Structure of the Antibody Combining Site. I. Hapten Stabilization of Antibody Conformation*

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ABSTRACT: The effect of ε-DNP-lysine on the tertiary structure of anti-2,4-dinitrophenyl antibodies and Fab fragments in solutions of 4 M guanidine-HCl was studied using far-ultraviolet optical rotatory dispersion. At pH 7.4 and 27° in 4 M guanidine-HCl, the optical rotary dispersion (ORD) curve of the ε-DNP-lysine–antibody complex was more levorotatory than that of the complex in buffer but significantly less levorotatory than the curve for antibody in 4 M guanidine-HCl. With the same conditions, Fab exhibited the same behavior except that the ORD curve of the ε-DNP-lysine–Fab complex in 4 M guanidine-HCl was almost the same as that shown by the complex in buffer. K_0, the average association constant, was unchanged in solutions of 4 M guanidine.

The binding of hapten to antibody, therefore, stabilizes the tertiary structure, particularly the Fab portion, in such a way as to cause a reduction in the number of possible unfolded conformations. This has been interpreted to mean that the amino acid residues involved in the binding site are located in at least several widely separated areas of the Fab portion which are brought into a close spatial relationship by the molecular conformation.

The question of how much of the immunoglobulin molecule is necessary for the function of antigen binding is of fundamental interest. Is the binding region composed of amino acid residues which are neighbors along the two chains comprising the site, or of residues which are only brought into a close spatial relationship by means of the secondary and tertiary folding?

It is now well established that both the heavy and light chains are in some way involved in the binding of hapten to antibody, although the function of each has not been clarified (Singer and Doolittle, 1966; Haber and Richards, 1966). The carboxy-terminal-half of the heavy chains (Fc)[1] can, by enzymic degradation, be shown to be unnecessary for antigen binding (Porter, 1959).

The classical studies of Landsteiner (1945) and later the work of Kabat (1960) have shown that probably only a small portion of the remaining antibody molecule comes into direct contact with antigen in the binding site. Karush (1962) has estimated that approximately 1% of the total number of amino acid residues is so involved. By analogy with enzyme systems (Koshland, 1959; Lumry, 1959), it is probable that the remainder of Fab is necessary for the maintenance of the proper conformation of the active site, but this has not yet been demonstrated for antibodies.

The residues making up the binding and catalytic sites of enzymes are generally separated from one another along the length of the polypeptide chains (Crestfield *et al.*, 1963; Walsh *et al.*, 1964). The unique conformation of these chains making up the active species brings these residues in close proximity (Blake *et al.*, 1965). It is not surprising, then, that the presence of the ligand stabilizes this conformation. Indeed, this has been experimentally demonstrated for several enzymes in urea and guanidine solutions (Sela *et al.*, 1957; Inagaki, 1959; Nelson *et al.*, 1962). On the other hand, if stabilization does not occur, then it is reasonable to assume that only a local area of the antibody molecule is involved in the binding site.

We present here the results of an investigation which explored these possibilities. The effect of the hapten ε-DNP-lysine on the tertiary structure of anti-DNP and its Fab fragment in solutions of guanidine-HCl was studied using far-ultraviolet optical rotatory dispersion as a probe. Although a detailed interpretation of optical rotary dispersion (ORD) data in terms of the structure of a protein, especially a predominantly nonhelical one such as an immunoglobulin, cannot be made at present, such data can be useful in detecting changes in the secondary structure (Schellman and Schellman, 1964; Cathou *et al.*, 1965; Steiner and Lowey, 1966).

Our results indicate that hapten significantly stabilizes the antibody molecule, particularly the Fab portion. Therefore, it is probable that the binding site is composed primarily of residues which are not vicinal on

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[1] Abbreviations used: Anti-DNP, antibodies directed against the DNP group; DNP-BGG, dinitrophenylated bovine γ-globulin; FDNB, fluorodinitrobenzene. The nomenclature used for immunoglobulins and the papain fragments Fab, Fc, and Fd are those adopted by the World Health Organization and summarized in *Bull. World Health Organ. 30*, 447 (1964).

any chain, but are held in a close contact by the conformation of the molecule.

Materials and Methods

Materials. Dinitrophenylated bovine γ-globulin (DNP-BGG) was prepared according to Eisen (1964). ε-DNP-lysine was obtained from Mann Research Laboratories. Tritiated ε-DNP-lysine was prepared by a micromodification of the method of Porter and Sanger (1948). [^{3}H]FDNB (10.4 c/mmole), obtained from Nuclear-Chicago, was the radioactive reagent. The final product was purified by electrophoresis at pH 1.9. *p*-Nitrophenol and 2,4-dinitrophenol were obtained from Distillation Products Industries. Guanidine-HCl was prepared by the method of Anson (1941) and dissolved in 0.01 M potassium phosphate–0.15 M NaCl buffer, pH 7.4 (phosphate-NaCl buffer). Papain was obtained from Worthington Biochemical Corp.

