A Kinetic Study of the Complementation of Fragments of Staphylococcal Nuclease

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SUMMARY

The complementation of fragments of staphylococcal nuclease to form an ordered structure was studied by stopped flow kinetic measurements. The increase in the intensity of tryptophan fluorescence of residue 140 on complementation was followed. The complementation of two fragments containing residues 6 to 48 and residues 49 to 149 (or 50 to 149) to form Nuclease-T' follows apparent first order kinetics, both with regard to variation of time and fragment concentration accessible to study. The first order rate constant of the formation of Nuclease-T' is in the range of 0.03 to 0.05 s⁻¹, which is much less than that of refolding of acid-denatured intact nuclease observed by Schechter et al. (Schechter, A. N., Chen, R. F., and Anfinsen, C. B. (1970) Science 167, 886-887). The change of temperature from 5° to 45° caused only a small increase in the rate of formation of Nuclease-T'. The presence of the ligands, thymidine 3',5'-diphosphate and Ca²⁺, and the change of pH of the reaction mixture from 5 to 8 had no effect on the rate constant. The complementation between the fragments of residues 1 to 126 and of residues 99 to 149 and between the fragments of residues 1 to 126 and of residues 49 to 149 (or 50 to 149) also fits first order kinetics and shows rate constants equivalent to that of Nuclease-T'. Examination of the kinetic equation for the simplest model, in which specific prefolding of each fragment is required for the productive collision, and the collision is the rate-limiting step of the complementation, fails to explain these results. However, the results may be interpreted by assuming the rate-limiting step either to be the prefolding of one of the two fragments, which then combines with the other fragment, or the folding of a disordered intermediate complex formed by the two fragments.

Several different studies have been concerned with the rates of folding and unfolding of protein upon rapid perturbation by temperature jumps (1) and pH changes (2-6). In the case of renaturation of acid-denatured staphylococcal nuclease, the over-all folding has been described by two first order processes: a rapid step (half-time, 55 ms), followed by a slower step (half-time, 350 ms) (2, 3). It has been suggested that the initial fast phase is related to a partial unfolding of the polypeptide chain with the formation of a number of intermediates (nucleation by helices), followed by numerous stabilizing interactions that occur as the slow step of the process (3).

The combination of two fragments, covering the amino acid sequence of staphylococcal nuclease, but lacking enzymic activity and ordered structure, form enzymically active complexes by noncovalent bondings (7-10) (Fig. 1). So far, two types of complexes have been observed. One active complex, called Nuclease-T'† (or type I complex), is formed by complementation of fragments of residues 0 to 48 and of residues 49 to 149 (or 50 to 149) (8). Another (type II complex), containing overlapping sequences, is formed from fragments of residues 1 to 126 and of residues 99 to 149 (9). The physical properties of the complexes resemble those of intact nuclease, although the level of biological activity is less. In particular, recent x-ray diffraction studies, comparing the crystals of Nuclease-T and Nuclease-T' with those of intact nuclease, have shown that these crystals are isomorphous (14).

The formation of Nuclease T' from Nuclease T (6-48) and Nuclease-T-(49,50-149) may be described by the reaction shown in Equation 1

\[
\text{Nuclease-T-(6-48)} + \text{Nuclease-T-(49,50-149)} \xrightarrow{k_f/k_r} \text{Nuclease-T'}
\]

\[(1)\]

† Fragments of nuclease have been designated by an adaptation of the rules of the Commission on Biochemical Nomenclature (13). 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-(40,50-149) is the mixture of Nuclease-T-(40-149) and Nuclease-T-(50-149) isolated from Nuclease-T (8). Nuclease-T, obtained from nuclease by limited digestion with trypsin, is composed of Nuclease-T-(6-48) and Nuclease-T-(49,50-149) (8). The reconstituted Nuclease-T from its component fragments is called Nuclease-T'.

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where $k_f$ and $k_r$ are the rate constants in the forward and reverse directions, respectively. The dynamic equilibrium state of the reaction (Equation 1) involving the unfolding and refolding of the three-dimensional structure of Nuclease-T, has been demonstrated by measurement of exchange of free Nuclease-T-(50-149) with labeled Nuclease-T-(50-140) incorporated in Nuclease-T' (15). It has been shown that the rate constant in the reverse direction of Equation 1 is highly temperature-dependent (half-time, 148 min at 10°C; 19 min at 20°C) (15). Since the rate-limiting step of the exchange between free Nuclease-T-(50-140) and the incorporated Nuclease-T-(50-140) is the dissociation of Nuclease-T (15), the experiment does not give quantitative information concerning the rate of association of the two fragments. In the present studies we have directly measured the rate constant in the forward direction for the complementation of nuclease fragments under various conditions. The nuclease molecule contains a single tryptophan at residue 140 (11). The fluorescence emission spectra of Trp-140 of the fragments in solution is the same as that of free tryptophan (9, 16). On the other hand, the tryptophan fluorescence of intact nuclease and of the ordered complexes shows an emission maximum with a much higher intensity and at a shorter wavelength than that of the disordered fragments (16), being consistent with the fact that in the three-dimensional structure of nuclease the side chain of Trp-140 resides in a nonpolar environment (17, 18). The progressive change of the intensity of tryptophan fluorescence was followed after mixing of two solutions each containing one of the two complementing fragments. Insofar as the increase in intensity of tryptophan fluorescence reflects the formation of the ordered structure, these studies give information that is useful to an understanding of the mechanism of folding of nuclease.

