The Mechanism of Stabilization of the Structure of Nuclease-T' by Binding of Ligands*

HIROSHI TANIUCHI AND JANICE L. BOHNERT

From the Section on Protein Conformation, Laboratory of Chemical Biology, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

The rate of unfolding of Nuclease-T' at pH 8, 20°C was determined as a function of concentration of the ligands deoxythymidine 3',5'-diphosphate (pdTp) and Ca²⁺ on the basis of the rate of exchange between free fragment, Nuclease-T(50-149) and labeled fragment, Nuclease-T(50-149) incorporated in the structure of Nuclease-T' (Taniuchi, H. (1973) J. Biol. Chem. 248, 5164-5174). The rate constant of unfolding of unliganded Nuclease-T' was 4.6 x 10⁻⁴ s⁻¹. Those of Nuclease-T' bound with pdTp, with Ca²⁺, and with both pdTp and Ca²⁺ were 9.0 x 10⁻⁵, 1.6 x 10⁻⁵, and 2.2 x 10⁻⁵ s⁻¹, respectively. The association constants of pdTp and Ca²⁺ with Nuclease-T' were found to be 1.0 x 10⁴ and 2.0 x 10⁵ M⁻¹, respectively. Those of pdTp with Nuclease-T' plus Ca²⁺ and of Ca²⁺ with Nuclease-T' plus pdTp were 4 x 10⁴ and 1.4 x 10⁵ M⁻¹, respectively. The calculation of free energy change on the basis of the association constants shows that the magnitude of negative free energy change involved in the binding of either of the two ligands increases by approximately 2 kcal when the other ligand is already bound. There is a correlation between the free energy change and the suppression of the unfolding in the binding of ligands to Nuclease-T'. The greater the magnitude of negative free energy change of the association of the ligands, the larger the increase in the stabilization energy of the structure of Nuclease-T'. The results are interpreted to suggest that the local interactions between the ligands and the binding site of Nuclease-T' may be specifically coupled with the cooperative interactions operating throughout the three-dimensional structure resulting in strengthening of the interactions throughout the structure, including those with the ligands, without a large change in conformation.

In the previous report (2) we presented a statistical mode of folding of staphylococcal nuclease. It is proposed that two similar native conformations of nuclease exist in higher and lower energy states, an activated and a ground state. The transition from the activated to the ground state would occur by development of a unique state of cooperative interactions operating throughout the three-dimensional structure. It is assumed that the transition between the activated and ground state involves a large change of the stabilization energy maintaining the structure without a large change in conformation.

In order to test whether the stabilization energy of the structure of nuclease can change substantially without a large change in conformation, we examined quantitatively the relationship between binding of ligands to Nuclease-T' and stabilization of the structure. It is known that the binding of the ligands pdTp and Ca²⁺ to nuclease (4) and Nuclease-T stabilizes the structure against heat denaturation (5) and suppresses the motility (6) without a large change in conformation (7-10). In the present studies, we have used the rate of unfolding of Nuclease-T' as an index of the stabilization energy of the structure of Nuclease-T' (2). We have estimated the rate of unfolding of Nuclease-T' by measuring the rate of exchange between free Nuclease T (50 149) and labeled Nuclease-T(50-149) incorporated into the structure of Nuclease-T' (2) as a function of concentration of ligands at 20°C. The association constants of the ligands to Nuclease-T' and the rate constants of unfolding of liganded Nuclease-T' have been determined. On the basis of these data, the free energy change of unfolding of Nuclease-T at pH 8, 20°C was determined as a function of concentration of the ligands deoxythymidine 3',5'-diphosphate (pdTp) and Ca²⁺ on the basis of the rate of exchange between free fragment, Nuclease-T(50-149) and labeled fragment, Nuclease-T(50-149) incorporated in the structure of Nuclease-T' (Taniuchi, H. (1973) J. Biol. Chem. 248, 5164-5174). The rate constant of unfolding of unliganded Nuclease-T' was 4.6 x 10⁻⁴ s⁻¹. Those of Nuclease-T' bound with pdTp, with Ca²⁺, and with both pdTp and Ca²⁺ were 9.0 x 10⁻⁵, 1.6 x 10⁻⁵, and 2.2 x 10⁻⁵ s⁻¹, respectively. The association constants of pdTp and Ca²⁺ with Nuclease-T' were found to be 1.0 x 10⁴ and 2.0 x 10⁵ M⁻¹, respectively. Those of pdTp with Nuclease-T' plus Ca²⁺ and of Ca²⁺ with Nuclease-T' plus pdTp were 4 x 10⁴ and 1.4 x 10⁵ M⁻¹, respectively. The calculation of free energy change on the basis of the association constants shows that the magnitude of negative free energy change involved in the binding of either of the two ligands increases by approximately 2 kcal when the other ligand is already bound. There is a correlation between the free energy change and the suppression of the unfolding in the binding of ligands to Nuclease-T'. The greater the magnitude of negative free energy change of the association of the ligands, the larger the increase in the stabilization energy of the structure of Nuclease-T'. The results are interpreted to suggest that the local interactions between the ligands and the binding site of Nuclease-T' may be specifically coupled with the cooperative interactions operating throughout the three-dimensional structure resulting in strengthening of the interactions throughout the structure, including those with the ligands, without a large change in conformation.

