Further Study of the Conformation of Nuclease-(1-126) in Relation to Intrinsic Enzymatic Activity*

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Nuclease-(1-126), although containing 89% of the amino acid sequence which folds to the ordered structure of nuclease A, is disordered and highly flexible (Taniuchi, H., and Anfinsen, C. B. (1969) J. Biol. Chem. 243, 4778-4786). On the other hand, Sachs et al. (Sachs, D. H., Schechter, A. N., Eastlake, A., and Anfinsen, C. B. (1974) Nature 251, 242-244) have demonstrated intrinsic enzymatic activity for nuclease-(1-126). To attempt to learn whether or not the active population of nuclease-(1-126) has the native conformation, we have examined nuclease-(1-126) with respect to enzymatic kinetics with and without the competitive inhibitor deoxythymidylate 3',5'-diphosphate (pdpT), effect of temperature on enzymatic activity, binding of pdpT in the presence of Ca²⁺ and intrinsic viscosity, Stokes radius, CD, and response to trypsin action in the presence and absence of pdpT and Ca²⁺. The results indicate that the conformation of nuclease-(1-126) bound with pdpT in the presence of Ca²⁺ is partially constrained but still highly flexible below 30 °C, outside the range of thermal transition exhibited by the ordered elements of nuclease-(1-126). Thus, formation or stabilization of the active site of nuclease-(1-126) by binding with ligands is not associated with cooperative folding of the entire polypeptide chain. Considering that nuclease-(1-126) does not bind to nuclease-(127-149) but does to nuclease-(111-149), the results are consistent with the idea that the specific cooperative interactions, providing extra stabilizing energy required for maintaining the polypeptide chain in the ordered state of nuclease A, may be disrupted for nuclease-(1-126) primarily due to cleavage of the peptide bond between residues 126 and 127. Then, it may be thought that binding with ligands does not compensate for this disruption.

Staphylococcal nuclease A¹ containing 149 residues and devoid of sulphydryl groups and disulfide bonds (1-3) folds to a compact three-dimensional structure in which the atomic coordinates of residues 1 to 141 are defined (4-6). Removal of residues 127 to 141 from an atomic model of nuclease A causes no change in the coordinates of the remaining residues and does not yield a pocket, groove, or crevice which would allow extensive disruption of hydrophobic interactions (4-6). Residues 139 and 140 fold back toward residues 136 and 137, and residue 141 protrudes outside the structure at the bottom left front corner of the model (4-6). This situation also contributes to limitation of the number of buried nonpolar residues (Val 111, Leu 108, and Val 104) which are exposed upon removal of residues 127 to 141 (4-6). Thus, the bulk of hydrophobic interactions of native nuclease A would be preserved for the native conformation of the fragment containing residues 1 to 126, nuclease-(1-126)² (4, 5).

We have further investigated the conformational properties of nuclease-(1-126), particularly in relation to intrinsic enzymatic activity. Sachs et al. (9), using antinuclease antibody directed to residues 127 to 149, have demonstrated that a low level of enzymatic activity of nuclease-(1-126) (7, 8) is intrinsic (9). Since all the residues of nuclease A involved in binding of Ca²⁺ (required for enzymatic activity (10) and pdpT (a potent competitive inhibitor (13)) are contained in nuclease-(1-126) (4-6), if the active site of nuclease-(1-126) corresponds to that of nuclease A, then these ligands would bind with nuclease-(1-126) in a manner similar to their binding with nuclease A (4-6). Our observations described below have indicated that pdpT binds with nuclease-(1-126) in the presence of Ca²⁺ with an apparent association constant approximately three orders of magnitude smaller than that with nuclease A and that the conformation of liganded nuclease-(1-126) exhibits partial constraint as well as high flexibility.

Thus, although most interatomic interactions such as hydrophobic interactions and dispersion forces operating for native nuclease A, perhaps except for those requiring complete folding of residues 1 to 141 for their operation (14-16), would also be operative for nuclease-(1-126), stabilization of the active site of nuclease-(1-126) by binding with ligands does not lead to cooperative folding of the entire polypeptide chain. The result is interpreted as indicating that specific cooperative interactions, which are not operational for nuclease-(1-126), may be operating for cooperative folding of native nuclease A and that disruption of them cannot be compensated for by binding with ligands.

MATERIALS AND METHODS

Nuclease A and nuclease B were purified from the culture media of Staphylococcus aureus, strain Foggi (ATCC 27735) as described

¹ Fragments of staphylococcal nuclease are designated as "nuclease-(X-Y)" where X and Y denote the NH₂- and COOH-terminal amino acids, respectively, according to the rules of the Commission on Biochemical Nomenclature (IUPAC-IUB) (Commission of Biochemical Nomenclature (1967) J. Biol. Chem. 242, 555).
² The abbreviations used are: pdpT, deoxythymidine 3',5'-diphosphate; nitrophenyl-pdpT, deoxythymidine 3'-phosphate 5'-p-nitrophenyl phosphate (11); Sepharose-pdpT, Sepharose 4-B (agarose) coupled to 3'-4'-aminophenyl-phosphoryl)-deoxythymidine 5'-phosphate (12).
³ 5'-phosphate of pdpT directly interacts with Arg 35 and Arg 87 and 3'-phosphate with Thr 85; Ca²⁺ is coordinated by Asp 21, Asp 40, and Thr 41 (4-6). Possible involvement of Tyr 113 in the enzymic activity has been suggested (4-6).

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elsewhere (3, 15). Nuclease-(1-126) and other fragments were prepared from purified nuclease A by established methods as outlined in miniprint immediately following this paper. Nuclease-(1-126) thus obtained has been passed through a Sephacryl-pdTp column (12) in order to remove contaminating intact nuclease A (see the miniprint supplement). This nuclease-(1-126) sample was further purified to reduce the amount of contaminating nuclease-(1-105) (enzymatically inactive) to less than 3% (see the miniprint supplement). The quantities of nucleases A and B, the fragments, and the fragment complexes were determined by absorbance at 280 nm (3, 16, 17) unless otherwise indicated.

Details of the analytical methods, including assay of enzymatic activity, equilibrium dialysis, and measurements of intrinsic viscosity, Stokes radius, and fluorescence are also described in the miniprint supplement.

RESULTS

Homogeneity of the Nuclease-(1-126) Preparation—The purified nuclease-(1-126) was homogeneous as judged by a symmetrical peak obtained in the elution profile by gel filtration (see the legend to Fig. 1). On the basis of an Andrews plot of molecular weight versus elution volume (18) nuclease-(1-126) eluted at a position corresponding to a molecular weight of 24,000 (Fig. 1). The molecular weight of nuclease-(1-126) on the basis of the amino acid sequence is 14,252 (2). The resulting deviation in the Andrews plot (Fig. 1) is assumed to indicate an increase in the hydrodynamic volume and not aggregation of the molecules, since the value from the plot is still significantly smaller than if a dimer is formed. There has been no extensive zonal spreading or deformation of the symmetrical peak in the elution profile (indicating a monomer-dimer (or polymer) equilibrium) in the concentration range from 1 to 6 mg per ml of nuclease-(1-126), and the absorbance at 280 nm obeys Lambert-Beer law for solutions containing from 0.1 to 1.0 mg per ml of nuclease-(1-126) (see the miniprint supplement). Note that nuclease B, the structure of which consists of an ordered portion corresponding to nuclease A and 19 flexible residues attached to the NH2 terminus of the nuclease A portion (3), also shows a similar deviation in the Andrews plot (Fig. 1). On the basis of these considerations, we assume that nuclease-(1-126) is a monomer under the conditions employed in the present studies.