**EXPERIMENTAL PROCEDURES**

Nuclease-T, Nuclease-T-(6-48), Nuclease-T-(49,50-149), and Nuclease-(1–126) were prepared from staphylococcal nuclease (Foggi strain) in a pure form by the procedures reported earlier (8, 9, 16). Nuclease-(99–140) was obtained by cyanogen bromide cleavage of Nuclease-T-(49,50-149) and purified on a column (1 X 200 cm) of Sephadex G-50 (fine) by the reported procedures (19). The molar quantity of Nuclease-T in solution was determined spectrophotometrically at 280 nm with an $E_{1}^{1}\text{cm}$ of 9.3 (7, 20). The molar amount of other nuclease fragments was estimated from amino acid analyses of 22-hour hydrolysates on a Beckman-Spinco analyzer (21).

**Measurement of Emission Spectra of Tryptophan Fluorescence**—Relative fluorescence intensity measurements were performed in a 3-ml quartz cuvette at 25°C with an Aminco-Bowman spectrophotofluorometer equipped with an RCA IP28 photomultiplier tube and a grating blazed at 300 nm (22). The excitation beam at 295 nm was passed through a horizontally polarizing filter to reduce scatter (22), and the unfiltered emission beam was recorded by an Aminco Instrument X-Y recorder. The bandwidth was 12 nm for both beams.

**Stopped Flow Kinetics**—The increase in relative intensity of tryptophan fluorescence of the reaction mixture was followed with a second Aminco-Bowman spectrophotofluorometer equipped with the rapid mixing device which has been described elsewhere (3, 23). Two solutions to be mixed were each contained in a 1-ml capacity syringe. The observation chamber was a 0.2-ml capacity quartz cuvette equipped with a Bel-F float. Several repetitive measurements could be taken with the same solutions. The temperature of the syringe contents and the cell compartment was kept constant by circulating water through the jackets with a Haake type F water bath for temperatures above 25°C and with a Formtemp, Jr. model 2903 bath for 25°C and below. The tryptophanyl residue of the sample was excited at 295 nm and the 340-nm fluorescence was monitored. The fluorescence emission signal, detected by an Aminco transistorized microphotometer (model 10-267), was recorded with an Aminco recorder at a chart speed of 0.1 inch per s, or on a Hewlett-Packard oscilloscope (model 141A) with scanning rate of 200 ms per cm (3). The oscilloscope trace was photographed with a Polaroid Land camera and an enlargement of the transparency was examined in a Nomars microcomparator.

The observed kinetic data were fitted to the first order kinetic Equation 2 (see "Results" below for the explanation)

$$\frac{I - I_{eq}}{I_0 - I_{eq}} = e^{-kt}$$

where $I_0$ and $I_{eq}$ are the relative fluorescence intensity at zero and infinite time, respectively. $\lambda$ is the sum of the rate constants of forward (k$_f$) and backward (k$_r$) reactions, that is $\lambda = k_f + k_r$ (see Equation 1). At and below 25°C the value for k$_r$ is small compared with k$_f$ (15) and the value for $\lambda$ is then, approximately equal to that for k$_f$. The value for $\lambda$ is obtained as the value which gives the best fit of Equation 2 to the observed data of relative fluorescence intensities and time values covering three or four half-lives of the reaction. The value for $\lambda$ is presented below as the first order rate constant (k$_f$).

All calculations and fitting of equations to data by the least squares method were performed with the aid of a PDP-10 computer system of the Division of Computer Research and Technology, National Institutes of Health.

**RESULTS**

**Complementation of Nuclease-T-(6-48) and Nuclease-T-(49,50-149)**

**Equilibrium Constant**—Fig. 2 shows the relative fluorescence intensity at 25°C of mixtures of Nuclease-T-(6-48) and Nuclease-T-(49,50-149) at equilibrium as a function of the molar ratio of the two. The equilibrium constant, $K$, for the reaction (Equation 1) is shown in Equation 3

$$K = \frac{a}{(1 - a)(R - a)} \cdot \frac{1}{C}$$

where $K$ is the molar ratio of Nuclease-T-(6-48) to Nuclease-T-(49,50-149), C is the initial concentration of Nuclease-T-(49,50-149), and a is the fraction of Nuclease-T’ produced. The latter is the value corresponding to that of the ordinate of Fig. 2. The data of a versus R were fitted to an equilibrium expression (Equation 3), and the corresponding value of the equilibrium constant at 25°C for the formation of Nuclease-T’ was 3.3 × 10$^{-4}$ M$^{-1}$ (Fig. 2).