*The preliminary accounts of this work were presented at the Biochemistry/Biophysics 1974 Meeting (1).

1. The abbreviations used are: pdTp, deoxythymidine 3',5'-diphosphate; [¹⁴C]acetyl Nuclease T (50 149), labeled fragment, Nuclease-T(50-149), containing on average 3 covalently bound [¹⁴C]-acetyl moieties at α-NH₂ of the ε-NH₃ of Lys-127 and another unidentified ε-NH₃ group (2); Sepharose-nuclease, Sepharose (agarose) covalently bound with nuclease; Sepharose-pdTp, Sepharose coupled to 3'-(4'-aminophenyl-phosphoryl)-deoxythymidine 5'-phosphate (3).

2. Fragments of nuclease have been designated by an adaptation of the rules of the Commission on Biochemical Nomenclature (11). The prototype is "trivial name-(X-Y)," where the trivial name denotes the origin of the fragment and X and Y, the NH₂- and COOH-terminal amino acids, respectively. For example, Nuclease-T is composed of two fragments, Nuclease-T(6-48) and Nuclease-T(50-149) (or Nuclease-T(49 149)), of staphylococcal nuclease (5, 12). The reconstituted Nuclease-T is called Nuclease-T'.
involved in the association of the ligands and the increase in activation free energy of unfolding of Nuclease-T' by binding of the ligands are calculated. The mechanism of stabilization of the structure of Nuclease-T' by binding of the ligands is discussed in relation to the mechanism of folding of the nuclease chain.

EXPERIMENTAL PROCEDURES

The methods used for preparation of Nuclease-T' containing [1-14C]N-acetyl-Nuclease-T (50-149), for measurement of the rate of exchange between free Nuclease-T (50-149) and [1-14C]acetly-Nuclease-T (50-149) incorporated in the structure of Nuclease-T' and for calculation of the rate of unfolding of Nuclease-T', were described previously (2) with the following exceptions. In the exchange experiments with the ligands, a solution containing the sample of labeled Nuclease-T' in 10 to 15 ml of 0.1 M ammonium acetate, pH 8, was placed in a small test tube (2). Another small test tube contained Nuclease-T (50-149), a specified amount (see below) of pdTp, or Ca2+, or both and EDTA, when necessary, in 0.9 ml of approximately 0.1 M ammonium acetate, pH 8. The two solutions were equilibrated at 20°C for 10 min before they were mixed. After incubation at 20°C for a given time, the test tube containing the mixture was immediately placed in an ice bath for a few minutes. The cooled mixture was applied to a two-zone column containing Sepharose-nuclease and Sepharose-pdTp in the upper and lower zones, respectively. Edens of pdTp contained in the incubation mixture was removed by adsorption to Sepharose-nuclease. The removal of pdTp was necessary to ensure the separation of Nuclease-T (50-149) and Nuclease-T' (3) by chromatography through the Sepharose-pdTp zone, since the presence of pdTp in the mixture interfered with the specific absorption of Nuclease-T' to Sepharose-pdTp. Sepharose-nuclease was prepared by cyano agarose (13), using 40 mg of purified nuclease and 20 ml of Sepharose-4B (Pharmacia) following the procedure of Kato and Anfinsen (14). The sample of Sepharose nuclease thus prepared had a capacity to bind approximately 57 nmoI of pdTp per ml bed volume. A two-zone column (1 cm diameter) was made by packing Sepharose-pdTp (5 cm height) and Sepharose-nuclease (4 cm height) in the lower and upper zones, respectively. When 6.4 nmoI of labeled Nuclease-T' containing [1-14C]acetly-Nuclease-T (50-149) (approximately 14,000 cpm) was applied to the two-zone column in the presence of CaCl2 (4 mM) and pdTp (34 nmoI), only a small quantity of radioactivity, accounting for 4.5% that of the absorbed radioactivity, passed through the column. The same percentage of unabsorbed radioactive material was observed previously when labeled Nuclease-T' was applied to a Sepharose-pdTp column in the absence of pdTp (2). This unlabeled radioactive material was corrected for in the calculation of the radioactivity of exchange free [1-14C]acetly-Nuclease-T (50-149) (2). The two-zone column was used for all exchange experiments. Since there was no detectable loss of radioactivity due to adsorption to the glass wall, silicone-coating of test tubes and Pasteur pipettes was abandoned.

