of starting buffer and 200 cc of 1.0 M ammonium acetate buffer, pH 8.0. In each case, an absorbance peak was observed eluting at a position very similar to that observed for native nuclease-T' (corresponding to buffer conductivity in the range of 26 to 31 mmbos). The peak fractions were pooled and lyophilized. A white powder was obtained in each case.

The complex of the native fragments nuclease-T-(6-48) and nuclease-T-(49-149) was prepared in essentially the same manner as the semisynthetic complexes, as described previously (4).

Stereo drawings were prepared with a Calcomp plotter, utilizing the high resolution coordinates for the nuclease-Ca ++ phan residue and producing a large fluorescence enhancement. Even those truncated peptides which are not capable of producing activity show the characteristic fluorescence behavior. The results are shown in Fig. 2. All of the synthetic peptides with truncations at the COOH terminus shift the emission maximum of the tryptophanyl residue in nuclease-T-(49-149) at position 140 when added to this peptide. This effect has been interpreted as being due to the insertion of the tryptophanyl residue into a nonpolar pocket in the active conformation of nuclease and nuclease-T'.

The synthetic peptides, therefore, were assayed for their ability to induce a specific conformation in nuclease-T-(49-149), as indicated by the fluorescence effect produced. The results are shown in Table I. All of the synthetic peptides with truncations at the COOH terminus shift the emission maximum of the tryptophanyl residue in nuclease-T-(49-149), producing significant levels of DNase and RNase activity. Synthetic-(6-43) and -(6-42) produce essentially no activity at this level of purity. [Asp43]synthetic-(6-43) also fails to produce enzymic activity, as might be expected from previous observations (10, 11). The presence of the active site ligand, Ca ++, and of the inhibitor, pdTp, in the incubation mixture enhances the DNase activity observed by nuclease-T-(6-48) and by synthetic-(6-47), [Ala4]synthetic-(6-46), and synthetic-(6-44). This effect has been interpreted as being due to the stabilization of the active complex formed (18). The slight inhibition of RNase activity observed is believed to reflect the ability of pdTp to compete effectively with RNA under the conditions used (18).

It appears that deletion of the terminal threonine 44 residue causes loss of the ability to effect normal activity in the incubation mixture. In order to determine whether this loss is a result of the loss of a function performed by the side chain of threonine 44 at the active site, the analogues synthetic-(9-44) and [Ala4]synthetic-(9-44) were prepared and assayed for DNase and RNase activity. The results are shown in Table I. The data indicate that the side chain of threonine 44 is not essential for enzymic activity. Therefore, the difference in the enzymic activities observed by synthetic-(6-44) and synthetic-(6-43) must be due, mainly, to factors other than the absence of the side chain of this amino acid.

The data in Table I also show that the analogue [Ala4]synthetic-(6-47) fails to produce enzymic activity. For this peptide, as well as for synthetic-(6-43), [Asp43]synthetic-(6-43), and synthetic-(6-42), the absence of significant activity is evident even at 20:1 molar ratios of synthetic peptide to nuclease-T-(49-149) in the presence of Ca ++ and pdTp.

Fluorescence of Semisynthetic Mixtures—Specific binding of synthetic-(6-47) analogue peptides to nuclease-T-(49-149) has been measured by determining the fluorescence spectral characteristics of mixtures of synthetic peptide with nuclease-T-(49-149) in the presence of Ca ++ and pdTp. Native nuclease-T-(6-48) (which contains no tryptophan) produces a blue shift and an enhancement of the fluorescence of the single-tryptophanyl residue in nuclease-T-(49-149) at position 140 when added to this peptide. This effect has been interpreted as being due to the insertion of the tryptophanyl residue into a nonpolar pocket in the active conformation of nuclease and nuclease-T'.

The synthetic peptides, therefore, were assayed for their ability to induce a specific conformation in nuclease-T-(49-149), as indicated by the fluorescence effect produced. The results are shown in Fig. 2. All of the synthetic peptides with truncations at the COOH terminus shift the emission maximum of the tryptophanyl residue and produce a large fluorescence enhancement. Even those truncated peptides which are not capable of producing activity show the characteristic fluorescence behavior. The results are taken to indicate that all of the truncated analogues bind nuclease-T-(49-149) to form semisynthetic complexes. It is noteworthy that the truncated sequence, synthetic-(6-42), still contains the information required for the stabilization of the specific three-dimensional structure of nuclease-T-(49-149) in nuclease-T'.

