Kinetics of Folding of Staphylococcal Nuclease

Abstract. *Staphylococcal nuclease undergoes a reversible structural transition between pH 3 and 4 which can be measured by changes in tryptophan fluorescence. A stopped-flow spectrofluorometer was used to study the kinetics of renaturation of nuclease from the acidified form on neutralization. The refolding is fast, and the data can be described as a sequence of two first-order processes, with half times of about 55 and 350 milliseconds, respectively.*

The folding of polypeptide chains is an intermediate step in making available the information of the genetic code for the biological function of the corresponding protein. Although many studies have been made of the kinetics of denaturation (*1*), few direct measurements exist of the folding of proteins from a disordered state to their native conformation. We present here measurements by stopped-flow spectrofluorometry of the renaturation of staphylococcal nuclease from acid solution.

Nuclease is a single polypeptide chain of 149 amino acids (molecular weight 16,800 daltons) of known sequence (*2*) which has been partially synthesized (*3*). There are no cysteine or cystine residues. There is a single tryptophan residue (position 140), located in the 2-Å Fourier map just on the COOH-terminal side of the last length of α-helix and largely shielded from water (*4*); the fluorescence of the tryptophan is sensitive to conformational changes in the protein (*5*). We have used the changes in the quantum yield of tryptophan fluorescence as an index of the renaturation of nuclease when the *p*H is changed from 3.2 to 6.7.

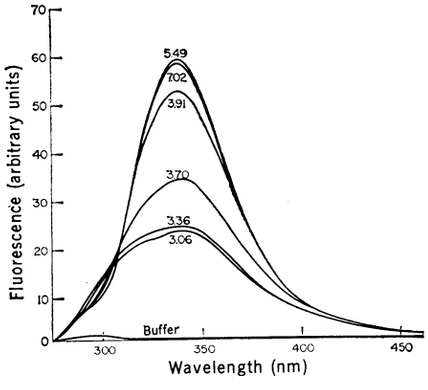

Fig. 1. Fluorescence emission spectra of staphylococcal nuclease at several *p*H values from 7.02 to 3.06 at 26°C. Protein ($8 \times 10^{-6}M$) in 0.01*M* sodium phosphate and 0.08*M* sodium chloride was adjusted to the specified *p*H values with 1*N* HCl, without significant change in volume. Excitation light, at 285 nm, was passed through a horizontal polarizing filter to minimize light scatter (*15*); emission was measured through a monochromator grating cut for maximum diffraction efficiency at 300 nm. The detector response curve of this instrument is linear between 300 and 400 nm, therefore these are true emission spectra (*15*).

The stopped-flow instrument has been described (*6*). With compressed air (2.72 atm) as the driving force on the syringes containing the reagents, we obtained a mixing time of 10 to 20 msec. The mixing chamber and the observation tube of quartz glass were similar to that described except that a four-jet ball mixer was employed instead of a "T" type mixer (*7*).

The observation tube was placed in an Aminco-Bowman spectrophotofluorometer fitted with an emission grating cut for maximum diffraction efficiency at 750 nm, which had good transmission in the second order. The R136 phototube was operated with a 900-volt battery pack, and the anode current was amplified and displayed on a variable persistence oscilloscope. An electronic time constant of 10 msec was employed. The oscilloscope tracings were photographed on Polaroid film and read on a Nikon profile projector at tenfold magnification.

The fluorescence emission spectra of staphylococcal nuclease (*8*), with excitation at 285 nm, at several values of *p*H are shown in Fig. 1. The major emission, centered between 330 and 350 nm, results from the tryptophan excited state; there is some tyrosine emission near 300 nm. There is little change in the tryptophan emission spectrum between *p*H 7 and 4, but between *p*H 4 and 3 there is a marked decrease in the amplitude of the fluorescence and a slight shift in the emission maximum toward longer wavelength. When fluorescence emission at 335 nm was measured as a function of *p*H by detailed titrations, the acid-induced spectral transition was fully reversible. The abruptness of the transition suggested that the protonation of one or several groups with *p*K's of about 3.8 caused the transition.

The intrinsic viscosity of the nuclease at *p*H 3 was about threefold greater than that of the native nuclease (*9*). This indicates that the spectral transition is accompanied by a general disruption of the native compact, globular structure. Studies by difference spectroscopy, optical rotation, and circular dichroism also denoted generalized conformational changes during the acid transition (*9*). A nuclear magnetic resonance study of the four histidine residues of nuclease has shown that their chemical shifts become equivalent to that of exposed histidine at *p*H 3.4 (*10*).

Figure 2A shows fluorescence emission during the mixing of nuclease at *p*H 3.2 with an acid buffer, with no significant change in *p*H. The dead time in this experiment is 25 to 30 msec.