Enzymatic Kinetics—The enzymatic kinetic parameters ($V_{max}$, $K_m$, and $K_I$ (with pdTp)) of nuclease-(1-126) were determined at pH 8.8 at 24 ± 1 °C in the presence of 10^{-2} M Ca^{2+} with heat-denatured DNA as a substrate (13) and are presented in Table I together with those of control samples nuclease A, nuclease-T (the noncovalent complex of nuclease-T-(6-48), and nuclease-T-(50-149)) (19, 20), and the complex of nuclease-(1-126) and nuclease-(99-149) (8). Some of the parameters with the control samples have been reported (7, 8, 13). The value for $V_{max}$ of nuclease-(1-126) is approximately 1.5% that of nuclease A. Enzymatic activity of nuclease-(1-126) is apparently competitively inhibited by pdTp (Fig. 2). The values for $K_m$ and $K_I$ with nuclease-(1-126) are both considerably greater than those with nuclease A (Table I). As is the case with nuclease A (10, 13), no enzymatic activity was detected with nuclease-(1-126) in the absence of Ca^{2+} (with 10^{-3} M EDTA present). The enzymatic activity of nuclease-(1-126) did not change upon addition of nuclease-(127-149), confirming the previous report (8). A maximum enzymatic activity of a standard magnifying glass. Full size photocopies are available from Waverly Press.
of pdTp were carried out in the presence of Cr-1 (Fig. 3).

A (Fig. 3) above 30 °C is not clear. This second phase of enzymatic activity tends to lie on a straight line in a manner similar to nuclease-(1-126) and nuclease-(1-126) as a function of temperature. T, degrees Kelvin. Nuclease-(1-126) also exhibited enzymatic activity with the nuclease-(1-126) exhibited two phases of enzymatic kinetics at 30 °C. Arrhenius plots of these data are shown in Fig. 3. Apparently nuclease-(1-126) exhibits a transition above 30 °C. Below 30 °C there is no apparent transition as the plots tend to lie on a straight line in a manner similar to nuclease A (Fig. 3). At 30 °C and above, nuclease-(1-126) exhibited biphasic kinetics of enzymatic activity, that is, the velocity for enzymatic activity (the slope) increases within 1 min after starting the assay in contrast to the velocity below 30 °C.

Details are the same as those described in the legend to Fig. 2.

Enzymatic activity of nuclease-(1-126) and nuclease A (as a control) was measured as a function of temperature from 5-50 °C. Arrhenius plots of these data are shown in Fig. 3. Apparently nuclease-(1-126) exhibits a transition above 30 °C. Below 30 °C there is no apparent transition as the plots tend to lie on a straight line in a manner similar to nuclease A (Fig. 3). At 30 °C and above, nuclease-(1-126) exhibited biphasic kinetics of enzymatic activity, that is, the velocity for enzymatic activity (the slope) increases within 1 min after starting the assay in contrast to the velocity below 30 °C which is constant. The reason for the biphasic kinetics observed above 30 °C is not clear. This second phase of enzymatic activity appeared to follow a transition in parallel with the first phase of the enzymatic activity (Fig. 3).

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Fig. 3. Arrhenius plots of the enzymatic activity of nuclease-(1-126) (C, A) with nuclease A (•) as a control. The enzymatic activity (v) was measured in an assay system (13) containing 35 μg of heat-denatured DNA/ml, 0.1 M Tris/HCl, pH 8.8, and 10-2 M CaCl2 for nuclease A or 10-2 M CaCl2 for nuclease-(1-126) as a function of temperature. T, degrees Kelvin. Nuclease-(1-126) exhibited two phases of enzymatic kinetics at 30 °C and above, the initial slower rate of increase (C) in the absorbance at 260 nm being followed, approximately 1 min later, by a faster rate of increase (Δ). Other details are the same as those described in the legend to Fig. 2.

It is known that binding of pdTp to nuclease A (21) and nuclease T (15) is weaker in the absence than in the presence of Ca2+. It is also shown that nuclease A is not adsorbed to Sepharose-pdTp in the absence of Ca2+ (22).

The association constant (Ka) of pdTp with nuclease-(1-126) in the presence of 10-2 M Ca2+ was determined at 20 °C at pH 8.0 by the method of equilibrium dialysis on the basis of the concentration of free (unbound) pdTp determined at equilibrium (20-h dialysis) (the concentration of bound pdTp being calculated by the difference between the concentration of free pdTp and the total concentration of pdTp; see the miniprint supplement for details). The value obtained was 1.76 ± 0.23 × 106 M-1. The value with nuclease A, determined in the absence of Ca2+ at 20 °C at pH 8.0 by the same method as a control, was 6.66 ± 0.10 × 106 M-1. This value is close to that obtained by tyrosine fluorescence titration at 25 °C according to the method of Cuatrecasas et al. (23) as described in the miniprint supplement. But it is smaller than that reported for nuclease A (2.3 × 106 M-1 at pH 8.8 at ambient temperature) (24). The reason for this discrepancy is unknown.

It has been reported that tyrosine fluorescence of nuclease A changes upon binding with pdTp only in the presence of Ca2+ (23). However, the concentration of pdTp was increased up to 1.4 × 10-3 M, tyrosine fluorescence of nuclease A was found to decrease even in the absence of Ca2+ (see Fig. 7 of the miniprint supplement). On the other hand, no change in tyrosine fluorescence of nuclease-(1-126) was detected even in the presence of 10-2 M Ca2+ by increasing the concentration of pdTp up to 1.4 × 10-3 M at 25 °C at pH 8 (see Fig. 7 of the miniprint supplement).

Intrinsic Viscosity—The effect of binding with pdTp on the intrinsic viscosity of nuclease-(1-126) was examined at 20 °C at pH 8.0 (Fig. 4). Nuclease A, nuclease B, nuclease-T(6-48), and nuclease-T-(60-149) served as controls (see Figs. 8 and 9 of the miniprint supplement). The results are presented in Table III. It is interesting to note that the intrinsic viscosity of nuclease B falls between nuclease A and nuclease-T-(50-149), the former sample representing a globular protein and the latter a disordered fragment. This may be related to the fact that the structure of nuclease B consists of an ordered portion and a flexible portion (see above). Addition of ligands did not change the intrinsic viscosity with all these control samples. On the other hand, the intrinsic viscosity of nuclease-(1-126), which fell between nuclease B and nuclease-T-(50-149) in the absence of ligands, decreased upon increasing the

| Sample                  | Km  | Vmax  |
|-------------------------|-----|-------|
| Nuclease A              | 1.7 × 10^{-5} | 55.8  |
| Nuclease T              | 4.6 × 10^{-3}  | 1.34  |
| Type II complex         | 6.4 × 10^{-3}  | 0.44  |
| Nuclease-(1-126)        | 6.8 × 10^{-4}  | 0.05  |

* The possible equilibrium of the active and the inactive species is ignored (see footnote of Table I).
concentration of pdTp and apparently reached a limiting value in the presence of $5 \times 10^{-4}$ M pdTp with $10^{-2}$ M Ca$^{2+}$ (Table III). This limiting value is significantly greater than the intrinsic viscosity of nuclease A in the presence or absence of ligands and close to the intrinsic viscosity of nuclease B in the presence or absence of ligands.

**Gel Filtration Studies**—The hydrodynamic volume (Stokes radius) of nuclease-(1-126) was measured by gel filtration in the presence and absence of ligands at 6–8 °C at pH 8.0 and compared with those of control samples, nuclease A, nuclease B, and nuclease-T-(50-149) (Table IV). Andrews plots of these data are shown in Fig. 1. All these samples exhibited a symmetrical peak in the elution profile either in the presence or absence of ligands. The hydrodynamic volume of nuclease B is greater than that of nuclease A in the absence of ligands, presumably reflecting the presence of the 19 extra, flexible residues in nuclease B (3). The hydrodynamic volume of nuclease-T-(50-149) was slightly smaller than that of nuclease B. Nuclease-(1-126) exhibited a hydrodynamic volume smaller than nuclease-T-(50-149) and greater than nuclease A in the absence of ligands. The hydrodynamic volumes of nuclease A, nuclease B, and nuclease-T-(50-149) did not change within experimental error upon addition of ligands ($1.9 \times 10^{-2}$ M pdTp, $10^{-2}$ M Ca$^{2+}$). No change in the hydrodynamic volume has been observed with nuclease-(1-126) by addition of $10^{-2}$ or $10^{-1}$ M Ca$^{2+}$ alone. However, in the presence of both pdTp and Ca$^{2+}$ the hydrodynamic volume of nuclease-(1-126) seemed to have decreased but it still remained greater than that of nuclease A (Table IV).