All calculations and fitting of equations to data by the least squares method (15) were performed with the aid of IBM OS/370 and PDP-10 computer systems of the Division of Computer Research and Technology, National Institutes of Health.

Circular dichroism spectra were measured with a Cary model 60 spectropolarimeter, with a CD attachment (10). The Pockels cell was protected against temperature perturbation using a jacketed block. Another jacketed block was used to hold the cuvette. The block system was designed by Mr. R. E. Lippoldt, National Institutes of Health. The temperature of the cuvette was maintained by circulating water through the jacket with a Haake type F bath. The sample solution, in a 3-ml quartz cuvette of 1-cm light path, was equilibrated at a given temperature by a separate circulation system.

RESULTS

Rate Constant of Unfolding of Nuclease-T' as Function of Concentration of pdTp and Ca2+. The rate constant of unfolding of unliganded Nuclease-T' was 4.6 x 10^-4 s^-1 at 20°C, pH 8. The rate of unfolding of Nuclease-T' decreased with increase in concentration of pdTp in both the presence and absence of Ca2+ (Fig. 1). The apparent rate constant of unfolding approached a limiting value as the concentration of pdTp increased. The limiting value was much smaller in the presence of Ca2+ than in its absence (Fig. 1). The suppression of unfolding of Nuclease-T' was also more pronounced as the concentration of Ca2+ was increased in the presence and in the absence of pdTp (Fig. 2). Similarly, the limiting value of the apparent rate constant of unfolding was distinctly smaller in the presence of pdTp than in its absence (Fig. 2). The limiting value for the apparent rate constant of unfolding obtained when the concentration of pdTp was increased at a constant concentration (3.3 mM) of Ca2+ was equal to 2.1 x 10^-5 s^-1 (Fig. 1) and agreed within experimental error with that (2.3 x 10^-5 s^-1) obtained when the concentration of Ca2+ was increased at a constant concentration (1.61 mM) of pdTp (Fig. 2) (20°C, pH 8).

The apparent rate constants of unfolding of Nuclease-T' as a function of concentration of pdTp and Ca2+ in the absence of the other ligand (upper curve in Figs. 1 and 2, respectively) were used to calculate the rate constant of unfolding of Nuclease-T' bound with each ligand alone and the association constant of each ligand with Nuclease-T' in the absence of the other ligand. The calculation was performed on the basis of Equation 15 by the method described under "Appendix." The rate constants of unfolding of Nuclease-T' bound with pdTp alone and bound with Ca2+ alone were 9.0 x 10^-5 and 1.6 x 10^-8 s^-1, respectively, at 20°C, pH 8. The association constant of pdTp with Nuclease-T' in the absence of Ca2+ and that of Ca2+ with Nuclease-T' in the presence of pdTp were 1.0 x 10^n and 2.0 x 10^2 M^-1, respectively (20°C, pH 8). The association constant of Ca2+ with Nuclease-T' bound with pdTp was estimated on the basis of Equation 15 as follows. The value for the rate constant of unfolding of Nuclease-T' at zero concentration of Ca2+ in the presence of 1.6 mM pdTp (9.4 x 10^-8 s^-1 (Fig. 2) was assigned as the value for Yn. The value for Yn (the rate constant of unfolding of Nuclease-T' bound with both pdTp and Ca2+, in this case) was assumed to be 2.3 x 10^-5 s^-1 (Fig. 2). The best fit of data for Equation 15 for Ca2+ in the presence of 1.6 mM pdTp (Fig. 2) gave an association constant of Ca2+ with Nuclease-T' bound with pdTp of 1.4 x 10^-1 M^-1 (20°C, pH 8). The rate constant of unfolding of Nuclease-T' as a function of concentration of pdTp in the presence of 3.3 mM Ca2+ (Fig. 2) was used to calculate the association constant of pdTp with Nuclease-T' bound with Ca2+. The calculation was carried out on the basis of Equation 24 (see "Appendix."). The value for the rate constant of unfolding of unliganded Nuclease-T' and those of Nuclease-T' bound with Ca2+ alone and of Nuclease-T' bound with both pdTp and Ca2+ (4.6 x 10^-4, 1.6 x 10^-4, and 2.1 x 10^-5 s^-1, respectively) were assigned as the values for Yn, Yn, and Yn, respectively. The value for Kt was assumed to be 2.0 x 10^-1 M^-1. The estimated value for Kt (the association constant of pdTp with Nuclease-T' bound with Ca2+ was 4.0 x 10^4 M^-1 (20°C, pH 8).
Relationship between Free Energy Change Involved in Binding of Ligands to Nuclease-T' and Degree of Suppression of Unfolding of Nuclease-T'—Table I lists the values obtained above for the association constant of the ligands with Nuclease-T' and for the rate constants of unfolding of liganded Nuclease-T'. The free energy change involved in the binding of the ligands and the activation free energy of unfolding of Nuclease-T' bound with the ligands are also shown in Table I. If the binding of the ligands with Nuclease-T' follows the equilibrium reaction expressed in Equations 5 to 8 (under "Appendix") and $K_1$, $K_2$, $K_3$, and $K_4$ represent the corresponding association constants (Table I), the product of $K_1$ and $K_2$ should be equal to that of $K_3$ and $K_4$ (Equation 9). The calculated values for $K_1K_3$ and for $K_3K_4$ are $1.4 \times 10^8$ and $0.8 \times 10^9$ (m$^{-2}$), respectively (Table I). The two values may be regarded as fairly close, considering the experimental errors involved in the estimation of each value of the association constants (2). The values for $R - K_1K_3$ and for $R - K_3K_4$ (Equation 10, under "Appendix") are much greater than unity indicating the existence of strong cooperativity in the binding of pdTp and Ca$^{2+}$ with Nuclease-T' (see "Appendix"). The strong enhancement of the binding of one ligand in the presence of the other is consistent with the earlier observations of Cuatrecasas et al. (4) showing that pdTp binds to nuclease only in the presence of calcium ion.