The oscilloscope tracing of the renaturation from acid solution of nuclease is shown in Fig. 2B. The acidified nuclease was mixed with an equal volume of phosphate buffer to yield a final *p*H of 6.70. The initial, rapid rise in fluorescence intensity, as the neutralized protein filled the cuvette, is followed by a slower increase in fluorescence as the nuclease molecules change their conformation to that which is stable at neutral *p*H. This change is clearly measurable over the first 400 msec of the reaction. The value of the amplitude of fluorescence after a minute of reaction is the upper part of the heavy white line, at about four

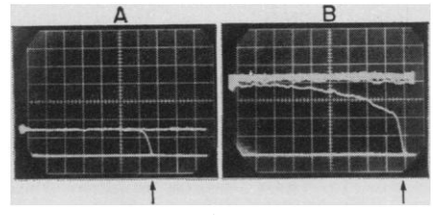

Fig. 2. (A) Photograph of the oscilloscope tracing of the mixing of nuclease (initial concentration, $1.2 \times 10^{-4}M$) in 0.002*M* citric acid and 0.09*M* sodium chloride (to keep ionic strength constant), *p*H 3.20, with an equal volume of the same buffer at 26°C. Time, increasing from right to left, is measured in 50 msec per division along the abscissa; fluorescence intensity (ordinate) at 335 nm is monitored with the second order transmission of the grating. At the arrow the mixed nuclease solution began to fill the observation cuvette and the fluorescence of acidified nuclease was registered. (B) Photograph of oscilloscope tracing of mixing experiment identical to the previous one except that the buffer syringe contained 0.05*M* sodium phosphate and 0.01*M* sodium chloride, *p*H 6.90. At the arrow there was a rapid rise in fluorescence as the mixed nuclease solution at *p*H 6.70 filled the cuvette; the following gradual increase in fluorescence is a measure of the renaturation of the protein.

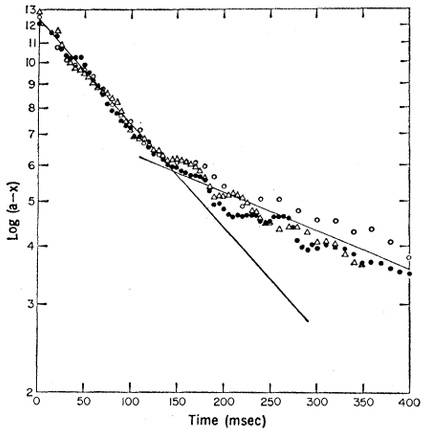

Fig. 3. First-order kinetic plot of the renaturation of acidified nuclease from *p*H 3.20 to *p*H 6.70. The value of log $(a - x)$ is plotted on the ordinate where a is the total increase in fluorescence from the beginning to the observed end of the reaction and x is the increase in fluorescence at any time.

and a half divisions on the ordinate above the base line.

The kinetics of this reaction have been analyzed by first-order plots, with the fluorescence of the acidified nuclease as the value for zero time and the final recorded value as that for infinite time. The first-order plots of three experiments at a final concentration of protein of $6 \times 10^{-5} M$ fit one line for about the first 150 msec of the reaction (Fig. 3). Subsequently, when the spectral change is more than 75 percent completed, they follow a different line. After we subtract the contribution of the second slope to the first, these lines are equivalent to processes with first-order rate constants (k_1) of 12.1 and 1.9 sec^{-1}, respectively.

This reaction has been studied at protein concentrations from 1.5 to $6.0 \times 10^{-5} M$. There was no significant change in the initial first-order rate constant.

The folding of nuclease from the extensively disrupted acidified state to the native form apparently can occur very rapidly. The kinetic analysis, by the simplest although not the only possible method, is compatible with an initial, very rapid folding, with a half time of about 55 msec (k_1 of 12.1 sec^{-1}), which is accompanied by a large change in tryptophan emission. This is followed by at least one slower conformational change, with a half time of about 350 msec (k_1 of 1.9 sec^{-1}), which causes further change in the environment of the tryptophan residue.

Most estimates of renaturation rates have been established only indirectly from the assumption of a two-state transition whose apparent equilibrium constant is the quotient of the renaturation and denaturation rate constants (*11*). However, Hauschka and Harrington (*12*) have recently directly measured the renaturation of *Ascaris* collagen, at *p*H 6.25, by optical rotation and have interpreted their data at 25°C in terms of three first-order processes, with half times of about 0.20, 2.5, and 11 minutes. Pohl (*13*) has used temperature-jump relaxation methods in the study of the reversible denaturation of chymotrypsin and trypsin. The half time values near 25°C of the renaturation of these enzymes are between about 7 and 70 seconds. The renaturation rates of staphylococcal nuclease appear significantly faster than the rates of conformational change in these proteins and faster than those in the several proteins whose renaturation rates have been estimated indirectly (*1*). The need for more than one first-order rate constant to fit our data indicates the existence of kinetic intermediates in the conformational changes of nuclease.