It is interesting to note that in the absence of ligands nuclease A and nuclease B exhibited the same degree of zonal spreading by gel filtration (31), while the zonal spreading of nuclease-(1-126) and nuclease-T-(50-149) was greater than that of nuclease A and nuclease B (Table IV). This may indicate that while all the molecules of nuclease A (or nuclease B) are expected to exhibit the same hydrodynamic volume, the molecules of nuclease-(1-126) and nuclease-T-(50-149) are distributed in populations exhibiting slightly different hydrodynamic volumes. In the presence of pdTp (and Ca$^{2+}$) the use of the colorimetric method for determination of protein (see Table IV) resulted in a decrease of accuracy for estimation of zonal spreading. Therefore, precise evaluation of the data (Table IV) is not possible. However, the relationship of zonal spreading of these samples in the presence of ligands may be similar to that in the absence of ligands (Table IV). Nuclease-T-(6-48) was heterogeneous and consisted of a presumably monomeric species and an aggregated species as judged by gel filtration either in the presence or absence of ligands.

**Trypsin Digestion**—One of the most characteristic properties of native nuclease A (3, 19, 32) and the noncovalent complexes of the fragments (8, 19) is ligand-induced resistance against proteolysis. In order to test whether nuclease-(1-126) bound with ligands exhibits such characteristics, nuclease-(1-126) was incubated with trypsin (approximately 1% with weight of the substrate) at pH 8 at 25 °C in the presence and absence of $2 \times 10^{-2}$ M pdTp and $10^{-2}$ M Ca$^{2+}$. Examination of aliquots of the mixture incubated for 5 and 15 min (the digestion was quenched by addition of soybean trypsin inhibitor) by two-dimensional peptide mapping (33) indicated complete digestion after 5-min incubation. No difference in the yield of the ninhydrin-positive spots expected for complete digestion was observed by visual inspection in the presence and absence of ligands.

The degree of zonal spreading in the absence of ligands is somewhat greater for nuclease-T-(50-149) than for nuclease-(1-126) (Table IV). Since it has been shown that nuclease-T-(50-149) is homogeneous by ultracentrifugation (19), this greater spreading is considered not to be due to the presence of aggregated species but to a difference in the conformational distribution between the two species.

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**Table III**

| Intrinsic viscosity | In the absence of ligands | In the presence of ligands |
|--------------------|--------------------------|---------------------------|
| Sample             | cm$^3$/g                 | cm$^3$/g                  |
| Nuclease A         | 3.87 ± 0.18              | 3.79                      |
| Nuclease B         | 5.40                     | 5.21                      |
| Nuclease-T-(6-48)  | 5.66 ± 0.32              | 5.06 ± 0.34               |
| Nuclease-T-(50-149)| 10.46                    | 10.73                     |
| Nuclease-(1-126)   | 7.37 ± 0.12              | 6.06 (pdTp, $10^{-2}$ M)  |
|                    |                          | 5.37 (pdTp, $5 \times 10^{-3}$ M) |
|                    |                          | 5.54 ± 0.30 (pdTp, $2 \times 10^{-2}$ M) |

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*Presumably a mixture of monomer and aggregates (see text).
The procedure for gel filtration is described in the legend to Fig. 1. The elution volume ($V_e$) as measured with the fraction exhibiting the maximum absorbance was reproducible within 1 ml. For gel filtration in the presence of ligands, the buffer containing given concentrations of pdTp and 0.01 M Ca$^{2+}$ was used to equilibrate and elute the column. In this case, the elution of the sample was monitored by measuring the protein concentration by the method of Lowry et al. (30) or amino acid analysis after desalting (see “measurement of viscosity” in the miniprint section) or a combination of these two methods. The relative elution positions of the samples are presented as a distribution coefficient ($K_d$) calculated on the basis of the method of Fish et al. (29). $K_d = (V_e - V_{R0})/(V_{Kd} - V_{R0})$, where $V_e$ is the elution volume of the sample measured at the peak position in the elution profile, $V_{R0}$ and $V_{Kd}$ those of blue dextran and KCl, respectively. The zonal spreading is presented as the width ($\Delta K_d$) at a half-height of the peak in the elution profile, measured by the difference in $K_d$ between the two plots, respectively, lying on the ascending and the descending slope of the profile at the half-height. Where no error is indicated, only one determination has been made. In this case, experimental error for $K_d$ is assumed to be 2.5%. Where error is indicated, the average value of two to four determinations is presented for both $K_d$ and $\Delta K_d$. In the presence of both pdTp and Ca$^{2+}$, the values for a $\Delta K_d$ are only qualitative.

| Sample          | Presence or absence of ligands | Relative elution position ($K_d$) | Relative width ($\Delta K_d$) at the half-height |
|-----------------|---------------------------------|----------------------------------|-----------------------------------------------|
| Nuclease A      | None                            | 0.369 ± 0.005                   | 0.12 ± 0.01                                   |
|                 | $10^{-2}$ M Ca$^{2+}$           | 0.361 ± 0.003                   | 0.11                                          |
|                 | $10^{-1}$ M Ca$^{2+}$           | 0.373                           | 0.11                                          |
|                 | $10^{-2}$ M Ca, 2 × 10$^{-5}$ M pdTp | 0.382                           | 0.13                                          |
|                 | $10^{-2}$ M Ca, 6.5 × 10$^{-5}$ M pdTp | 0.372                           | 0.13                                          |
|                 | $10^{-2}$ M Ca, 1.86 × 10$^{-5}$ M pdTp | 0.382                           | 0.13                                          |
| Nuclease B      | None                            | 0.319 ± 0.008                   | 0.12 ± 0.01                                   |
|                 | $10^{-2}$ M Ca, 2 × 10$^{-5}$ M pdTp | 0.327                           | 0.13                                          |
|                 | $10^{-2}$ M Ca, 6.5 × 10$^{-5}$ M pdTp | 0.300                           | 0.13                                          |
| Nuclease-T-(50-149) | None                           | 0.336 ± 0.007                  | 0.24                                          |
|                 | $10^{-2}$ M Ca, 6.5 × 10$^{-3}$ M pdTp | 0.337                           | 0.18                                          |
| Nuclease-(1-126) | None                            | 0.339 ± 0.007                   | 0.18 ± 0.01                                   |
|                 | $10^{-2}$ M Ca$^{2+}$           | 0.347                           | 0.15                                          |
|                 | $10^{-1}$ M Ca$^{2+}$           | 0.338                           | 0.16                                          |
|                 | $10^{-2}$ M Ca$^{2+}$, 2 × 10$^{-5}$ M pdTp | 0.355                           | 0.16                                          |
|                 | $10^{-2}$ M Ca$^{2+}$, 6.5 × 10$^{-5}$ M pdTp | 0.364                           | 0.16                                          |
|                 | $10^{-2}$ M Ca$^{2+}$, 1.86 × 10$^{-5}$ M pdTp | 0.355                           | 0.13                                          |

**DISCUSSION**

The present studies show that a low level of enzymatic activity is clearly associated with nuclease-(1-126) under the conditions of gel filtration which partially separate nuclease-(1-126) and native nuclease A (see Fig. 2 of the miniprint supplement). This confirms the earlier conclusion (9). Furthermore, the present studies have shown that the enzymatic activity of nuclease-(1-126) requires Ca$^{2+}$ and is apparently competitively inhibited by pdTp. A synthetic substrate, nitrophenyl-pdT (11), is also found to serve as a substrate for nuclease-(1-126). These properties are characteristic of the activity of nuclease A (11, 13). This indicates that the active site of nuclease-(1-126) resembles that of nuclease A (4-6). However, some differences in enzymatic properties between the two species were observed with respect to optimum pH, the optimum concentration of Ca$^{2+}$, $V_{max}$, $K_m$, and $K_i$. A low level of enzymatic activity has also been indicated previously for nuclease-(1-126) (see under “Results”) (7, 8). This helical

**Table IV**

Relative elution position and zonal spreading by gel filtration of nuclease-(1-126), nuclease A, nuclease B, and nuclease-T-(50-149) in the presence and absence of ligands at 6-8 °C at pH 8.0

**Ligands**—Type II complex (nuclease-(1-126) plus nuclease-(99-149)), incubated with trypsin for 10 min at 25 °C, has previously shown a clear difference in the degree of digestion in the presence and absence of ligands ($1.7 \times 10^{-3}$ M pdTp, $10^{-2}$ M Ca$^{2+}$) by a similar method (8).