**Kinetics of Complementation**—The kinetic curve of the increase in tryptophan fluorescence intensity after stopped flow mixing of Nuclease-T’-(6-48) and Nuclease-T-(49,50-149) is shown in Fig. 3. The fluorescence intensity at the end of the reaction was equivalent to the amount of Nuclease-T’ expected for the reactants present. The kinetic data invariably showed a good fit to first order kinetics (Fig. 4). The value for the rate constant calculated on the basis of first order kinetics had little depend-
though the difference may be within the experimental error of an oscilloscope trace (Fig. 5), the value calculated for the first order rate constant was 0.09 s\(^{-1}\) and the half-time was 7.7 s. The reaction appears to be more rapid in the early phase, although the difference may be within the experimental error of the early measurements. Here the precision is poor when looking at only a small per cent of the total change during the early part of a relatively slow process.

**Effect of Ligands**—It is known that the binding of calcium ions and pdTp\(^{6}\) to nuclease and Nuclease-T stabilizes the structures against heat denaturation (7), presumably not by acceleration of the folding but by suppression of the unfolding reaction (15). Table III shows the kinetic analysis of the effect of the ligands on the rate of formation of Nuclease-T'. The apparent first order rate constant for the reaction does not change by the addition of one or both ligands, being consistent with the idea that ligands do not influence folding.

**Effect of pH**—Previous studies indicated that the equilibrium constant for the formation of Nuclease-T' from the two fragments is dependent on pH such that the formation of Nuclease-T' is essentially absent at pH 4.0 and reaches a maximum at pH 6, as the pH of the mixture increases (9). However, the rate of formation of Nuclease-T' from the two fragments (Table I) shows only a small increase with a 2-fold increase in the initial concentrations of both fragments (Table I). On the other hand, an attempt to fit the rate data to second order kinetics resulted in a large variation of the value for the rate constant (Table II), reflecting a poor fit of an individual set of kinetic data to second order kinetics. Therefore, the apparent kinetic behavior of the formation of Nuclease-T' from the two fragments is best described as a first order process, even though the reaction requires both components. All rate constants described below are calculated on the basis of first order kinetics (see "Experimental Procedures"). The value for the equilibrium constant (\(K\), Equation 3) was calculated as a value to give a best fit of Equation 3 to the presented data, using the least squares method. The theoretical curve obtained on the basis of the calculated value for \(K\) is shown as the solid line.

![Fig. 2. Intensity of tryptophan fluorescence as a function of the ratio of Nuclease-T-(6-48) to Nuclease-T-(49,50-149). The two fragments were mixed in 0.05 M Tris-HCl, pH 8, containing 0.1 M NaCl. The initial concentration of Nuclease-T-(49,50-149) was 6.4 \(\times\) 10\(^{-5}\) M. The ordinate is the relative fluorescence intensity as 328 nm expressed as the fraction of the maximum intensity observed at a molar ratio of Nuclease-T-(6-48) to Nuclease-T-(49,50-149) equal to 3.85. The observed values for the fluorescence intensity used in the calculations were corrected for the fluorescence of Nuclease-T-(49,50-149) alone. The emission maxima of tryptophan fluorescence of Nuclease-T and Nuclease-T-(49,50-149) were 328 and 347 nm, respectively, in the present experiment. The value for the apparent kinetic behavior of the formation of Nuclease-T' from the two fragments is best described as a first order process, even though the reaction requires both components. All rate constants described below are calculated on the basis of first order kinetics. The reaction appears to be more rapid in the early phase, although the difference may be within the experimental error of the early measurements. Here the precision is poor when looking at only a small per cent of the total change during the early part of a relatively slow process.

![Fig. 3. The stopped flow kinetics of the formation of Nuclease-T' from Nuclease-T-(6-48) and Nuclease-T-(49,50-149). Nuclease-T-(6-48) (3.23 \(\times\) 10\(^{-5}\) M) and Nuclease-T-(49,50-149) (1.69 \(\times\) 10\(^{-5}\) M) were mixed at pH 8.0 in 0.05 M Tris-0.1 M NaCl. Increase in intensity of tryptophan fluorescence with time after mixing the two fragments was followed (see "Experimental Procedures"). Temperature, 25°.](http://www.jbc.org/)

![Fig. 4. A plot of the natural logarithm of Nuclease-T-(49,50-149) concentration (c) versus time. The data is from the experiment given in Fig. 3. The following assumptions were made in order to calculate the value for c: Nuclease-T' was quantitatively formed at the end of reaction; the increase in intensity of the fluorescence is proportional to the amount of Nuclease-T' formed; the difference between the initial concentration of Nuclease-T-(49,50-149) and that of Nuclease-T' at a given time is the value for c at that time.](http://www.jbc.org/)
The observed data (see Fig. 3 and Table I) were treated on the basis of second order kinetics

\[ \ln \frac{1 - x/P_2}{1 - x/P_3} = (P_3 - P_2)k_2 t \quad (4) \]

where \( x \) is the concentration of Nuclease-T' at time \( t \) calculated from the increase in the relative intensity of the tryptophan fluorescence, and \( [P_2] \) and \( [P_3] \) are the initial molar concentrations of Nuclease-T-(6-48) and Nuclease-T-(49-50-149), respectively from the increase in the relative intensity of the tryptophan fluorescence, and \( [P_2] \) and \( [P_3] \) are the initial molar concentrations of Nuclease-T-(6-48) and Nuclease-T-(49-50-149), respectively.