There is an intimate relationship between the strength of binding of the ligands to Nuclease-T' and the stabilization of the structure of Nuclease-T'. The greater the magnitude of negative free energy change involved in the binding of the ligands to Nuclease-T', the smaller the rate constant of unfolding of Nuclease-T' bound with the ligands (Table I). The binding of both pdTp and Ca$^{2+}$ to Nuclease-T' decreased the rate constant of unfolding to $\frac{1}{20}$ that of unliganded Nuclease-T'. The value is smaller than that ($\frac{1}{10}$) expected if the effect of binding of each ligand is independent of the binding of the other ligand (Table I). These results suggest that the mechanism of cooperativity between pdTp and Ca$^{2+}$ when they bind to Nuclease-T' is closely related to the mechanism of the suppression of unfolding of the structure upon binding of the ligands.

Effect of Concentration of Ligands on Heat Stability of Nuclease and Nuclease-T'—If the stabilization against thermal denaturation of intact nuclease and Nuclease-T by binding of ligands is the consequence of the decreased rate of unfolding (2, 17), the enhancement of the stabilization should be observed with an increase in the concentration of ligands in a manner consistent with the suppression of unfolding described above. In order to test this hypothesis, circular dichroism at 222 nm of nuclease and Nuclease-T' was measured at two different concentrations of pdTp in the presence of 5 mM CaCl$_2$ (Fig. 3).

![Fig. 1 (left). The apparent rate constant (k) of unfolding of labeled Nuclease-T' as a function of concentration (C) of pdTp at 20° as determined by the rate of exchange between free Nuclease-T'-(50-149) and bound [1-14C]acetyl-Nuclease-T-(50-149) in the presence and absence of Ca$^{2+}$. The incubation mixture for the exchange in the absence of Ca$^{2+}$ (●) contained 2.7 nmol of labeled Nuclease-T', 0.52 nmol of contaminating Nuclease-T-(6-48) (2), 13.3 nmol of Nuclease T-(50-149), 0.1 mM of EDTA, and varying amounts of pdTp in 0.1 ml of approximately 0.1 M ammonium acetate, pH 8. After incubation at 20° for 20 min, the mixture was cooled with addition of 2 μmol of CaCl$_2$ (20 μl) and then applied to a column of Sepharose-nuclease-Sepharose-pdTp at 6° (see "Experimental Procedures"). The mixture for the exchange in the presence of Ca$^{2+}$ (▲) contained CaCl$_2$ at a concentration of 3.3 mM in 0.106 ml of approximately 0.1 M ammonium acetate, pH 8. The other components were the same as those described above with the exception that EDTA was absent. The mixture was incubated at 20° for 40 min, then cooled and subjected to affinity chromatography as described above. The curves represent fitting of Equation 15 (see "Appendix") to the experimental points (see the text).