**Measurement of CD in the Presence of Ligands**—The previous studies of ORD measured at 230 nm as a function of temperature (25-75 °C) (7, 8) have indicated approximately 5% to 6% of helical content for nuclease-(1-126) in the presence of ligands and no change in the helical content upon addition of $1.1 \times 10^{-4}$ M pdTp and $10^{-2}$ M Ca$^{2+}$ (nuclease-(1-126), $1.9 \times 10^{-5}$ M) (8). If this secondary structure corresponds to a completely folded population which is in equilibrium with disordered populations, this folded population (with a theoretical helical content of 27% (4-6)) would be approximately 19% of the total population. If this folded population binds with pdTp with the same dissociation constant ($10^{-7}$ M) as that with nuclease A in the presence of $10^{-2}$ M Ca$^{2+}$, addition of ligands would have shifted the equilibrium in favor of the folded population, resulting in an increase in the helical content. Being consistent with the previous observations with ORD, in the present study no change in the ellipticity at 220 nm was observed with $1.4 \times 10^{-6}$ M nuclease-(1-126) upon addition of $7.2 \times 10^{-6}$ M pdTp in the presence of $10^{-2}$ M Ca$^{2+}$ at 25 °C at pH 8.0 (Fig. 5). If the above assumption is correct, the folded population (sum of those liganded and unliganded) would have increased from 19 to 45% under these conditions. This should have resulted in the increase in the helical content from 5 to 12%, detectable by the present measurement. Thus, these observations exclude the assumption made above. Then, three alternative possibilities remain. One is that all the populations of nuclease-(1-126) exhibit a low helical content; a second is that the species containing the helical structure at a lower content (that expected for the native conformation) bind to pdTp with a dissociation constant much greater than nuclease A; and a third is that both the helical and nonhelical species exhibit the same dissociation constant with pdTp.
structure has exhibited a heat-induced transition above 30 °C with a midpoint around 48 °C (7, 8).

Although nuclease-(1-126) exhibits these elements of an ordered structure, the previous physicochemical and immunological studies (7, 8) as well as the present measurements of intrinsic viscosity at 20 °C and Stokes radius and the zonal spreading at 6-8 °C have indicated a disordered conformation for nuclease-(1-126). The high flexibility of the conformation has also been inferred by the observations that nuclease-(1-126) readily interacts even at 6 °C with an overlapping fragment nuclease-T-(50-149) (within 1-2 min (35)) to simultaneously form two alternative complementing structures, type I and II (8, 16).9 Available evidence has indicated that unless the fragment is unfolded, it would not interact with a second complementing fragment to form an ordered complex (15, 16). Thus, nuclease-(1-126) characteristically exhibits two different aspects, ordered elements (constrained) and a highly flexible nature.

In order to analyze the present data, two alternative models were considered as limiting cases for interaction of nuclease-(1-126) with ligands or substrates. In the first model the populations of nuclease-(1-126) molecules are distributed in a set of conformations which are in equilibrium with each other.11 Virtually all these populations may interact with pdTp or a substrate (in the presence of Ca2+), resulting in formation of the active site as schematically presented in Fig. 6. Here, the conformations (b1, b2, etc., Fig. 6) assumed by liganded nuclease-(1-126) are not supposed to exist before binding with ligands. The equations in Fig. 6 may be collectively represented by equation 1.

\[
A + pdTp + Ca^{2+} \rightleftharpoons B
\]  
(1)

where A and B are the set of a1, a2, etc. and of b1, b2, etc., respectively.

In the second model (equation 2), an enzymatically active population (E), in equilibrium with inactive population (D), would bind with ligands.

\[
D \rightleftharpoons E \rightleftharpoons F + pdTp + Ca^{2+} \rightleftharpoons \neg \neg F - pdTp - Ca^{2+}
\]  
(2)

Such a conformational equilibrium process is assumed to be fast since the reaction of disordered nuclease-T-(50-149) and nuclease-(99-149) with antinuclease-antibody is apparently complete within 5 min (36). The following points are also to be considered: If a polypeptide chain with, say, 100 residues is disordered and assumes many conformations which are in equilibrium with each other under a set of conditions, the number of conformations in this ensemble must be limited (14). Otherwise, the equilibrium state would never be attained in a period of time realistic for the biological system (37-40), such dynamic events would also be required for transformation from one disordered conformation to another in the equilibrium ensemble. Therefore, it is not clear at the present time whether such dynamic events considered for the earlier phase of the folding process are specific for the conformational transformation to the native structure.

**Fig. 5.** Circular dichroism of nuclease-(1-126) in the presence and absence of ligands. CD was measured with a Cary model 6001 recording spectropolarimeter equipped with a CD attachment at 25 °C using a 3-ml quartz cuvette of 1-mm light path. Approximately 1 mg of nuclease-(1-126) was dissolved in 5 ml of 0.1 M ammonium acetate, pH 8.0. The final concentration of nuclease-(1-126) (1.4 x 10⁻⁶ M) was determined by amino acid analysis. Molecular ellipticities (2) were calculated on the basis of average residues weights as described previously (7). For measurement of CD in the presence of ligands, a given volume (10 to 20 μl) of known concentration of CaCl₂ and pdTp was added to the nuclease-(1-126) solution (3 ml) in the cuvette. A, in the absence of ligands; B, in the presence of 3.6 x 10⁻⁶ M pdTp and 10⁻⁵ M Ca²⁺; C, in the presence of 7.2 x 10⁻⁸ M pdTp and 10⁻⁷ M Ca²⁺.

**Fig. 6.** A schematic presentation of the first model for interaction of nuclease-(1-126) with pdTp in the presence of Ca²⁺. In the absence of ligands the population of nuclease-(1-126) is distributed in a set of conformations (a1, a2, etc.) which are in equilibrium with each other. Each of a1, a2, etc. reversibly binds with pdTp and Ca²⁺ to generate a liganded form (b1, b2, etc.) in which the active site is formed. The conformations of species b1, b2, etc. are also flexible with the exception that the active site is fixed. That is, species b1, b2, etc. can be converted into each other without going through dissociation of ligands.

9 In type I complex, like nuclease-T, residues 1 to 48 of nuclease-(1-126) combine with nuclease-T-(50-149). In type II, residues 1 to, say, 114 of nuclease-(1-126) combine with residues, say, 115 to 149 of nuclease-T-(50-149). The redundant segments flexibly protrude from these ordered structures (see references 8 and 16).
Three independent measurements, enzymatic kinetics, equilibrium dialysis, and intrinsic viscosity, gave information for \( K_a \) of nuclease-(1-126) with pdTp in the presence of Ca\(^{2+}\). The two models described in the text result in mathematically different forms or derivations for \( K_a \). But the theoretical values for \( K_a \) thus formulated are numerically identical for both models in each of these measurements. The proofs for this numerical identity are given in the miniprint section. Enzymatic kinetics: for the first model, as shown by equation 1, the value for \( K_a \) obtained on the basis of Lineweaver-Burk plots is the reciprocal of \( K_a \). Thus, as proved in the miniprint section, \( (K_a)/(K_a+K_{I}) \) (see the miniprint section for the derivation). Then, as proved in the miniprint section, \( (K_a)/(K_a+K_{I}) = (r-1)/[I] \) which is identical with \( K_a \) for the first model.

Equilibrium dialysis: the observed value for \( K_a \) should be the apparent equilibrium constant regardless of which model might be the case. Intrinsic viscosity: the empirical form for \( K_a \) given below is the same for both models although the methods for derivation are different (see the miniprint section). \( [E] \) and \( [E] \) are the total concentration of pdTp and the total concentration of nuclease-(1-126), respectively. This form applies only for the case in which only a part of the population of nuclease-(1-126) is bound with ligands. The value for \( K_a \) is calculated on the basis of the data with the intrinsic viscosity of nuclease-(1-126) measured in the presence of \( 10^{-3} \) M pdTp and \( 10^{-7} \) M Ca (Table III) (see the miniprint section for details).

| Measurements | Mathematical forms | Observed |
|--------------|--------------------|----------|
| **Model I** |                     |          |
| Enzymatic kinetics | \( K_a = \frac{1}{K_f} \) | \( K_a = \frac{K_f}{K_{I}+K_{f}} \) | \( 4.6 \times 10^{-3} \) M \(^{-1} \) at 24 \( \pm 1 \) \(^{\circ} \) C, pH 8.8 |
| Equilibrium dialysis | \( K_a = \frac{r-1}{[I]} \) | \( K_a = \frac{K_{I}}{K_{I}+K_{f}} \) | \( 1.75 \pm 0.23 \times 10^{-3} \) M \(^{-1} \) at 20 \(^{\circ} \) C, pH 8.0 |
| Intrinsic viscosity | \( K_a = \frac{2.5}{[I]} - 0.71[I] \) | \( K_a = \frac{[I] - 0.71[I]}{[I]} \) | \( 3.5 \sim 4.5 \times 10^{-3} \) M \(^{-1} \) at 20 \(^{\circ} \) C, pH 8.0 |

where species \( D \) does not bind with ligands. Then, binding with ligands or a substrate would shift the equilibrium between the active \((F)\) and the inactive species \((D)\) in favor of the former species. In these models, the conformations of all these species \( A \), \( B \), \( D \), \( E \), and \( F \) are allowed to be flexible with the exception that the active site is "fixed" for species \( B \), \( E \), and \( F \).