The reaction mixture was at pH 8.0 in 0.05 M Tris-0.1 M NaCl. The illustration is an enlargement of a photograph of an oscilloscope trace showing the change in relative fluorescence intensity. Other details are under "Experimental Procedure." Temperature, 25°C.

**Table II**

**Trial fit for second order kinetics**

<table>
<thead>
<tr>
<th>Concentration of Nuclease-T-(6-48) ( \times 10^5 )</th>
<th>Concentration of Nuclease-T-(49,50-149) ( \times 10^5 )</th>
<th>( k_2 ) ( \times 10^{-1} ) ( s^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 1.78 )</td>
<td>( 1.29 )</td>
<td>( 7890 )</td>
</tr>
<tr>
<td>( 2.67 )</td>
<td>( 1.94 )</td>
<td>( 5443 )</td>
</tr>
<tr>
<td>( 3.56 )</td>
<td>( 2.58 )</td>
<td>( 390 )</td>
</tr>
<tr>
<td>( 5.34 )</td>
<td>( 3.87 )</td>
<td>( 327 )</td>
</tr>
</tbody>
</table>

**Table III**

**Effect of pdTp and Ca\(^{2+}\) on rate of formation of Nuclease-T'**

The reaction mixture was at pH 8.0 in 0.05 M Tris-0.1 M NaCl. Temperature, 25°C.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Concentration of Nuclease-T-(6-48) ( \times 10^5 )</th>
<th>Concentration of Nuclease-T-(49,50-149) ( \times 10^5 )</th>
<th>( k_f ) ( \times 10^{-3} )</th>
<th>( t_{1/2} ) ( s )</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>( 1.78 )</td>
<td>( 1.29 )</td>
<td>( 0.040 )</td>
<td>( 17.33 )</td>
</tr>
<tr>
<td>( 10^{-2} ) M pdTp</td>
<td>( 1.78 )</td>
<td>( 1.20 )</td>
<td>( 0.013 )</td>
<td>( 16.12 )</td>
</tr>
<tr>
<td>( 5 \times 10^{-4} ) M CaCl(_2)</td>
<td>( 1.78 )</td>
<td>( 1.20 )</td>
<td>( 0.040 )</td>
<td>( 17.33 )</td>
</tr>
<tr>
<td>( 10^{-2} ) M pdTp ( 5 \times 10^{-4} ) M CaCl(_2)</td>
<td>( 1.45 )</td>
<td>( 1.45 )</td>
<td>( 0.039 )</td>
<td>( 17.77 )</td>
</tr>
</tbody>
</table>

Nuclease-T' was determined over the temperature range of 5 to 45°C in the absence and in the presence of pdTp and Ca\(^{2+}\) (Table V). The values for the rate constant are at the same level at all temperatures, whether the ligands were present or not, although the rate constant may be decreasing slowly as temperature decreases (Table V). Above 40°C the rate of unfolding of Nuclease-T' apparently exceeded that of the forward reaction (Equation 1), since no increase in the intensity of the fluorescence was observed after mixing the two fragments in the absence of the ligands. However, when both ligands were present, the forward reaction could be examined at 45°C and still be detected at 52°C (Table V), presumably as a result of the suppression of the unfolding (15).

**Table IV**

**Effect of pH on rate of formation of Nuclease-T'**

The initial concentrations of Nuclease-T-(6-48) and Nuclease-T-(49-50-149) in the reaction mixture were \( 1.48 \times 10^{-6} \) and \( 1.45 \times 10^{-5} \) M, respectively. Temperature, 25°C.

<table>
<thead>
<tr>
<th>pH</th>
<th>Buffer</th>
<th>( k_f ) ( \times 10^{-4} )</th>
<th>( t_{1/2} ) ( s )</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0</td>
<td>Tris-0.1 M NaCl</td>
<td>0.046</td>
<td>15.07</td>
</tr>
<tr>
<td>5.52</td>
<td>NH(_4)Ac-0.1 M NaCl</td>
<td>0.052</td>
<td>11.18</td>
</tr>
<tr>
<td>4.89</td>
<td>NH(_4)Ac-0.1 M NaCl</td>
<td>0.055</td>
<td>12.60</td>
</tr>
</tbody>
</table>

**Table V**

**Effect of temperature on rate of formation of Nuclease-T' in presence and absence of pdTp and Ca\(^{2+}\)**

The initial concentrations of Nuclease-T-(6-48) and Nuclease-T-(49-50-149) in the reaction mixture were \( 1.48 \times 10^{-5} \) and \( 2.97 \times 10^{-5} \) M, respectively, in the experiments without ligands and \( 1.48 \times 10^{-5} \) and \( 1.45 \times 10^{-5} \) M, respectively, in the mixture containing \( 10^{-4} \) M Ca\(^{2+}\) and \( 10^{-4} \) M pdTp.