The [Ala4]synthetic-(6-47) analogue shows a smaller fluorescence effect than does synthetic-(6-47), although the effect is still considerable. [Asp43]synthetic-(6-43), not shown in the figure for the sake of clarity, gives a fluorescence enhancement of the same magnitude as that of synthetic-(6-42). Clearly, this enhancement is at least as great as that of synthetic-(6-43) and -(6-47).

Trypsin Stability of Mixtures of Synthetic Peptides and Nu-
Fig. 2. Effect of native nuclease-T-(6-48) and of the synthetic peptides on the fluorescence emission spectrum of nuclease-T-(49-149). The curves represent the fluorescence emission spectra, upon excitation at 295 nm, of nuclease-T-(49-149) alone (V) or in the presence of nuclease-T-(6-48) (O), synthetic-(6-47) ( ), [Ala46]synthetic-(6-46) ( △ ), synthetic-(6-44) ( ● ), synthetic-(6-43) ( △ ), [Ala46]synthetic-(6-42) ( △ ), and [Ala46]synthetic-(6-47) ( ▲ ). Ca++ and pdTp were present in all cases. Experimental conditions were as described in the text under “Experimental Procedures.”

TABLE II

| Peptide species incubated with nuclease-T-(49-149) | Percentage of DNase activity remaining at indicated time after addition of trypsin |
|--------------------------------------------------|----------------------------------------------------------------------------------|
| Synthetic-(6-47) .................................. | (100) 120 107 100 100 |
| [Ala46]Synthetic-(6-46) ......................... | (100) 103 95 104 97 |
| Synthetic-(6-44) ................. | (100) 100 98 101 98 |

Fig. 3. Stability to trypsin of the fluorescence emission spectra of mixtures of nuclease-T-(49-149) and (A) native nuclease-T-(6-48), (B) synthetic-(6-44), (C) synthetic-(6-43), and (D) synthetic-(6-42) in the presence (●) and absence (〇) of Ca++ and pdTp. Experimental conditions were as described under “Experimental Procedures.”

**Table II**

**Resistance of enzymic activity of semisynthetic complexes to trypsin in the presence of Ca++ and pdTp**

Synthetic peptide was incubated with nuclease T-(49-149) in the presence of CaCl2 and pdTp, in Tris-Cl buffer, pH 8.0. Trypsin was added, and the DNase activity of the mixture was followed as a function of time. See “Experimental Procedures” for other details. (In the absence of ligands, essentially all activity was lost within 1 hour after addition of trypsin.)

The data in Table II show that full activity is present even after 3 hours of incubation of synthetic-(6-47), [Ala46]synthetic-(6-47), and synthetic-(6-44) with nuclease-T-(49-149) and trypsin in the presence of ligands. In the absence of ligands, on the other hand, enzymic activity disappeared within 1 hour. The resistance of the enzymic activity of the semisynthetic mixtures to tryptic attack in the presence of ligands parallels that observed for the native mixture under similar conditions.
has perhaps been altered relative to binding for the active complexes. That the inactive semisynthetic complexes formed do bind ligands, as suggested by the mode of binding for these complexes. The lowered absolute resistance to trypsin in the presence of ligands suggests that the mode of binding for these complexes is stable, as expected, for both active semisynthetic mixtures. The initial rapid fluorescence decrease is apparent also for the inactive mixes for synthetic-(6-43) and -(6-42) (Fig. 3, C and D, respectively). However, here, the fluorescence emission surviving the initial period of digestion continues to decrease steadily, apparently due to a decreased resistance of the nuclease-T-like complexes produced in these cases. Thus, while the stabilization of the fluorescence effect by ligands in Fig. 3, C and D indicates that the active semisynthetic complexes formed do bind ligands, the lowered absolute resistance to trypsin in the presence of ligands suggests that the mode of binding for these complexes has perhaps been altered relative to binding for the active complexes. This behavior has been found previously for [Asp]synthetic-(6-47) (11).