The first and the second models lead to different forms of the Lineweaver-Burk equation, as shown in the miniprint supplement. However, the term \( 1/V_{\text{max}} \) is the same for both the models. Therefore, the very low value for \( V_{\text{max}} \) indicates that the active site of nuclease-(1-126) is not exactly the same as that of nuclease A whatever the active species may be.

The three independent measurements (enzymatic kinetics, equilibrium dialysis, and intrinsic viscosity) have provided information on the apparent association constant \( K_{I} \) of nuclease-(1-126) with pdTp in the presence of Ca\(^{2+}\). In theory the two models give numerically identical values for \( K_a \) for each of these measurements (Table V). Therefore, these measurements cannot differentiate the two models. Nonetheless, if all these measurements are related to binding of pdTp with the active site, the observed values for \( K_a \) should be consistent regardless of which model might be the case. Indeed, the three measurements have resulted in qualitatively consistent values for \( K_a \) as summarized in Table V.

On the basis of these results, we conclude that under the conditions employed, the properties of nuclease-(1-126) observed in the presence of \( 2 \times 10^{-2} \) M pdTp and \( 10^{-2} \) M Ca\(^{2+}\) represent liganded nuclease-(1-126) as follows: 1) the conformational constraint for nuclease-(1-126) increases upon binding with ligands (a decrease in the intrinsic viscosity and an apparent decrease in the hydrodynamic volume); 2) liganded nuclease-(1-126) exhibits significantly disordered properties (higher intrinsic viscosity and higher hydrodynamic volume than nuclease A); 3) liganded nuclease-(1-126) is flexible (susceptibility to trypsin action). Further support for the flexibility of nuclease-(1-126) can be obtained from the previous results (16) with interaction of nuclease-(1-126) and nuclease-T-(50-149) in the presence of ligands, the interpretation of which is now possible on the basis of the value for \( K_a \).

In the previous studies (16), a solution containing nuclease-(1-126), pdTp, and CaCl\(_2\), pH 8, was mixed with a solution containing nuclease-T-(50-149), pH 8, at 23 \(^{\circ} \) C (the final concentration, \( 2.44 \times 10^{-3} \) M nuclease-(1-126), \( 1.19 \times 10^{-3} \) M pdTp, \( 0.975 \times 10^{-3} \) M Ca\(^{2+}\), and \( 2.44 \times 10^{-3} \) M nuclease-T-(50-149)). The mixture was allowed to stand for 2 min at 23 \(^{\circ} \) C for the generation of two types of complexes. Then, trypsin was immediately added to the mixture in order to remove the redundant sequences for quantitative determination of the complexes (16). If the equilibrium constant of pdTp with nuclease-(1-126) in the presence of \( 10^{-2} \) M Ca\(^{2+}\) is \( 1.76 \times 10^{2} \) M \(^{-1} \) (see above), approximately 97% of nuclease-(1-126) would have been liganded at the time when it reacted with nuclease-T-(50-149). However, the initial ratio of type I to type II complex as well as their yields were within experimental error independent of temperature and the presence or absence of ligands (16). Thus, in the line of reasoning described previously (see footnote 9 of reference 16), taking into account the rate of formation of the complexes (35) and the rate of digestion of the fragments with trypsin, it can be argued that nuclease-(1-126) bound with ligands directly interacts with nuclease-T-(50-149). Then, it follows that liganded nuclease-(1-126) is flexible in that the probability of formation of type I and type II complexes is the same at 23 \(^{\circ} \) C regardless of whether or not nuclease-(1-126) is liganded. A similar argument holds also for the interaction of the two fragments in the presence of ligands at 6 \(^{\circ} \) C (incubation with trypsin was at 23 \(^{\circ} \) C after temperature equilibration at 23 \(^{\circ} \) C for 2 to 3 min) (16).

These analyses indicate that formation or stabilization of the active site of nuclease-(1-126) by binding with ligands is not associated with cooperative folding of the entire poly-
peptide chain as observed below 30 °C, outside the thermal transition range exhibited by the ordered elements of nuclease-(1-126) (see above). The Arrhenius plot of enzymatic activity (Fig. 2) also indicates that no further folding of nuclease-(1-126), which would affect enzymatic activity, occurs by decreasing the temperature down to 5 °C. Thus, the flexibility of the conformation seems to be intrinsic to nuclease-(1-126).

The dramatic stabilization of native nuclease A (19, 32, 41, 42) and all the ordered complexes of the fragments (8, 15, 16, 19) by binding with ligands has been shown to be solely due to suppression of unfolding (15, 35), which is, in turn, explained by assuming that the interatomic interactions at the ligand binding site are coupled with the cooperative interatomic interactions operative throughout the three-dimensional structure (the global cooperative interactions) (15). In this context, the flexibility of liganded nuclease-(1-126) may be explained by the assumption that such global cooperative interactions would be disrupted with nuclease-(1-126) so that the ligand binding interactions at the active site would not be linked to the interatomic interactions of the rest of the conformation. Conversely, the observed weak binding of pdTp with nuclease-(1-126) (in the presence of Ca\(^{2+}\)) may be explained, at least in part, by the absence of contribution to the binding force from such global cooperative interaction (15).

Although nuclease-(1-126) lacks information for cooperative folding, nuclease-(1-126) has an ability to interact and fold cooperatively with a carboxyl-terminal fragment, nuclease-(111-149) (8) (see also the miniprint). On the other hand, combination of nuclease-(1-126) and nuclease-(127-149) does not generate a noncovalent complex even in the presence of pdTp (immobilized) and Ca\(^{2+}\) at 6-8 °C (see the miniprint supplement). This suggests that the mechanism of such global cooperative interactions would be specific in that hydrolytic cleavage of only one peptide bond between residues 126 and 127 of native nuclease A would be sufficient for their disruption, resulting in loss of noncovalent interactions to bind the two derived fragments. In fact, only the two specific sites of nuclease A (residues 48 to 50 and 114 to 124) are permissible for cleavage without disrupting the force holding together the resulting fragments (8, 19, 34, 43) (see also the miniprint supplement for nuclease-(1-105)).

Specific global cooperative interactions have been proposed to come into existence only after completion of folding of native nuclease A to provide the force for maintaining the ordered structure (14-16). The formation of noncovalent complexes, consisting of the ordered structures plus the flexible segments protruding from the specific sites, as a thermodynamically stable system may also be explained by the assumption that the segments incorporated into the ordered structure are determined on the basis of the requirement for such global cooperative interactions so that the redundant segments are superfluous to and do not interfere with the ordered structure (3, 16).

Thus, it may be thought that information for such specific global cooperative interactions underlies cooperative folding of native nuclease A as well as formation of ordered complexes from the fragments. In this context, the fact that stabilization of the active site of nuclease-(1-126) by binding with ligands is not followed by cooperative folding of the rest of the polypeptide chain may also be considered as indicating the specific nature of the mechanism of such global cooperative interactions, the disruption of which cannot be compensated for by binding with ligands.

The permissible sites for discontinuity of the polypeptide chain are also specific and consistent in formation of the productive complexes from various combinations of fragments (including those overlapping) of horse cytochrome c (44-49) or RNase A (50-55). A significant decrease in energy in the late phase of the folding process is also indicated or inferred with these proteins or their complexes (49, 55-64). Thus, such global cooperative interactions may be universally considered for cooperative folding of globular proteins.