The combination of Nuclease-(1-126) and Nuclease-(99-149) forms the ordered complex of type II having an enzymic activity of approximately 15% that of intact nuclease (9). The ordered complex presumably excludes the segments of overlapping amino acid sequence, which may occur somewhere between residues 114 and 124, with the redundant portion protruding from the ordered structure (9).
The concentrations of pdTp and Ca$^{2+}$ where present in the mixture were $10^{-4}$ and $10^{-2}$ M, respectively. Temperature, 25°C.

### Table VI

<table>
<thead>
<tr>
<th>Concentrations of fragments</th>
<th>Ligands</th>
<th>$k_f$</th>
<th>$k_{-f}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-(99-149)</td>
<td>Nuclease-(l-126)</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>$M \times 10^3$</td>
<td>1.2</td>
<td>0.67</td>
<td>0.026</td>
</tr>
<tr>
<td>2.4</td>
<td>1.33</td>
<td>0.027</td>
<td>26.67</td>
</tr>
<tr>
<td>4.8</td>
<td>2.66</td>
<td>0.038</td>
<td>18.24</td>
</tr>
<tr>
<td>4.8</td>
<td>2.57</td>
<td>0.076</td>
<td>9.12</td>
</tr>
</tbody>
</table>

### Table VII

<table>
<thead>
<tr>
<th>Concentrations of fragments</th>
<th>Ligands</th>
<th>$k_f$</th>
<th>$k_{-f}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-(49,50-149)</td>
<td>Nuclease-(l-126)</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>$M \times 10^3$</td>
<td>0.58</td>
<td>0.64</td>
<td>0.031</td>
</tr>
<tr>
<td>1.49</td>
<td>1.26</td>
<td>0.062</td>
<td>21.66</td>
</tr>
<tr>
<td>2.90</td>
<td>2.57</td>
<td>0.066</td>
<td>19.25</td>
</tr>
<tr>
<td>1.45</td>
<td>1.28</td>
<td>0.049</td>
<td>14.14</td>
</tr>
</tbody>
</table>

### Discussion

It has been shown that the three-dimensional structures of nuclease and Nuclease-T involve a dynamic equilibrium of folding and unfolding under physiological conditions (13). The rate of dissociation of Nuclease-T, which includes the unfolding step, is highly dependent on temperature (14), as is generally the case with heat denaturation of proteins (25). The binding of pdTp and calcium ion to Nuclease-T suppresses the dissociation (15). In contrast, the present results show that the rate of association of Nuclease-T-(6-48) and Nuclease-T-(49,50-149) to form Nuclease-T is essentially independent of temperature in the temperature range studied. This is consistent with the observation by Epstein et al. that the initial rate of refolding of acid-denatured nuclease is independent of temperature (3). The present observations indicate that pdTp and calcium ion also have no effect on the rate of formation of Nuclease-T'. Therefore, the ligand-induced resistance of nuclease and Nuclease-T against heat denaturation (7) is the result of suppression of the unfolding rather than acceleration of the folding. The fact that the rate of formation of Nuclease-T' is independent of temperature may suggest that the rate-limiting step for the formation of Nuclease-T' is under the control of an entropic energy barrier. Therefore, the slower rate of formation of Nuclease-T' compared with the refolding of acid-denatured nuclease may be explained in terms of a lower probability of two polypeptide chains folding to a specific spatial arrangement relative to the refolding of a single polypeptide chain. However, the actual difference in magnitude of the entropic energy barrier that occurs between these two systems depends on the actual mechanism of folding.

The observed first order kinetic behavior for the complementation of disordered fragments under a variety of experimental conditions limits the number of plausible mechanisms that should be considered to explain the folding process. The first mechanism to be considered is that Nuclease-T-(6-48) and Nuclease-T-(49-149) independently fold into the "native" conformations and then collide with each other to form Nuclease-T'. Let the concentrations of Nuclease-T-(6-48) and Nuclease-T-(49-149) having non-native conformations or disordered structures be $A$ and $C$, respectively, and those of the native conformations be $B$ and $D$, respectively. $T$ is the concentration of Nuclease-T'.

\[
\begin{align*}
A & \xrightarrow{k_1} B \\
C & \xrightarrow{k_3} D \\
B + D & \xrightarrow{k_5} T
\end{align*}
\]

where $k_i$, etc. denote the rate constants of the indicated steps. The rate equations are

\[
\begin{align*}
\frac{dA}{dt} &= -k_2A + k_1B \\
\frac{dB}{dt} &= k_1A + k_i - k_2B - k_5B
\end{align*}
\]