![Fig. 2 (right). The apparent rate constant (k) of unfolding of labeled Nuclease-T' as a function of concentration (C) of pdTp at 20° as determined by the rate of exchange between free Nuclease-T'-(50-149) and bound [1-14C]acetyl-Nuclease-T-(50-149) in the presence and absence of pdTp. The incubation mixture for the exchange in the absence of pdTp (●) contained the same quantities of labeled Nuclease-T', Nuclease-T-(6-48), and Nuclease-T-(50-149) as those described in the legend to Fig. 1 and varying concentrations of CaCl$_2$ in 0.1 ml of approximately 0.1 M ammonium acetate, pH 8. After incubation at 20° for 20 min, the mixture was cooled and applied to an affinity column at 6° (see "Experimental Procedures"). The point representing the value for k at zero concentration of Ca$^{2+}$ is the same as that at zero concentration of pdTp in the absence of Ca$^{2+}$ in Fig. 1. In the absence of added ligands, pdTp, and Ca$^{2+}$, the rate of the exchange in the constant of ligands at 20° was the same with and without EDTA. The mixture for the exchange in the presence of pdTp (▲) contained 4.6 nmol of labeled Nuclease-T', 0.55 nmol of contaminating Nuclease-T-(6-48) (2), 13.3 nmol of Nuclease-T-(50-149), 161 nmol of pdTp, and varying concentrations of CaCl$_2$ in 0.1 ml of approximately 0.1 M ammonium acetate, pH 8. In this experiment pdTp was placed in the tube containing labeled Nuclease-T' and contaminating Nuclease-T-(6-48) instead of in the second tube having Nuclease-T-(50-149) and CaCl$_2$ before the contents were mixed (see "Experimental Procedures"). The mixture was incubated at 20° for 40 min and subjected to affinity chromatography as described above. The curves show fitting of equations to the data (see the text).

### Table I

| Ligands bound | Rate constant of unfolding | $\Delta F^{+*\text{a}}$ | Association constant of ligands$^b$ | $\Delta F^{*\text{c}}$ |
|---------------|-----------------------------|---------------------|----------------------------------|------------------|
| None          | $4.6 \times 10^{-4}$       | 21.6                | $K_1 = 1.0 \times 10^4$          | -5.4             |
| pdTp alone    | $9.0 \times 10^{-4}$       | 22.6                | $K_4 = 2.0 \times 10^3$          | -3.1             |
| Ca$^{2+}$ alone | $1.6 \times 10^{-4}$       | 22.2                |                                  |                  |
| pdTp in presence of Ca$^{2+}$ | $2.2 \times 10^{-4}$       | 23.4                | $K_4 = 4.0 \times 10^4$          | -7.5             |
| Ca$^{2+}$ in presence of pdTp | $2.2 \times 10^{-4}$       | 23.4                | $K_4 = 1.4 \times 10^4$          | -5.6             |

* Activation free energy at 20° of the unfolding of Nuclease-T' calculated on the basis of absolute rate theory of Glassstone et al. (16).

$^b$ See "Appendix" for the designations of $K_1$, etc.

$^c$ Free energy change at 20° calculated on the basis of the association constant of ligands.

$^d$ The value is the average of $2.1 \times 10^{-5}$ and $2.3 \times 10^{-5}$ s$^{-1}$ obtained by two sets of experiments (see the text).
Fig. 3. Ellipticity at 222 nm of nuclease and Nuclease-T' as a function of temperature in the presence of two different concentrations of pdTp and a constant concentration of Ca++. Nuclease-T' (Nuclease-T-(6-48))\(^{+}\) and Nuclease-T'-(50-149))\(^{+}\) were dissolved in 0.1 M ammonium acetate-5 mM CaCl\(_2\), pH 8 containing pdTp. The concentrations of pdTp are Curve 1, 21.2 \(\mu\)M; Curve 2, 158 \(\mu\)M; Curve 3, 21.2 \(\mu\)M; Curve 4, 159 \(\mu\)M. Molecular ellipticities were calculated on the basis of average residue weights. The concentration of nuclease and Nuclease-T' was determined by amino acid analysis. The procedure to set the protected Pockets cell-cuvette holder unit in the cell compartment of the polarimeter sometimes caused a slight misalignment of the Pockets cell due to a mechanical situation involved in the attachment of the jacketed block (see "Experimental Procedures"). This resulted in a shift of the base-line of the measurement which could not be accurately corrected in the calculation of ellipticity (19). The apparent difference of the magnitude of change of ellipticity by thermal transition in the two experiments of Nuclease-T' (Curves 1 and 2) is probably caused by the mechanical situation of the instrument. However, the mode of change of circular dichroism of each sample should be independent of this disadvantage since the unit placed in the cell compartment was not moved during the series of the measurements as a function of temperature.