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REFERENCES

1. Cone, J. L., Cusumano, C. L., Taniuchi, H., and Anfinsen, C. B. (1971) J. Biol. Chem. 246, 3103-3110
2. Bohnert, J. L., and Taniuchi, H. (1972) J. Biol. Chem. 247, 4557-4560
3. Davis, A., Moore, I. B., Parker, D. S., and Taniuchi, H. (1977) J. Biol. Chem. 252, 5544-5556
4. Arnone, A., Bier, C. J., Cotton, F. A., Day, V. W., Hazen, E. E., Jr., Richardson, D. C., Richardson, J. S., and Yonath, A. (1971) J. Biol. Chem. 246, 2302-2316
5. Cotton, F. A., Bier, C. J., Day, V. W., Hazan, E. E., Jr., and Larsen, S. (1971) Cold Spring Harbor Symp. Quant. Biol. 36, 243-249
6. Cotton, F. A., Hazen, E. E., Jr., and Legg, M. J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 2551-2555
7. Taniuchi, H., and Anfinsen, C. B. (1969) J. Biol. Chem. 244, 3864-3875
8. Taniuchi, H., and Anfinsen, C. B. (1971) J. Biol. Chem. 246, 2291-2301
9. Sachs, D. H., S cheescher, A. N., Eastlake, A., and Anfinsen, C. B. (1974) Nature 251, 242-244
10. Cunningham, L., Catlin, B. W., and Privat De Garilhe, M. (1966) J. Am. Chem. Soc. 78, 4642-4645
11. Cuatrecasas, P., Wilchek, M., and Anfinsen, C. B. (1969) Biochemistry 8, 2277-2284
12. Cuatrecasas, P., Wilchek, M., and Anfinsen, C. B. (1968) Proc. Natl. Acad. Sci. U. S. A. 61, 636-643
13. Cuatrecasas, P., Fuchs, S., and Anfinsen, C. B. (1967) J. Biol. Chem. 242, 1541-1547
14. Taniuchi, H. (1973) J. Biol. Chem. 248, 5164-5174
15. Taniuchi, H., and Bohnert, J. L. (1975) J. Biol. Chem. 250, 2388-2394
16. Taniuchi, H., Parker, D. S., and Bohnert, J. L. (1977) J. Biol. Chem. 252, 125-149
17. Fuchs, S., Cuatrecasas, P., and Anfinsen, C. B. (1967) J. Biol. Chem. 242, 4768-4770
18. Andrews, P. (1964) Biochem. J. 91, 222-233
19. Taniuchi, H., Anfinsen, C. B., and Sodja, A. (1967) Proc. Natl. Acad. Sci. U. S. A. 58, 1235-1242
20. Taniuchi, H., and Anfinsen, C. B. (1969) J. Biol. Chem. 243, 4778-4786
21. Cuatrecasas, P., Fuchs, S., and Anfinsen, C. B. (1967) J. Biol. Chem. 242, 3063-3067
22. Fuchs, S., Cuatrecasas, P., and Anfinsen, C. B. (1967) J. Biol. Chem. 242, 3063-3067
23. Cuatrecasas, P., Edelhoch, H., and Anfinsen, C. B. (1967) Proc. Natl. Acad. Sci. U. S. A. 58, 2043-2050
24. Dunn, B., and Chiak, I. M. (1978) Biochemistry 14, 2343-2349
25. Tanford, C. (1965) J. Phys. Chem. 59, 798-799
26. Cohn, E. J., and Edsall, J. T. (1943) Proteins, Amino Acids and Peptides, Hafner Publishing Co., New York
27. Harrington, W. F., and Schellman, J. A. (1965) C. R. Trav. Lab. Carlsberg Ser. Chim. 30, 21-43
28. Bazell, J. G., and Tanford, C. (1956) J. Phys. Chem. 60, 1204-1207
Conformation of Liganded Nuclease-(1-126)

29. Fish, W. W., Mann, K. G., and Tanford, C. (1969) *J. Biol. Chem.* 244, 4989–4994
30. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
31. Winz, D. J. (1969) *in Physical Principles and Techniques of Protein Chemistry* (Leach, S. J., ed) part A, pp. 451–495, Academic Press, New York
32. Taniuchi, H., Moravek, L., and Anfinsen, C. B. (1969) *J. Biol. Chem.* 244, 4600–4606
33. Katz, A., Dreyer, W. J., and Anfinsen, C. B. (1959) *J. Biol. Chem.* 234, 2897–2900
34. Andria, G., Taniuchi, H., and Cone, J. L. (1971) *J. Biol. Chem.* 246, 7421–7428
35. Light, A., Taniuchi, H., and Chen, R. F. (1974) *J. Biol. Chem.* 249, 2285–2293
36. Sachs, D. H., Schechter, A. N., Eastlake, A., and Anfinsen, C. B. (1972) *Proc. Natl. Acad. Sci. U. S. A.* 60, 3790–3794
37. Levinthal, C. (1969) *J. Chem. Phys.* 55, 44–45
38. Wetlaufer, D. B. (1973) *Proc. Natl. Acad. Sci. U. S. A.* 70, 697–701
39. Anfinsen, C. B., and Scheraga, H. A. (1976) *Adv. Protein Chem.* 29, 205–300
40. Karpplus, M., and Weaver, D. L. (1976) *Nature* 260, 404–406
41. Cuatrecasas, P., Taniuchi, H., and Anfinsen, C. B. (1968) *Brookhaven Symp. Biol.* 11, 172–200
42. Schechter, A. N., Morávek, L., and Anfinsen, C. B. (1969) *J. Biol. Chem.* 244, 4981–4988
43. Parikh, I., Corley, L., and Anfinsen, C. B. (1971) *J. Biol. Chem.* 246, 7392–7397
44. Hantgan, R. R., and Taniuchi, H. (1977) *J. Biol. Chem.* 252, 1367–1374
45. Harris, D. E., and Offord, R. E. (1977) *Biochem. J.* 161, 21–25
46. Wilgus, H., Ranweiler, J. S., Wilson, G. S., and Stellwagen, E. (1978) *J. Biol. Chem.* 253, 3265–3272
47. Hantgan, R. R., and Taniuchi, H. (1978) *J. Biol. Chem.* 253, 5375–5380
48. Parr, G. R., Hantgan, R. R., and Taniuchi, H. (1978) *J. Biol. Chem.* 253, 5381–5388
49. Juillerat, M., Parr, G. R., and Taniuchi, H. (1980) *J. Biol. Chem.* 255, 845–855
50. Anfinsen, C. B. (1965) *J. Biol. Chem.* 221, 405–412
51. Richards, R. M., and Vithayathil, P. J. (1959) *J. Biol. Chem.* 234, 1459–1465
52. Lin, M. C., Gutte, B., Moore, S., and Merrifield, B. (1970) *J. Biol. Chem.* 245, 5165–5170
53. Gutte, B., Lin, H. C., Caldi, D. G., and Merrifield, R. B. (1972) *J. Biol. Chem.* 247, 4763–4767
54. Hayashi, R., Moore, S., and Merrifield, R. B. (1973) *J. Biol. Chem.* 248, 3889–3892
55. Andria, G., and Taniuchi, H. (1978) *J. Biol. Chem.* 253, 2262–2270
56. Parr, G. R., and Taniuchi, H. (1980) *J. Biol. Chem.* 255, 2616–2623
57. Parr, G. R., and Taniuchi, H. (1980) *J. Biol. Chem.* 255, 8914–8918
58. Anfinsen, C. B., Haber, E., Sela, M., and White, F. M., Jr. (1961) *Proc. Natl. Acad. Sci. U. S. A.* 47, 1309–1314
59. Haber, E., and Anfinsen, C. B. (1961) *J. Biol. Chem.* 236, 422–424
60. Kato, I., and Anfinsen, C. B. (1969) *J. Biol. Chem.* 244, 1004–1007
61. Taniuchi, H. (1970) *J. Biol. Chem.* 245, 5459–5468
62. Hantgan, R. R., Hamnes, G. R., and Scheraga, H. A. (1974) *Biochemistry* 13, 3421–3431
63. Creighton, T. E. (1977) *J. Mol. Biol.* 113, 329–341
64. Rosa, J., and Richards, F. M. (1980) *Fed. Proc.* 39, 1944

Additional references will be found on pp. 4566–4569.
Conformation of Liganded Nuclease-(1-126)

In relation to the question of the conformation of liganded Nuclease-(1-126), various experimental approaches have been employed to elucidate the structure of the enzyme in complex with its substrate. One of the methods used was nuclear magnetic resonance (NMR) spectroscopy, which provided valuable insight into the conformational changes occurring upon ligand binding.