Preparation and Purification of Antibody. Anti-DNP antibodies were prepared by the method of Farah *et al.* (1960) as modified by Cheng and Talmage (1965). Rabbits were immunized in the footpads with a total of 10 mg of DNP-BGG in complete Freunds' Adjuvant. After 2 weeks the rabbits were given intravenous booster doses of 5 mg of alum-precipitated DNP-BGG (20 mg/ml) suspended in 0.15 M NaCl; similar booster doses were given weekly thereafter. The rabbits were bled each week beginning with the third week and blood from several rabbits was pooled weekly. Antibodies for the experiments described in this paper were prepared from bleedings of about 8 weeks and later. The solvent for purified antibody and for all experiments was phosphate-NaCl buffer. Antibodies so prepared contained negligible DNP and exhibited binding constants, K_0, with ε-DNP-lysine at 23° and at pH 7.4 of greater than 10^8 l./mole.

Papain Fragments. Fab fragments were obtained by papain digestion of anti-DNP according to the method of Porter (1959) as modified by Nisonoff (1964).

Concentration Determinations. Concentrations of antibody and papain fragments were determined spectrophotometrically at 280 mμ, using the following extinction coefficients for 1% solutions and a path length of 1 cm: anti-DNP, 16.5 and Fab, 18.1 (Steiner and Lowey, 1966). These values were checked by means of the micro-Kjeldahl procedure for nitrogen analysis, and amino acid analyses, and found to be the same for our preparations. Concentrations of haptens were also determined spectrophotometrically, using the following molar extinction coefficients: ε-DNP-lysine, ϵ_{363} 17,530 (Eisen and Siskind, 1964) and *p*-nitrophenol, ϵ_{360} 7760.

Optical Rotation. Measurements in the 280–210-mμ range were made using a Cary 60 recording spectropolarimeter at 27° in a 1-mm cell. Samples contained either approximately 6×10^{-6} M antibody or 1×10^{-5} M Fab. Rotations are expressed in terms of the molar rotation [φ] which is defined (Cathou *et al.*, 1965) as

$$[\phi] = \frac{10^2\alpha}{lc} \quad (1)$$

where α is the observed rotation in degrees, l is the path length in centimeters, and c is the concentration of optically active solute in moles per liter. The molecular weight of antibody was taken as 150,000 and that of Fab as 50,000 (Noelken *et al.*, 1965). No corrections have been made for the refractive index of the solvents. Unfortunately, information on the dispersion of the refractive index of guanidine solutions is currently unavailable. However, rigorous comparisons can be made with uncorrected data in the same solvent. Although comparison of such data obtained in different solvents may introduce error, we feel that our general conclusions are valid for the following reasons. (1) The refractive index of 4 M guanidine and water at 589 mμ differs by less than 5% (Kielley and Harrington, 1960). (2) The dispersion of other solvents such as urea behaves in a similar manner to that of water (Fasman, 1963). (3) The experimental error introduced in measuring ORD is of the order of 3%.

Concentrated antibody–hapten complexes were prepared by mixing a 6 molar hapten excess with antibody. These solutions were then diluted with either phosphate–NaCl buffer or guanidine-HCl to give 1.5 ml of solution with an antibody concentration of approximately 6×10^{-6} M and a guanidine-HCl concentration of 4 M. These solutions were then dialyzed *vs.* 10 ml of buffer or 4 M guanidine-HCl, respectively, containing the same concentration of hapten, at 22° for 6 hr, a time previously determined to be sufficient for equilibration. A solution of antibody in 4 M guanidine-HCl was similarly dialyzed *vs.* 4 M guanidine-HCl in the absence of hapten. After 6 hr the contents of the dialysis bags were removed and the concentration of antibody was determined spectrophotometrically at 280 mμ using the outer dialysis solution as a blank; the absorbancy at 280 mμ of bound ε-DNP-lysine in the antibody–hapten complex was subtracted from the total absorbancy to obtain the absorbancy due to antibody. The optical rotation of the solution was then measured using the outer dialysis solution as a blank. Solutions containing Fab were prepared in the same way.

Measurement of Binding Ratio of ε-DNP-lysine and Antibody by Equilibrium Dialysis. The molar ratio of ε-DNP-lysine bound to antibody in hapten excess as a function of guanidine-HCl concentration was determined essentially by the same method as that used for the preparation of samples for measurement of optical rotation.