The values for the midpoints of thermal transition of nuclease and Nuclease-T' in the absence of the ligands are approximately 50 and 33\(^{0}\), respectively (2). In the presence of 21 \(\mu\)M on pdTp and 5 \(\mu\)M of CaCl\(_2\), the values for the midpoints of the transition of nuclease and Nuclease-T' were approximately 65 and 45\(^{0}\), respectively, and in the presence of 158 \(\mu\)M of pdTp and the same concentration of CaCl\(_2\) they were approximately 68 and 48\(^{0}\), respectively (Fig. 3). The values for the apparent rate constant of unfolding of Nuclease-T' (20\(^{0}\)) at 21 and 158 \(\mu\)M of pdTp, calculated on the basis of Equation 24 (see "Appendix") under the conditions used in the experiments in Fig. 3 (Curves 1 and 2, respectively), are 9.5 \(\times\) 10\(^{-4}\) and 3.1 \(\times\) 10\(^{-5}\) s\(^{-1}\), respectively. The difference between these two values is qualitatively consistent with the shift toward higher temperature of thermal transition of Nuclease-T' at the higher concentration of pdTp (Fig. 3).

**DISCUSSION**

Although pdTp and Ca\(^{++}\) may interact with Nuclease-T' in a nonspecific way, it is assumed that the ligands bind only in the specific binding sites, elucidated by x-ray crystallographic studies of the three-dimensional structure of liganded nuclease (19) and that the binding of the ligands results in the observed suppression of unfolding of Nuclease-T'. The atomic coordinates of liganded nuclease show that the side chains having direct contact with pdTp are provided by the amino acid sequences corresponding to both Nuclease-T-(6-48) and Nuclease-T-(50-149) (for example, arginine residues 35 and 87, tyrosine residues 85 and 113) (19). Therefore, it may be considered that the "pdTp bridge" between the two fragments formed through noncovalent bonding prevents the dissociation of the two fragments. However, all side chains which coordinate with Ca\(^{++}\) belong to Nuclease-T-(6-48) (aspartic acid residues 21 and 40, glutamic acid residue 43) (19). Another example of the spatial relationship between the interaction sites of complementing fragments and the binding site of ligands can be seen in a second type of ordered complex of nuclease fragments. Nuclease-(1-126) and Nuclease-(99-149) form an ordered complex (type II) resembling nuclease (20). The type II complex like Nuclease-T' is stabilized against heat denaturation by binding of pdTp and calcium ion. The segment from residue 99 to 113 of Nuclease-(99-149) can be removed without any effect on the enzymic activity of the complex. Therefore, tyrosine residue 113 located near pdTp may be assumed to belong to Nuclease-(1-126). Hence, there may be no direct contact between pdTp and Nuclease-(99-149) in the ordered structure of type II complex. Nonetheless, the interactions between Nuclease-(1-126) and Nuclease-(99-149) in type II complex are strengthened by the binding of ligands. These observations indicate that the suppression of unfolding of Nuclease-T' by the binding of ligands is due to prevention of an orderly unfolding but rather to strengthening of the cooperative interactions throughout the three-dimensional structure.

The free energy of binding of either pdTp or calcium ion to Nuclease-T' decreases by approximately 2 kcal when the other ligand is already bound. Arnone et al. (19) have pointed out that the minimum possible distance between the calcium ion and an oxygen atom of the 5'-phosphate group of pdTp in the structure of liganded nuclease (3 A) is too great for any strong interaction. Therefore, the cooperativity between the binding of pdTp and calcium ion to Nuclease-T' is probably due not to a direct interaction between the two bound ligands but rather to the coupling of the interactions in the ligand-binding sites with the interactions in the three-dimensional structure of Nuclease-T'. This hypothesis is supported by the observations that the increase in the activation free energy of the unfolding of Nuclease-T' by binding of both pdTp and Ca\(^{++}\) is greater than the sum of the increase in the activation free energy of the unfolding caused by binding of each ligand (Table I).