The rate process for the formation of the ordered complexes from the two fragments in the presence of pdTp and Ca$^{2+}$ was again fitted to first order kinetics as shown in Table VII. In the absence of ligands, however, the rate constant appeared to increase slightly, as noted before for the complementation of Nuclease-(1-126) and Nuclease-(99-149). The observations might suggest that the ligands bind weakly to Nuclease-(1-126), interfering with the formation of the ordered complex by complementation.
The solution of these differential equations is complicated by the number of parameters involved. However, if only the initial rate of formation of Nuclease-T' is considered, the following approximate treatment may be allowed.

\[ T = 0 \]

and

\[ -\frac{dA}{dt} = -\frac{dC}{dt} = \frac{dT}{dt} \]
then,

\[ \frac{dB}{dt} = 0 \]

and

\[ \frac{dD}{dt} = 0 \]

Accordingly,

\[ k_1A - k_2B - k_5B\cdot D = 0 \]

\[ k_3C - k_4D - k_5B\cdot D = 0 \]

\[ \frac{dT}{dt} = k_5B\cdot D \]

From Equations 13 and 15

\[ b = k_2 \left( k_1A - \frac{dT}{dt} \right) \]

Similarly

\[ d = k_4 \left( k_3C - \frac{dT}{dt} \right) \]

From Equations 16, 16, and 17,

\[ \left( \frac{dT}{dt} \right)^2 - \left( k_1A + k_3C + \frac{k_2k_4}{k_5} \right) \frac{dT}{dt} + k_1k_3A\cdot C = 0 \]

The solution of the quadratic equation for \( \frac{dT}{dt} \) leads to Equation 19

\[ \frac{dT}{dt} = \frac{1}{2} \left[ (k_1A + k_3C + \frac{k_2k_4}{k_5}) \pm \sqrt{(k_1A + k_3C + \frac{k_2k_4}{k_5})^2 - 4k_1k_3A\cdot C} \right] \]

where the negative sign is chosen by considering that the observed value for the initial rate of formation of Nuclease-T' is not greater than that for the rate of formation of prefolded intermediates B and D from A and C, respectively, that is, \( dT/dt \leq k_1A \) and \( dT/dt \leq k_3C \). As seen in Equation 19, the initial rate of formation of Nuclease-T' does not exactly follow either first order or second order kinetics by this mechanism. However, Equation 19 can be simplified if the following extreme cases are considered.

If

\[ \frac{4k_1k_3A\cdot C}{k_1^2 + k_2k_4 + \frac{k_2k_4}{k_5}} \ll 1, \]

by Maclaurin's series

\[ \left( k_1A + k_3C + \frac{k_2k_4}{k_5} \right)^2 - 4k_1k_3A\cdot C \]

\[ \frac{dT}{dt} = \frac{k_1k_3A\cdot C}{k_1A + k_3C + \frac{k_2k_4}{k_5}} \]

Then, from equation 19

\[ \frac{dT}{dt} = \frac{k_1k_3A\cdot C}{k_1A + k_3C + \frac{k_2k_4}{k_5}} \]

Case 1,

\[ \frac{k_2k_4}{k_5} \gg k_1A \quad \text{and} \quad \frac{k_2k_4}{k_5} \gg k_3C \]

and

\[ k_1A \gg 4k_3C \quad \text{and} \quad k_1A \gg \frac{k_2k_4}{k_5} \]

The latter condition is to satisfy Equation 20. Then, from Equation 21

\[ \frac{dT}{dt} = k_5k_1k_2A\cdot C \]

where

\[ K_1 = k_1A \quad \text{and} \quad K_2 = \frac{k_2k_4}{k_5} \]

Case 2,

\[ k_1A \gg 4k_3C \quad \text{and} \quad k_1A \gg \frac{k_2k_4}{k_5} \]

The coefficient 4 in the equality equation is introduced to be consistent with Equation 20. Then, from Equation 21

\[ \frac{dT}{dt} = k_3C \]

Case 1 may describe the situation where the equilibrium relationships between A and B and between C and D occur very rapidly, as compared to the collision between B and D to form Nuclease-T', that is, Step 5 is the rate-limiting process (\( k_2 \gg k_1, k_4 \gg k_3, k_5 \gg k_2, k_5 \gg k_2 \)). In this case, the initial rate of formation of Nuclease-T' should be proportional to the product of the situations would not be sufficient to contribute to the observed increase in the intensity of tryptophan fluorescence upon mixing the two solutions.
initial concentrations of both Nuclease-T-(6–48) and Nuclease-
T-(49–149). The experimental results rule out this mechanism.