Properties of Semisynthetic Complexes—It has been shown previously that the resistance to trypsin of the enzymic activity and of the fluorescence emission properties of mixtures of crude synthetic-(6-47)-related peptides and native nuclease-T-(49-149) in the presence of Ca++ and pdTp provides the basis for the isolation of semisynthetic analogues of the nuclease-T complex (4, 11). Inasmuch as the peptides involved in the COOH-terminal activity transition, namely, synthetic-(6-44), -(6-43), and -(6-42), effect trypsin-resistant nuclease-T-like properties when mixed with native nuclease T-(49-149), such semisynthetic complexes were prepared for these peptides using the functional method (see "Experimental Procedures"). The resultant complexes were all isolated on phosphocellulose and identified by enzymic activity when appropriate and by amino acid composition. The yields were 4.0, 0.7, and 1.0 mg, respectively, for the [Des 45-47], [Des 44-47], and [Des 43-47] complexes. All of the semisynthetic complexes were assayed for DNase and RNase activity, along with native nuclease-T', with the results shown in Table III. [Des 45-47]semisynthetic nuclease-T', containing synthetic-(6-44) in place of native nuclease-T-(6-48), is approximately 82% as active catalytically as native nuclease-T'. The [Des 44-47] and [Des 43-47] semisynthetic complexes show approximately 3 to 5% and 0.4 to 0.8%, respectively, of the enzymic activity of nuclease-T'. Normal semisynthetic nuclease-T' (containing synthetic-(6-47) of normal sequence), prepared by the same method as in previous work, showed about 90% the enzymic activity of nuclease-T' (4).

The sharp transition in the enzymic activity upon loss of threonine 44 observed with crude peptides (Table I) is again quite apparent with the purified semisynthetic complexes. This observation is in agreement with the preliminary conclusion (based on activity measurements before purification) that threonine 44 performs an important role in this portion of the active site of nuclease-T'. On the other hand, both [Des 44-47] and [Des 43-47] semisynthetic nuclease-T' do exhibit low levels of enzymic activity, in contrast to the essentially total lack of activity shown by the crude synthetic-(6-43) and -(6-42) peptides in the presence of nuclease T-(49-149) (Table I). Such low activities do not appear to be due to dissociation of otherwise highly active complexes, since fluorescence emission properties of these two analogues appear to be of the same character as those of nuclease-T' and of [Des 45-47]semisynthetic nuclease-T', namely, a blue shift in the emission maximum and a large intensification of the emission spectrum of the single tryptophyl residue. Based on our previous experience (11), it is probable that the very low level of enzymic activity associated with the [Des 43-47]semisynthetic nuclease-T' complex is due to the presence of a trace amount of native nuclease-T', produced during the trypsin purification step as a result of the presence of either native nuclease or nuclease-T-(6-48) in the nuclease-T-(49-149) used. The activity associated with [Des 44-47]semisynthetic nuclease-T' is subject to similar considerations, although, again based on our previous experience, the percentage relative to nuclease-T' appears to be too high to be fully accounted for in the same manner. Rather, we feel that [Des 44-47] semisynthetic nuclease-T' does in fact possess intrinsic enzymic activity, the low level relative to nuclease-T' being due to the loss of the peptide bond between positions 43 and 44. The lack of significant activity effected by mixtures of crude synthetic-(6-43) with nuclease-T-(49-149) (Table I) probably reflects competition of nonfunctional peptide impurities for available nuclease-T-(49-149).

The effect of elevated temperature on the enzymic activity of the purified complexes was determined as previously described (4). As already found for native nuclease-T' and normal semisynthetic nuclease-T' (4), the DNase activity associated with [Des 45-47] semisynthetic nuclease-T' varies biphasically, with a drastic falloff occurring above 45°C, presumably due to dissociation of the complex. A similar behavior was found for the [Des 44-47] and [Des 43-47] semisynthetic complexes. For the latter two cases, the data indicate that the low levels of enzymic activity are not due to contamination of the isolated complexes by native nuclease.