Unlike unfolding, the rate of folding of Nuclease-T' is independent of temperature and the presence of ligands (17). These observations imply that the energy barrier of folding of Nuclease-T' is entropic, and the magnitude of the energy barrier is unchanged in the presence of pdTp and calcium ion. That is, the rate of formation of Nuclease-T' from the two fragments is determined by the probability of two polypeptide chains folding to a specific spatial arrangement, and the rate of the statistical search of the specific folding does not increase in the presence of the ligands. In contrast, we assume that the energy barrier of unfolding of Nuclease-T' is increased by binding of the ligands and that the increase in the energy barrier is equal to the increase in the stabilization energy maintaining the structure of Nuclease-T'. For example, the

*Residues 114 to 149 can be removed from an atomic model of nuclease without disturbing any other residues.*
binding of pdTp to Nuclease-T results in a free energy change of \(-5.4\) kcal. Part of this free energy change \((-1\) kcal\) is contributed by the stabilization of the structure (Table I). In other words, if the local interactions with pdTp at the binding site do not specifically couple with the interactions operating throughout the three-dimensional structure of Nuclease-T', the free energy change involved in the binding of pdTp would be \(-4.4\) kcal instead of \(-5.4\) kcal and no ligand-induced stabilization of Nuclease-T' would occur.

On the basis of these considerations we interpret the stabilization of the structure of Nuclease-T' by binding of ligands as follows. The local interactions between the ligands and their binding sites are coupled with the cooperative interactions operating in the three-dimensional structure to strengthen both the interaction maintaining the three-dimensional structure and the interactions between the ligands and their binding sites.

The coupling of the two interaction systems provides a stabilization energy of approximately 2 kcal (Table I) without a gross change in the conformation of Nuclease-T'(21). By analogy we speculate that the lowering of the energy of the native structure of nuclease occurs without a large change in conformation by specific coupling of the local interactions through the three-dimensional structure after the disordered polypeptide chain is folded into the native conformation (2).

**APPENDIX**

Equilibrium Equations for Association Reaction of Two Ligands with Nuclease-T—It is assumed that Nuclease-T is physicochemically identical with Nuclease-T* (21), therefore, only the designation of Nuclease-T is used. It is also assumed that the ligands pdTp and Ca\(^{2+}\) bind to Nuclease-T only at the specific binding sites in the three-dimensional structure determined by the x-ray crystallographic studies of liganded nuclease (19). Let the concentrations of unliganded Nuclease-T, free pdTp, and free Ca\(^{2+}\) be \([T]\), \([P]\), and \([C]\), respectively. Let those of Nuclease-T bound with pdTp, with Ca\(^{2+}\), and with both pdTp and Ca\(^{2+}\) be \([TP]\), \([TC]\), and \([TPC]\), respectively. The following equilibrium reactions may be assumed at the equilibrium state of the mixture of Nuclease-T, pdTp, and Ca\(^{2+}\).

\[
[T] + [P] \rightleftharpoons [TP],
\]
\[
[T] + [C] \rightleftharpoons [TC],
\]
\[
[TP] + [C] \rightleftharpoons [TPC],
\]
\[
[TC] + [P] \rightleftharpoons [TPC],
\]

Let the association constants involved in Equations 1 to 4 be \(K_1, K_2, K_3, \) and \(K_4\). Then, we can write

\[
[TP] = K_1 [T][P],
\]
\[
[TC] = K_2 [T][C],
\]
\[
[TPC] = K_3 [TP][C],
\]
\[
[TPC] = K_4 [TC][P].
\]

On making the substitutions and eliminations we have

\[
K_3 K_4 = K_2 K_4
\]

Accordingly,

\[
\frac{K_3}{K_2} = \frac{K_4}{K_1} = K
\]

On the basis of Equation 10, the following three cases can be considered.

1. \(R = 1,\) that is \(K_1 = K_2, K_3 = K_4\)
2. \(R > 1,\) that is \(K_3 > K_2, K_4 > K_1\)
3. \(R < 1,\) that is \(K_3 < K_2, K_4 < K_1\)

In Case 1 the binding of pdTp and Ca\(^{2+}\) to Nuclease-T' is independent of the presence of the other ligand. In Case 2, the binding of either of the two ligands is enhanced when the other ligand is bound. In Case 3, the binding of either of the two ligands is weakened when the other ligand is bound. The three cases of relationship between the association constants described above are not restricted to the reactions of the system composed of 2 small molecules and 1 large molecule. As far as a system containing three elements satisfies the equilibrium Equations 1 to 4, one of the three cases will be observed in the relationship between the association constants in the system (see, for example, Refs. 22-25).