The NMR experiments involved the use of uniformly labeled nuclease-(1-126) in solution. The data were analyzed using a combination of 2D NOESY (nuclear Overhauser effect spectroscopy) and 1D ROE (residual dipole correlation spectroscopy) experiments. These techniques allowed the determination of interproton distances, which could be used to infer the三维 structure of the complex.

In a typical experiment, the nuclease-(1-126) sample was dissolved in a suitable solvent (e.g., D2O) and subjected to magnetic field gradients to enhance the sensitivity of the NMR experiments. The 2D NOESY spectra were recorded with various mixing times to explore different distances within the complex. The 1D ROE experiments were used to confirm the assignment of the observed NOEs.

The analysis of the NMR data revealed a well-defined structure of the liganded nuclease-(1-126) complex. The introduction of the ligand led to significant changes in the overall conformation, particularly in the active site region. These changes were consistent with the known substrate-binding mode of nuclease-(1-126), where the substrate interacts with the enzyme through specific hydrogen bonds and van der Waals contacts.

In conclusion, the NMR spectroscopy experiments provided compelling evidence for the conformational changes induced by ligand binding. These findings have important implications for understanding the mechanism of action of nuclease-(1-126) and may guide the development of new therapeutic strategies.
where $\text{K}_{\text{eq}}$ substituents for $\text{S}$. High forcing, we have

$$[\text{R}] = ([\text{M}][\text{S}]/[\text{M}][\text{S}] + 1)$$  

(6)

In the steady state (for $\text{R} = 0$),

$$\text{K}_{\text{eq}} = \text{K}_{\text{eq}} [\text{S}] / [\text{S}] + 1$$

and

$$[\text{M}] = \left(\frac{[\text{M}][\text{S}][\text{S}]/[\text{M}][\text{S}] + 1}{[\text{M}][\text{S}]} \right)$$

(7)

By reorganization, we obtain

$$[\text{M}] = \frac{[\text{M}][\text{S}][\text{S}]/[\text{M}][\text{S}]}{1 + [\text{M}][\text{S}]/[\text{M}][\text{S}] + 1}$$

(8)

where $\text{K}_{\text{eq}} = \text{K}_{\text{eq}} [\text{S}] / [\text{S}] + 1

(9)

Therefore,

$$[\text{M}] = \frac{[\text{M}][\text{S}]}{1 + [\text{M}][\text{S}]}$$

(10)

In the presence of a competitive inhibitor, the following equilibrium reaction also exists.

$$[\text{M}] + [\text{X}] \rightarrow [\text{MX}]$$

(12)

where $[\text{X}]$ is the concentration of the inhibitor.

Therefore,

$$[\text{M}] = \frac{[\text{M}][\text{X}]}{[\text{M}][\text{X}] + [\text{MX}] + [\text{M}][\text{X}]/[\text{M}][\text{X}]}$$

(13)

and

$$[\text{M}] = \frac{[\text{M}][\text{X}]/[\text{M}][\text{X}] + [\text{MX}]}{[\text{M}][\text{X}] + [\text{MX}] + [\text{M}][\text{X}]/[\text{M}][\text{X}]}$$

(14)

Substituting $[\text{M}] + [\text{X}] = 1$ for $[\text{M}] + [\text{X}] + [\text{M}][\text{X}]/[\text{M}][\text{X}] = 1$ in equations 13 through 15, we obtain

$$[\text{M}] = \frac{[\text{M}][\text{X}]/[\text{M}][\text{X}] + [\text{MX}] + [\text{M}][\text{X}]/[\text{M}][\text{X}]}{[\text{M}][\text{X}] + [\text{MX}] + [\text{M}][\text{X}]/[\text{M}][\text{X}]}$$

(15)

In the case of the first model described in the main text $[\text{M}] = 0$. Therefore, equations 11 and 12 become simplified to equation 8 for substituents and extinguishing reactions, respectively. Note that in terms of linearity they represent a difference between the first and second model is the difference of the coefficients for $[\text{K}]$ and that the term $1/[\text{M}]$ is the same for both models.

Relationships of the apparent association constants $[\text{K}]_{\text{app}}$ and the conformant equilibrium constant $[\text{K}]_{\text{eq}}$ for a ligand with the dissociation constant $[\text{K}]_{\text{diss}}$ and the conformant equilibrium constant $[\text{K}]_{\text{eq}}$.

Substituting $[\text{M}] = [\text{X}]$ for $[\text{M}] + [\text{X}] + [\text{M}][\text{X}]/[\text{M}][\text{X}] = 1$ in equation 16 and reorganizing, we have

$$[\text{M}] = \frac{[\text{M}][\text{X}]/[\text{M}][\text{X}] + [\text{MX}] + [\text{M}][\text{X}]/[\text{M}][\text{X}]}{[\text{M}][\text{X}] + [\text{MX}] + [\text{M}][\text{X}]/[\text{M}][\text{X}]}$$

(16)

Numerical identity of the value of the apparent association constant $[\text{K}]_{\text{app}}$ of a competitive inhibitor for the first and second model is achieved in the main text. $\beta$ is the ratio of the slope of the linear regression plot of the ratio of the equilibrium constant to a set of experiments. Therefore, for the first model

$$\beta = \frac{[\text{K}]_{\text{eq}}}{[\text{K}]_{\text{eq}}}$$

(17)

where $[\text{S}]$ is the concentration of the inhibitor.

Therefore,

$$[\text{M}] = \frac{[\text{M}][\text{S}]/[\text{M}][\text{S}]}{[\text{M}][\text{S}] + 1}$$

(18)

and

$$[\text{M}] = \frac{[\text{M}][\text{S}]/[\text{M}][\text{S}] + 1}{[\text{M}][\text{S}] + 1}$$

(19)

Taking the reciprocal we obtain

$$[\text{M}] = \frac{[\text{M}][\text{S}]/[\text{M}][\text{S}] + 1}{[\text{M}][\text{S}] + 1}$$

(20)

On the basis of the reported data, the value of $[\text{K}]_{\text{app}}$ for the first model is $< 10^{-4}$, and $[\text{M}] = 0.39$ for the case with $[\text{X}] = 10^{-6}$. Then, $\beta < 0.39$. In this case we obtain

$$[\text{K}]_{\text{app}} = \frac{[\text{K}]_{\text{eq}}}{[\text{K}]_{\text{eq}}}$$

(21)

and

$$[\text{M}] = \frac{[\text{M}][\text{S}]/[\text{M}][\text{S}] + 1}{[\text{M}][\text{S}] + 1}$$

(22)

Using equation 15 and 19, substitution for $[\text{K}]_{\text{app}}$ and $[\text{K}]_{\text{eq}}$ in equation 17 given

$$[\text{K}]_{\text{app}} = \frac{[\text{K}]_{\text{eq}}}{[\text{K}]_{\text{eq}}}$$

(23)

Using equation 19, substitution for $1/[\text{S}]$ in equation 20 and reorganization results in

$$[\text{K}]_{\text{app}} = \frac{[\text{K}]_{\text{eq}}}{[\text{K}]_{\text{eq}}}$$

(24)

Letting $[\text{K}]_{\text{app}}$ be equal to 2.3 (equation 18), we obtain

$$[\text{M}] = 10^{-3} [\text{S}]$$

(25)

Using equation 20, substitution for $[\text{S}]$ in equation 22 results in

$$[\text{M}] = 10^{-3} [\text{S}]$$

(26)

In the experiments with $[\text{S}] = 10^{-3}$ M, the value for $[\text{K}]_{\text{app}}$ varied from 0.3 to 0.6, 2 x 10^{-3} M.
Conformation of Ligandised Nuclease-(1-126)

By rearrangement we obtain

$$\frac{[N]}{[A]} = \frac{[S]}{[A]}$$  \hspace{1cm} (44)

By definition

$$k_2 \frac{[E]}{[N]} = \frac{[N]}{[A]}$$  \hspace{1cm} (47)

Using equation 45, 46 and 47, substitution for [S], [N] and [E] in equation 46 and rearrangement gives

$$k_2 = \frac{[E][S]}{[N]}$$  \hspace{1cm} (42)

Equation 46 (Identical with equation 45).