The rate of renaturation of nuclease is slower by several orders of magnitude than those established by temperature-jump methods for the conformational isomerizations of enzymes during their catalytic function, and many orders slower than those values established for the helix-coil transformation (*14*). These results for the renaturation of nuclease give an approximate idea of the time required for this polypeptide chain of 149 amino acids to assume a large enough subset of all possible backbone and side-chain dihedral angles so that the presumably statistical process of folding reaches the thermodynamically stable, native conformation.

The measurement of the renaturation kinetics of several spectral and hydrodynamic properties of nuclease, in addition to tryptophan fluorescence, is necessary for an understanding of the sequence of processes in folding. The correlation of these results with the structural information in the 2-Å model of nuclease offers an approach to an understanding of the mechanism of folding of this protein.

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References and Notes

1. For reviews see J. Steinhardt and E. M. Zaiser, *Advan. Protein Chem.* **10**, 152 (1955); M. Joly, *A Physico-Chemical Approach to the Denaturation of Proteins* (Academic Press, New York, 1965), pp. 191–260; C. Tanford, *Advan. Protein Chem.* **23**, 122 (1968).
2. H. Taniuchi, C. B. Anfinsen, A. Sodja, *J. Biol. Chem.* **242**, 4752 (1967).
3. C. B. Anfinsen, *Pure Appl. Chem.* **17**, 461 (1968); D. A. Ontjes and C. B. Anfinsen, *Proc. Nat. Acad. Sci. U.S.*, in press.
4. A. Arnone, C. J. Bier, F. A. Cotton, E. E. Hazen, D. C. Richardson, J. S. Richardson, *ibid.*, in press, and personal communication.
5. P. Cuatrecasas, H. Taniuchi, C. B. Anfinsen, *Brookhaven Symp. Biol.* **21**, 172 (1968); H. Edelhoch, R. L. Perlman, M. Wilchek, *Ann. N.Y. Acad. Sci.* **158**, 391 (1969); H. Taniuchi and C. B. Anfinsen, *J. Biol. Chem.* **244**, 3964 (1969). For a review of the physical basis of such fluorescence changes in proteins see R. F. Chen, H. Edelhoch, R. F. Steiner, in *Physical Principles and Techniques of Protein Chemistry*, S. J. Leach, Ed. (Academic Press, New York, 1969), p. 171.
6. R. Harvey, *Anal. Biochem.* **29**, 58 (1969); R. F. Chen, A. N. Schechter, R. L. Berger, *ibid.*, p. 68.
7. R. L. Berger, B. Balko, H. F. Chapman, *Rev. Sci. Instrum.* **39**, 493 (1968).
8. Highly purified nuclease was prepared from the growth medium of *Staphylococcus aureus* (Foggi strain) as described by S. Fuchs, P. Cuatrecasas, C. B. Anfinsen [*J. Biol. Chem.* **242**, 4768 (1967)] and L. Morávek, C. B. Anfinsen, J. L. Cone, H. Taniuchi [*ibid.* **244**, 497 (1969)].
9. D. C. Richardson, A. N. Schechter, C. B. Anfinsen, unpublished data. The value of the intrinsic viscosity of the acidified nuclease is very sensitive to changes in ionic strength.
10. J. S. Cohen, personal communication.
11. See for example, C. Tanford, R. H. Pain, N. S. Otchin, *J. Mol. Biol.* **15**, 489 (1966).
12. P. Hauschka, thesis, Johns Hopkins University (1969).
13. F. M. Pohl, *Eur. J. Biochem.* **4**, 373 (1968); *ibid.* **7**, 146 (1969).
14. M. Eigen and G. Hammes, *Advan. Enzymol.* **25**, 1 (1963); G. Hammes, *Advan. Protein Chem.* **23**, 1 (1968); R. Lumry, R. Legare, W. G. Miller, *Biopolymers* **2**, 489 (1964); E. Hamori and H. A. Scheraga, *J. Phys. Chem.* **71**, 4147 (1967).
15. R. F. Chen, *Anal. Biochem.* **14**, 497 (1966); *ibid.* **20**, 339 (1967).

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Substrate Stabilization: Genetically Controlled Reciprocal Relationship of Two Human Enzymes

Abstract. *5-Phosphoribosyl-1-pyrophosphate, a substrate shared by adenine phosphoribosyltransferase and hypoxanthine-guanine phosphoribosyltransferase, accumulates in human erythrocytes lacking hypoxanthine-guanine phosphoribosyltransferase. 5-Phosphoribosyl-1-pyrophosphate added to purified adenine phosphoribosyltransferase stabilizes it against heat inactivation. The increased activity of adenine phosphoribosyltransferase seen in erythrocytes deficient in hypoxanthine-guanine phosphoribosyltransferase may result from substrate stabilization of this enzyme in vivo.*

The regulation of enzyme activity in mammalian cells is often discussed in terms of the mechanisms of induction,