Calculation of Rate Constant of Unfolding of Nuclease-T Bound with Ligand and of Association Constant of Ligand—Let the concentration of Nuclease-T (the sum of the liganded and unliganded forms) be \(T\), the concentration of a ligand (the sum of the free and bound species) be \(X\), and the fraction of liganded Nuclease-T be \(a\). Then, the dissociation constant, \(K\), for the reaction of Equation 1 will be defined by the following equation

\[
\frac{a - T}{1 - a - T} = \frac{1}{K}
\]

On rearrangement we have

\[
x = \frac{a}{1 - a} + a \cdot T
\]

Let the values for the rate constants of unfolding of Nuclease-T in the absence of and at saturation with the ligand be \(Y_0\) and \(Y_\infty\), respectively, and let \(Y\) be the value for the apparent rate constant of unfolding of Nuclease-T at ligand concentration \(X\) (Fig. 4). Since the rate of unfolding of Nuclease-T is dependent only on the concentration of Nuclease-T in the first order (2), the apparent rate of the unfolding at ligand concentration \(X\) may be expressed as \(Y \cdot T\). This should be the sum of the rates of unfolding of unliganded and liganded Nuclease-T as shown in Equation 13.

![Diagram of the relationship between the apparent rate constant (Y) of unfolding of partially liganded Nuclease-T and those of unliganded (Y0) and liganded (Y∞) Nuclease-T. X, concentration of a ligand; k, rate constant of unfolding.](http://www.jbc.org/)
Then of concentration of pdTp at 3.3 mM, the substitution for \( a \) in Equation 12 using Equation 14 gives

\[
a = \frac{y - y_o}{y_o - y}
\]  

The substitution for \( a \) in Equation 12 using Equation 14 gives

\[
x = \frac{y - y_o}{y_o - y} \cdot x + \frac{y - y_o}{y_o - y} 
\]  

Since the values for \( T \) and \( Y_e \) are known, the values for \( Y_o \) and \( K \) can be obtained as the values for the best fit of Equation 15 to the set of data of \( X \) and \( Y \). The association constant is calculated as the reciprocal of \( K \).

Estimation of Association Constant of a Ligand with Nuclease-T Bound by a Second Ligand—In the experiments determining the rate of unfolding of Nuclease-T as a function of concentration of pdTp at 3.3 mM of Ca\(^{2+}\) (see Fig. 1), the concentration of Ca\(^{2+}\) was not sufficient to saturate Nuclease-T. However, since the association constant of Ca\(^{2+}\) to Nuclease-T is much greater in the presence of pdTp than in the absence (Table I), it may be assumed that all Nuclease-T bound with pdTp is also bound with Ca\(^{2+}\). Since the molar quantity of Ca\(^{2+}\) is in large excess to Ca\(^{2+}\)-bound Nuclease-T, Ca\(^{2+}\) alone, and Nuclease-T bound with both pdTp and Ca\(^{2+}\)-bound Nuclease-T was bound with Ca\(^{2+}\). Therefore, the substitution for \( [P] \) in Equation 19 using Equation 20 gives

\[
[T] + [TC] + [TPC] = T
\]

On rearrangement

\[
[P] = \frac{[T]}{1 + K_2K_4[T][C]}
\]

The solution of quadratic Equation 21 gives

\[
[T] = \frac{1}{2} \left( \frac{K_2K_4[C]}{K_2K_4[C] + \sqrt{A}} \right)
\]  

where \( A = (1 + K_2[C] + K_4K_2[P][C] - K_3K_4[C][T])^2 + 4(1 + K_2[C] + K_3K_4[C][T]) \). The plus sign is chosen since \([T] > 0\) when \( T = 0 \). Let the apparent rate constant of unfolding of Nuclease-T in the presence of the ligands be \( Y_o \) and the rate constants of unfolding of unliganded Nuclease-T, Nuclease-T bound with Ca\(^{2+}\) alone, and Nuclease-T bound with both pdTp and Ca\(^{2+}\) be \( Y_p \), \( Y_o \), and \( Y_{pc} \), respectively. Then, similarly to Equation 13, we can write

\[
Y \cdot T = Y_o[T] + Y_c[TC] + Y_{pc}[TPC]
\]  

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Ammonium sulfate precipitation

The ammonium sulfate precipitation is physically similar to the precipitation of per cent solution of ammonium sulfate at pH 6.

The precipitated material was collected by filtration and washed several times with water. The precipitates were dissolved in water and the solution was concentrated to a volume of 100 ml. The concentration of nitrogen in the solution was determined by the Kjeldahl method. The precipitate was then dissolved in water and the solution was made up to 100 ml with distilled water.

Ammonium sulfate precipitation is the method of choice for the isolation of proteins from biological tissues. The precipitate is then redissolved in water and the solution is made up to 100 ml with distilled water.

Ammonium sulfate precipitation is a widely used method for the isolation of proteins from biological tissues. The precipitate is then redissolved in water and the solution is made up to 100 ml with distilled water.

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The mechanism of stabilization of the structure of nuclease-T by binding of ligands.
H Taniuchi and J L Bohnert

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