**DISCUSSION**

The arrangement of the side chain carboxyl groups of residues aspartic acid 19, 21, and 40, and glutamic acid 43 around the essential Ca++ at the active site of the nuclease-Ca++-pdTp complex is shown in a stereo drawing in Fig. 1. The location of the bound inhibitor, pdTp, is also shown. Because studies in solution (2-5) and in the crystal state (6, 7) have shown that the two enzyme species are closely related in over-all structure, the relative position of residues at the active site of nuclease-T' is believed to be very similar to that shown in the figure. Any major differences would be expected to exist near the points of trypsin cleavage. By analogy with the ribonuclease-ribonuclease-S system (25, 26), the residues in the COOH-terminal
region of nuclease-T-(6-48) and in the NH2-terminal region of nuclease-T-(49-149) would be expected to be arranged in a more disordered manner in nuclease-T' than in nuclease, where the peptide bond between residues 48 and 49 restricts the orientations which the polypeptide chain can assume in this region. The finding that residues lysine 45, histidine 46, proline 47, and lysine 48 can be left out of the COOH terminus of nuclease-T-(6-48) without significant loss of activity is in agreement with the idea of a random, essentially unimportant conformation in this portion of the nuclease-T' molecule. The fluorescence experiments (Figs. 2 and 3) indicate that, in addition to the above residues, threonine 44 and glutamic acid 43 can also be left out of nuclease-T-(6-48) without a significant change in the over-all conformation of the complex formed.  

The fact that enzymic activity is almost completely lost when residues threonine 44 through lysine 48 are missing, while a nuclease-T'-like complex is still formed, suggests that loss of threonine 44 causes a critical perturbation of glutamic acid 43. The results with the [Ala44]synthetic-(9-44) analogue indicate that lack of activity is not due to the absence of the side chain of threonine 44. Rather, threonine 44 appears to maintain the critical properties of the γ-COOH group. The somewhat decreased stability to trypsin of the fluorescence of mixtures of synthetic-(9-13) and native nuclease-T (10-110) in the presence of ligands very likely reflects altered ligand binding upon loss of threonine 44. In fact, the trypsin stability of the fluorescence effect for synthetic-(6-43) is very similar to that for synthetic-(6-42), which lacks glutamic acid 43 and therefore would not interact with Ca++ through the side chain of this residue. These findings strongly support the view that glutamic acid 43 is performing an important role during enzymic catalysis by nuclease-T', either directly as a catalytic group or indirectly through the essential calcium ion.

One structural element common to all of the truncated analogues which bind native nuclease-T-(49-149) efficiently is the sequence of residues 13 through 35. This segment in nuclease (and presumably in nuclease-T', as well) forms a triple-stranded anti-parallel β-pleated sheet array. It is likely that this β-structure contributes, in a significant manner, towards the stabilization of the nuclease-T' complex. We have found that synthetic-(9-44) brings about a fluorescence effect with nuclease-T-(49-149) in the presence of ligands, whereas synthetic-(9-35) and synthetic-(9-37) do not. Since synthetic-(6-42) also gives rise to a fluorescence effect, it is possible that a particular residue in the sequence 38 through 41 is important for complex formation with nuclease-T-(49-149). The functioning of aspartic acid 40 as an "anchoring" residue is an interesting possibility.

The finding that replacement of aspartic acid 19 with alanine leads to loss of activity parallels the results of substitutions (with and without preservation of charge) of aspartic acid residues 21 and 40 and glutamic acid 43 in synthetic-(6-47) (9, 10). Based on the high resolution crystal structure of the nuclease-Ca++-pdTp complex (7), the side chain carboxyls of these 4 residues (Fig. 4) are believed to be sufficiently close to the calcium ion to be able to interact with it, although the exact nature of this interaction is not known (14). The results with the [Ala43] analogue of synthetic-(6-47), together with the earlier observations (9-11), indicate the need for the presence of these four carboxyl groups around the essential Ca++ during the catalytic process.

The results presented here, and those previously reported (9-11), have provided a qualitative indication of the importance of aspartic acid 19, 21, and 40 and glutamic acid 43 in the active site of nuclease-T'. Purification of some of these synthetic analogues, as reported previously (11) and in the present work, suggests that when large differences in properties exist between analogue and normal synthetic peptides at the crude level of purity such differences remain after purification. As described earlier (11), the availability of these purified semisynthetic analogues of nuclease-T', with modifications at key loci in the active site region in the molecule, should allow a quantitative description of the participation of certain residues in ligand binding and enzymic activity.

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