Equilibrium dialyses were carried out in duplicate on solutions containing approximately 1×10^{-6} M antibody and 6×10^{-6} M ε-DNP-lysine-^{3}H (specific activity of about 2 c/mole) at guanidine concentrations varying from 0 to 7.5 M for 6 hr at 22°. Antibody concentrations were determined as before. Duplicate 0.5-ml aliquots of both inside and outside solutions

were counted in Bray's solution containing (Cab-o-sil), a thixotropic agent on an ANS, Inc., scintillation counter, Model 1300. Control solutions containing only ε-DNP-lysine-^{3}H showed deviations of counts between inside and outside solutions of no more than 1–2%. The concentrations of free and bound hapten were calculated from the radioactivity of the solutions employing the specific activity of hapten determined at each concentration of guanidine-HCl.

Fluorescence Measurements. Binding constants of antibody and *p*-nitrophenol were determined by the method of fluorescence quenching. Fluorescence was measured on an Aminco-Bowman spectrofluorometer using an Osram XBO 150 W/1 Xenon lamp as light source. Constant temperature of the sample was maintained with a specially built cell holder and a circulating water bath. An activation wavelength of 282 mμ and a fluorescence wavelength of 345 mμ were employed. The solvent for all solutions was phosphate-NaCl buffer. The maximum quenching of fluorescence, Q_{max}, experimentally determined for the antibody ε-DNP-lysine system, was used to analyze the results obtained for antibody and *p*-nitrophenol. The results were analyzed essentially as described by Eisen and Siskind (1964) to obtain K_0, the average association constant, employing a PDP-1 computor.

Results

The number of moles of hapten bound per mole of antibody in hapten excess at various guanidine-HCl concentrations is given in Table I. Complete saturation of binding sites occurs up to guanidine-HCl concentrations of 4 M. In 5 M guanidine-HCl there is a 25% loss of binding sites. In 6 M guanidine-HCl only a small residual amount of bound hapten remains, while in 7.5 M guanidine-HCl, hapten is no longer bound. Guanidine-HCl (4 M) was chosen as the concentration for study, since full binding capacity was retained. Guanidine (4 M) is sufficient to denature ribonuclease (Haber and Anfinsen, 1962).

As a further check on the effect of 4 M guanidine-HCl on the binding of hapten to antibody, the binding constant, K_0, was determined. Since this is a high-affinity antibody, determination of precise values for K_0 by means of equilibrium dialysis is difficult; however, in both 4 M guanidine-HCl and phosphate-NaCl buffer, K_0 was found to be of the order of 10^8. A preferable method, which is more sensitive, is that of fluorescence quenching (Velick *et al.*, 1960). Unfortunately, titration of this high affinity antibody with the most tightly bound hapten, ε-DNP-lysine, gives results which cannot be analyzed by the usual methods. In the range of protein concentrations in which the method of fluorescence quenching can be used free hapten concentration is very low. Binding constants, can, however, be easily determined for analogous but more weakly bound haptens, such as *p*-nitrophenol. Determination of K_0 for both antibody and its Fab fragment give identical values within experimental error in the absence and presence of 4 M guanidine-HCl. The results are shown in Table II.

TABLE I: Binding of ε-DNP-lysine by Anti-DNP at 20°, pH 7.4.[a]

Guanidine Concn (M)	Moles of ε-DNP-lysine Bound/Mole of Anti-DNP
0	1.95
3.0	1.96
4.0	2.00
5.0	1.49
6.0	0.30
7.5	0.05

[a] Experimental details are given in the text. Representative results and calculations for a solution containing 4 M guanidine-HCl are: specific activity of ε-DNP-lysine-^{3}H = 5.49×10^{11} cpm/mole; anti-DNP concentration = 0.68×10^{-6} M, radioactivity of inner solution = 1615 cpm, radioactivity of outer solution = 1261 cpm, h_b, concentration of bound hapten = 1.28×10^{-6} M, $(h_b)/(Ab) = 1.89$. The standard deviation of the counting rate was less than 1%.

TABLE II: Binding Constant, K_0, of Anti-DNP and Fab with *p*-Nitrophenol at 23°, pH 7.4.[a]

Species	Guanidine Concn (M)	K_0 (l./mole $\times 10^{-5}$)
Anti-DNP	0	1.90
	4	1.37
Fab	0	2.81
	4	3.51

[a] Experimental details are given in the text.