Case 2 can occur under conditions in which Step 3 (the forma-
tion of the native conformation of one of the two disordered
fragments) is the rate-limiting process instead of Step 5 ($k_8 \gg k_9$). In this case, the initial rate of formation of Nuclease-
T' will be limited by the initial concentration of only one fragment
($C$) which shows a slow rate of conformational transition. There-
fore, the formation of Nuclease-T' could follow quasi-first order
kinetics. However, the inequality expressed in Equation 23 is
not valid if the value for $A$ is very small. If both $A$ and $C$ are at
very low concentrations, the relationship of Equation 22 will be
established instead, even though Step 3 remains the rate-limiting
step. Consequently, the initial rate of formation of Nuclease-T'
would depend on the initial concentrations of both fragments.
Unfortunately, because of technical difficulties our studies could
not reach extremely low concentrations of fragments and we were
unable to test this possibility.

If prefolding (nucleation) of one fragment is the rate-limiting
step in the formation of Nuclease-T' and a similar nucleation is
also the rate-limiting step in the refolding of acid-denatured
nuclease (2, 3), one has to assume that the segment involved in
the nucleation of the fragment is not the same as that with acid-
denatured nuclease in order to explain the large difference in the
rate of folding.\(^{\text{a}}\)

\(^{\text{a}}\) Sachs et al. have estimated the value for the equilibrium
constant of Equation 6 ($k_A/k_B$ referred to as $K_{\text{eq}}$) from the ob-
served value for the binding constant between Nuclease-T-(50–
149) and anti nuclease antibody (26). It is assumed that the
binding constant between the fragment of “native conformation”
and the antibody is the same as that between intact nuclease
and the antibody (26). If the estimated value (2.0 \times 10^{-10}) for $K_{\text{eq}}$
(26) is used as the value for both $k_A/k_B$ and $k_B/k_A$ and the value
for the over-all association constant ($K$) of the two fragments is
given as $10^6$ M\(^{-1}\) (see the text), one can attempt to calculate the
value for the second order rate constant, $k_9$, as follows. In the
equilibrium state of the association and dissociation reaction of
the two fragments expressed by Equations 5, 6, and 7,

$$
\frac{dA}{dt} = \frac{dC}{dt} = \frac{dT}{dt} = 0
$$

Then, from Equations 8, 9, and 12

$$
k_2 \cdot \frac{k_4}{k_3} A + \frac{k_9}{k_8} C = k_6 T
$$

Accordingly,

$$
\frac{k_4}{k_2} \cdot \frac{k_3}{k_4} \cdot \frac{k_9}{k_8} = \frac{T}{A \cdot C} = K
$$

If the value for the dissociation rate constant, $k_6$, at $25^\circ$ is
$1.0 \times 10^{-8}$ s\(^{-1}\) (15), one obtains

$$
k_4 = 2.5 \times 10^{-6}$ M\(^{-1}\) s\(^{-1}\)

This value is equivalent to the fastest bimolecular ionic reactions
involving protons (24). However, it is unlikely for a bimolecular
interaction involving the three-dimensional arrangement of
polypeptide chains to have such an extremely large rate constant.
Therefore, the following possibilities exist if the mechanism of
complementation is valid. First, the association and dissociation
reaction expressed by Equation 7 involves more than one step
($k_1 = k_1' + k_1'',\ldots$, $k_4 = k_4' + k_4'',\ldots$) Therefore, the observed
value for the dissociation rate constant is the value only for the
rate-limiting step among these sequential steps and not equal to
the true value for $k_6$. Second, the value for $K_{\text{eq}}$ ($k_4/k_3$) con-
cerning Nuclease-T-(6–48), which has not been estimated, is larger
than $2.0 \times 10^{-4}$.

Another possible model of the folding process is one consistent
with the statistical mode of protein folding proposed previously
(15) where Nuclease-T-(6–48) and Nuclease-T-(49–149) may
interact to form an intermediate complex as shown in Equations
24 and 25.

$$
A + C \overset{k_7}{\underset{k_8}{\rightleftharpoons}} \begin{array}{c} \text{[A-C]} \end{array}
$$

(24)

$$
[A-C] \overset{k_9}{\underset{k_10}{\rightarrow}} T
$$

(25)

The fragments are assumed to be generally disordered structures
(8, 9) at the time they collide and interact. The presumed inter-
mediate is represented as $[A-C]$ in the equations and its forma-
tion and breakdown are shown as reversible reactions. It is
assumed that the state of equilibrium, described by Equation 24, is
reached quickly after the fragments are mixed, implying that both
association and dissociation are rapid reactions. As a complex, many
spatial orientations of the polypeptide chains should be possible
in $[A-C]$ and rapid changes of the conformation of $[A-C]$ will
occur statistically, but in a finite number of ways until the com-
plex reaches the structure of Nuclease-T (15). During the forma-
tion of the ordered structure of the complex, some mole-
cules of the complex may also dissociate to the fragments $A$ and
$C$. This mechanism may be related to the refolding of acid-
denatured nuclease when the single polypeptide chain should refold
faster than the complementation of fragments to form Nuclease-
T'. The disordered nuclease molecules would change statistically
from one conformation to another within the ensemble of
conformations occurring under physiological conditions until the
final structure is reached (15), with the process proceeding with-
out interruptions since dissociation to two interacting species is
not possible with intact nuclease.