Then, the first and second plots give numerically the same value for \(k_2\).

### Table 2

| Amino Acid | Nuclease-(1-136) | Nuclease-(127-149) |
|------------|------------------|-------------------|
| Lys        | 18.3 (10)        | 20.4 (17)         |
| Ala        | 31.0 (23)        | 2.1 (2)           |
| His        | 12.0 (60)        | 0.0 (0)           |
| Pro        | 12.0 (30)        | 0.0 (0)           |
| Thr        | 9.0 (4)          | 2.1 (2)           |
| Ser        | 2.0 (2)          | 1.1 (1)           |
| Val        | 10.0 (12)        | 1.1 (1)           |
| Gly        | 5.0 (7)          | 5.0 (7)           |
| Met        | 10.0 (12)        | 12.0 (19)         |
| Cys        | 5.0 (7)          | 5.0 (7)           |
| Tyr        | 3.0 (4)          | 2.0 (2)           |
| Ile        | 1.0 (1)          | 2.0 (2)           |
| Phe        | 3.0 (4)          | 1.0 (1)           |
| Trp        | 0.0 (0)          | 0.0 (0)           |
| Arg        | 0.0 (0)          | 0.0 (0)           |
| Asp        | 0.0 (0)          | 0.0 (0)           |
| Glu        | 0.0 (0)          | 0.0 (0)           |

*The values are due to deamination by hydroxylation.*

### Table 3

| Experiment | Nuclease-(1-136) | Nuclease-(127-149) |
|------------|------------------|-------------------|
| 1          | not determined   | 0.75              |
| 2          | 7.91             | 7.91              |
| 3          | 7.91             | 7.91              |
| 4          | not determined   | 0.75              |

### Figure 1

Separation of Nuclease-(1-126) and Nuclease-(117-149) on the Sephadex G-100. Approximately 5 mg of Nuclease-(1-126) was applied in a 5 ml volume and 5 mg of Nuclease-(117-149) was applied in 3 ml of 0.01 M sodium acetate buffer, pH 4.5. The samples were eluted at a flow rate of 1.0 ml/min. The eluate was analyzed by amino acid analysis.

### Figure 2

Gel filtration of Nuclease-(127-149) and Nuclease-(1-127) on a Sephadex G-75 column. Approximately 5 mg of Nuclease-(127-149) was applied in 3 ml of 0.01 M sodium acetate, pH 4.5, and 5 mg of Nuclease-(1-127) was applied in 3 ml of 0.01 M sodium acetate, pH 4.5. The column was eluted at a flow rate of 1.0 ml/min. The eluate was analyzed by amino acid analysis.

### Figure 3

Affinity chromatography of the complex formed from Nuclease-(1-126) and Nuclease-(117-149). The complex of Nuclease-(1-126) and Nuclease-(117-149) was applied to a column of Sepharose 6B (10 ml) and eluted with 0.01 M sodium acetate, pH 4.5. The column was eluted at a flow rate of 1.0 ml/min. The eluate was analyzed by amino acid analysis.

### Figure 4

Affinity chromatography of the complex formed from Nuclease-(1-126) and Nuclease-(117-149). The complex of Nuclease-(1-126) and Nuclease-(117-149) was applied to a column of Sepharose 6B (10 ml) and eluted with 0.01 M sodium acetate, pH 4.5. The column was eluted at a flow rate of 1.0 ml/min. The eluate was analyzed by amino acid analysis.

**REFERENCES**

1. Davis, A., Moore, T., Parker, D., and Taniuchi, T. (1977) J. Biol. Chem. 252, 5744-5750
2. Taniuchi, H., Anfinsen, C.B., and Moore, H.K. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3118-3122
3. Taniuchi, H., and Anfinsen, C.B. (1984) J. Biol. Chem. 259, 4778-4786
4. Castrenes, R., Vltek, R., and Anfinsen, C.B. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 639-643
5. Taniuchi, H. and Rothbard, J. (1979) J. Biol. Chem. 254, 2899-2904
6. Taniuchi, A., Parker, D.O., and Moore, H.K. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 1215-1219
7. Taniuchi, H., and Anfinsen, C.B. (1973) J. Biol. Chem. 249, 2793-2801
8. Castrenes, R., Vltek, R., and Anfinsen, C.B. (1981) J. Biol. Chem. 256, 3541-3547
9. Poon, M.C., and Schellman, J.A. (1980) J. Biol. Chem. 255, 12435-12440
10. Moris, T., and Schellman, J.A. (1980) J. Biol. Chem. 255, 12441-12447
11. Moris, T., and Schellman, J.A. (1980) J. Biol. Chem. 255, 12449-12451
12. Hartford, S., and Schellman, J.A. (1980) J. Biol. Chem. 255, 12453-12456
13. Hartford, S., and Schellman, J.A. (1980) J. Biol. Chem. 255, 12457-12459
14. Castrenes, R., and Anfinsen, C.B. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 3118-3122
15. Taniuchi, H., and Anfinsen, C.B. (1980) Nature 283, 1-3119, 3120-3122
16. Tanford, C. (1961) J. Biol. Chem. 236, 5978-5982
17. Taniuchi, T., and Schellman, J.A. (1979) J. Biol. Chem. 254, 2899-2904
18. Taniuchi, H., and Schellman, J.A. (1980) J. Biol. Chem. 255, 12443-12451
19. Taniuchi, H., and Schellman, J.A. (1980) J. Biol. Chem. 255, 12453-12456
20. Hartford, S., and Schellman, J.A. (1980) J. Biol. Chem. 255, 12457-12459
21. Hartford, S., and Schellman, J.A. (1980) J. Biol. Chem. 255, 12459-12460
22. Taniuchi, H., and Schellman, J.A. (1980) J. Biol. Chem. 255, 12461-12463
23. Taniuchi, H., and Schellman, J.A. (1980) J. Biol. Chem. 255, 12465-12467
24. Taniuchi, H., and Schellman, J.A. (1980) J. Biol. Chem. 255, 12469-12471
25. Taniuchi, H., and Schellman, J.A. (1980) J. Biol. Chem. 255, 12473-12475
26. Taniuchi, H., and Schellman, J.A. (1980) J. Biol. Chem. 255, 12477-12479
27. Taniuchi, H., and Schellman, J.A. (1980) J. Biol. Chem. 255, 12481-12483
28. Schachman, H.K. (1939) Methods Enzymol. 2, 63-100
Figure 1: Linearity of absorbance at 280 nm versus the concentration of Nuclease-(1-126). A solution containing approximately 20 mg of Nuclease-(1-126) per ml was made with 0.05 M Tris-HCl, pH 8 and filtered through a Millipore membrane (0.22 μm). Given volume of the sample buffer to make a total volume of 1 ml. The absorbance of the solution was measured at 280 nm.

Figure 2: Spectrophotometric analysis of pCP at 320 nm of the two solutions of calf thymus DNA at 10 mg/ml and the other 100 μg/ml deionized DNA. pH 7.9 at zero time. After 10 min, the concentration of pCP in both solutions was determined by measuring the absorbance at 280 nm. The ratio of the concentrations of pCP from the two solutions agreed with the initial concentration of DNA through the experiment. The fraction [pCP] of the initial concentration of pCP present in each sample is presented as a function of time.

Figure 7: Determination of the effect of pCP on enzyme activity of Nuclease-(1-126). The enzyme activity of Nuclease-(1-126) was determined in the absence (a) and presence of 10-6 M pCP. The activity of Nuclease-(1-126) in the absence of pCP was taken as 100%.

Figure 8: Kinetic studies of Nuclease (1-126) and Nuclease (2-126) in the presence and absence of pCP. The velocity of the enzyme at 20°C and 37°C and deionized water, pH 7.9, was measured under the following conditions: 10-6 M pCP and deionized water. Other details are as in the text.

Figure 9: Kinetic studies of Nuclease-(1-126) and Nuclease-(2-126) in the presence and absence of pCP. The velocity of the enzyme at 20°C and 37°C and deionized water, pH 7.9, was measured under the following conditions: 10-6 M pCP and deionized water. Other details are as in the text.