The optical rotatory dispersion of the antibody–ε-DNP-lysine complex in the range of 220–260 mμ is given by curve 1 of Figure 1. Identical results were obtained for antibody alone. In agreement with the results obtained by Steiner and Lowey (1966), the curve has a pronounced negative trough at 224 mμ, a dip at 230 mμ, and a positive shoulder at about 237 mμ. As the depth of the trough at 224 mμ varied somewhat from preparation to preparation an average curve would be meaningless, and, therefore, results of a representative sample are shown. However, the results in any individual experiment were internally consistent. The standard deviation of the difference in rotations at 224 mμ of the antibody–hapten complex and antibody in 4 M guanidine-HCl was 9%, while that of the antibody–hapten complex in buffer and of antibody in 4 M guanidine-HCl was 13%. The optical

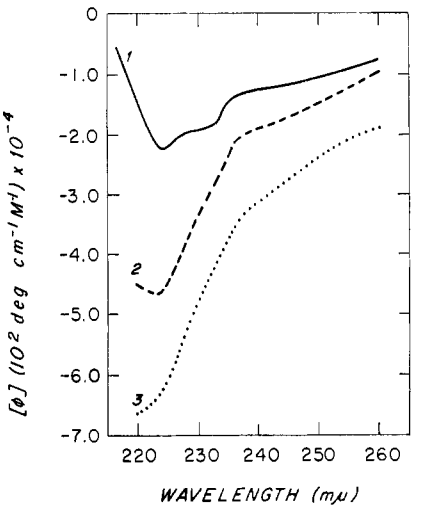

FIGURE 1: Ultraviolet rotatory dispersion of anti-DNP and the anti-DNP-ε-DNP-lysine complex at pH 7.4. Curve 1 (———): anti-DNP-ε-DNP-lysine in phosphate–NaCl buffer; curve 2 (— — —): anti-DNP-ε-DNP-lysine in 4 M guanidine-HCl; and curve 3 (· · · · ·): anti-DNP in 4 M guanidine-HCl, no hapten present.

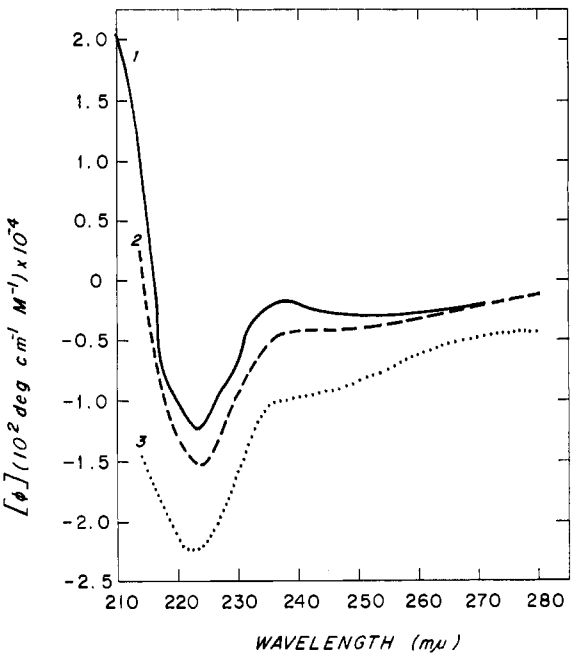

FIGURE 2: Ultraviolet rotatory dispersion of Fab and the Fab-ε-DNP-lysine complex at pH 7.4. Curve 1 (———): Fab-ε-DNP-lysine in phosphate–NaCl buffer; curve 2 (— — —): Fab-ε-DNP-lysine in 4 M guanidine-HCl; and curve 3 (· · · · · · ·): Fab in 4 M guanidine-HCl, no hapten present.

rotatory dispersion of the antibody-ε-DNP-lysine complex in 4 M guanidine-HCl is given by curve 2 of Figure 1. The values of the rotation are more negative than those observed for the antibody–hapten complex in buffer alone. The negative trough is still centered at 224 mμ and there is still a positive shoulder at 237 mμ but the dip at 230 mμ is no longer evident. The optical rotation of antibody in the absence of any hapten in 4 M guanidine-HCl is shown in curve 3 of Figure 1. A negative trough at 224 mμ is no longer seen, and the shoulder at 237 mμ is minimized. The entire curve has been shifted to even more negative values than those observed for the antibody–hapten complex in 4 M guanidine-HCl. ε-DNP-lysine at a comparable concentration as that used in all of these experiments had no discernible optical rotation in the range 300–210 mμ.