In this mechanism the concentration of Complex $[A-C]$ is
dependent on both $A$ and $C$, which should be seen experimentally.
However, the kinetic behavior depends on the value of the equi-
librium constant of Equation 24 and the initial concentrations of
the fragments. For example, if the association constant of
Equation 24 is equivalent to or larger than $10^6$ M\(^{-1}\) and the initial
concentrations of Nuclease-T-(6–48) and Nuclease-T-(49–149)
are $10^{-5}$ M or greater (the level of those in the present experi-
ments), the population of the disordered fragments would exist
mainly in the form of the intermediate complex before conversion
to Nuclease-T' would occur. Under such conditions, the con-
centration of the intermediate complex would then become the
rate-limiting factor, as shown below.

The rate equations appropriate for this mechanism (Equations
24 and 25) may be expressed on the basis of the treatment of
Adrovich (27), assuming the value for $k_6 T$ to be negligible for
the initial phase of the complementation.

\[
\frac{dx}{dt} = -k_7 x(x+m) + k_8 y
\]

(26)

\[
\frac{dy}{dt} = k_7 x(x+m) - (k_8 + k_9) y
\]

(27)

\[
\frac{dT}{dt} = k_9 y
\]

(28)

where $x = A$, $x + m = C$, and $y = [A-C]$. Since it is assumed
that the equilibrium state of Equation 24 is rapidly established,

\[ \frac{dx}{dt} = 0 \]

then,

\[ y = \frac{k_7}{k_g} x(x+m) \]  

(29)

From Equations 27 and 29

\[ \frac{dy}{dt} = -k_y y \]  

(30)

Then,

\[ y = y_0 e^{-k_y t} \]  

(31)

where \( y_0 \) is the value for \( y \) at \( t = 0 \).

From Equations 28 and 31

\[ \frac{dT}{dt} = -k_y y_0 e^{-k_y t} \]  

(32)

From Equation 29,

\[ y_0 = \frac{k_7}{k_g} x_0(x+m) \]  

(33)

where \( x_0 \) and \( x_0 + m_0 \) are the concentrations of unbound Nuclease-T-(6-48) and Nuclease-T-(49-149), respectively, at zero time.\(^6\)

Let

\[ x_0 + y_0 = P_2 \]  

(34)

\[ x_0 + y_0 + m = P_3 \]  

(35)

\[ \frac{k_8}{k_7} = K_3 \]  

(36)

where \( P_2 \) and \( P_3 \) correspond to the concentrations of Nuclease-(6-48) and Nuclease-(49-149), respectively, before any reaction between the two fragments occurs. From Equations 33 to 36 one obtains

\[ y_0 = \frac{P_2 + P_3}{2} \]  

since

\[ \frac{P_2 + P_3}{2} = \frac{P_2 + P_3 + K_3}{2} - \frac{(P_2 + P_3 + K_3)^2 - 4P_2P_3}{2} \]  

(37)

As shown in Table VIII, when the value for \( K_3 \) is \( 10^{-6} \) M, a 3-fold increase in the initial concentration of Nuclease-T-(49-149) (\( P_3 \)), with the initial concentration of Nuclease-T-(6-48) (\( P_2 \)) kept constant, would result in only a 1.3-fold increase in the concentration of Complex [A-C], on which the initial rate of formation of Nuclease-T' depends (Equation 32).\(^7\) On the other hand, if the value for \( K_3 \) is larger than \( 10^{-6} \) M, the initial rate of formation of Nuclease-T' would respond to the increase in the initial concentration of Nuclease-T-(49-149) in a manner approaching a second order reaction (Table VIII).

In summary, the kinetic studies reported above consistently followed apparent first order kinetics with each complementing system. It is clear, however, that further studies are required before one of these suggested mechanisms of fragment complementation may be proved.

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\(^6\) A possibility for the existence of a weak intermolecular interaction between the same fragments is ignored.

\(^7\) In the equilibrium state of the reaction expressed by Equations 24 and 25,

\[ K = \frac{k_9k_{90}}{k_8k_9} = \frac{1}{K_1} \cdot \frac{k_9}{k_9} \]

where \( K \) is the over-all association constant of Nuclease-T-(6-48) and Nuclease-(49-149) (Equation 1). If \( k_9 = 0.043 \text{ s}^{-1} \) at 25° (see above), \( k_9 = 1.0 \times 10^{-2} \text{ s}^{-1} \) at 25° (15), and \( K_1 = 10^{-4} \text{ M} \) at 25°, the value for \( K \) will be \( 4.3 \times 10^{-6} \text{ M}^{-1} \). This value is greater than any of those experimentally estimated. The latter are \( 6.3 \times 10^{-6} \text{ M}^{-1} \) in the present report, \( 4.0 \times 10^{-4} \text{ M}^{-1} \) or greater in the previous report (15). The discrepancy between these observed values must be clarified before it is possible to understand the significance of the difference between the theoretical value and those observed.
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