The optical rotatory dispersion of the papain fragment Fab of anti-DNP and the Fab–hapten complex are given in curve 1 of Figure 2; both had identical rotations. The curve has a negative trough at 224 mμ and a positive shoulder at about 235 mμ; there is only the slightest suggestion of a dip at 230 mμ; the curve in the region 245–280 mμ is much flatter than that of antibody. The ORD curve of Fab–hapten in 4 M guanidine-HCl is shown as curve 2 of Figure 2; rotations are only slightly more negative than those in curve 1, and the minimum is still at 224 mμ. Values for the rotation of the Fab fragment in 4 M guanidine-HCl are shown in curve 3 of Figure 2. A striking change has occurred. The trough has been shifted to 221 mμ and again, as in the case of whole antibody, the rotations are more negative.

Discussion

In 4 M guanidine-HCl antibody, in the absence of hapten, exhibits a large increase in levorotation with respect to the rotation observed in buffer. This general effect of guanidine-HCl on the ORD of antibody has been previously observed (Noelken and Tanford, 1964) and interpretated as an unfolding of the molecule. These authors have shown, and we have confirmed (R. E. Cathou, unpublished results) that the extent of unfolding depends on the concentration of guanidine-HCl. In solutions of up to about 2 M guanidine-HCl, little change is observed in the optical rotatory dispersion; at higher concentrations of guanidine-HCl, the rotation becomes increasingly more levorotatory, reaching a maximum at about 5–6 M.

Although denaturation of free antibody occurs in solutions of guanidine-HCl at concentrations greater than 2 M, the binding capacity of anti-DNP for ε-DNP-lysine is not at all affected by guanidine-HCl concentrations as high as 4 M and only slightly affected by a concentration of 5 M as shown by the binding experiments. Not only are there still 2 moles of hapten bound/mole of antibody in 4 M guanidine-HCl, but also the binding constant, K_0, is unchanged. The ORD curve of the anti-DNP–hapten complex in 4 M guanidine-HCl is significantly less levorotatory than that of free antibody in the same solvent, indicating that less unfolding of the molecule occurs in the presence

of hapten. Since ORD is generally not very sensitive to localized changes in structure (Cathou *et al.*, 1965; Fasella and Hammes, 1965), then the difference in ORD curves exhibited by antibody in the presence and absence of hapten must reflect a more general change in structure. The fact that some unfolding still occurs even in the presence of hapten will be discussed below.

The Fab fragment of anti-DNP exhibits the same general behavior as the parent molecule in solutions of 4 M guanidine-HCl, although there are striking quantitative differences. The ORD of Fab–hapten complex in 4 M guanidine closely resembles Fab in phosphate–NaCl buffer. However, in the absence of hapten a major degree of levorotation occurs. This implies that the binding of ε-DNP-lysine to Fab prevents, to a large extent, the unfolding of the protein. The properties of Fab are not significantly changed on cleavage of anti-DNP by papain (Steiner and Lowey, 1966; Porter, 1959; Karush, 1959; Nisonoff *et al.*, 1960; Weltman and Sela, 1964). Therefore, the greater unfolding in the anti-DNP–hapten complex can be considered to be localized primarily in Fc. This is understandable since Fc is not involved in hapten binding and according to the current model of γ-globulin structure (Noelken *et al.*, 1965) is rather loosely associated with Fab.

The stabilization of enzyme conformation accompanying the binding of small molecules at substrate and allosteric sites is now well established (*cf.* Lumry, 1959; Stadtman, 1966). Although conformational isomers in the stable native state may exist, there is probably a single, dominant, folded species (or a group of closely related conformers with similar thermodynamic properties in equilibrium with each other) which favors binding. Unfolded species on the other hand, do not allow binding. Denaturation, or unfolding of the molecule, undoubtedly proceeds through several species, the first of which may be reversibly formed, such as the expanded form of serum albumin (Foster, 1960). If the association constant is greater than the equilibrium constant of the reaction associated with unfolding, then substrate stabilization occurs.

The pronounced hapten stabilization of antibody conformation implies that in the presence of hapten the various regions of the polypeptide chains which contain the active site are held together in such a way as to reduce the number of possible unfolded conformations. This can occur only if the active site involves amino acid residues located on at least several widely separated areas of the molecule (presumably both on the light chains and the Fd portion of the heavy chain) and which come together to make up the active site.

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Some Properties of the Products of Reaction of Tadpole Collagenase with Collagen*

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ABSTRACT: Cleavage products of the action of tadpole collagenase on mammalian and tadpole collagens have been isolated and separated by ammonium sulfate fractionation, and have been analyzed for amino acid composition, size, shape, molecular weight, conformation, and stability. Molecular weight determinations by sedimentation equilibrium ultracentrifugation agree closely with the earlier observations that the two fragments produced by enzyme action represent three-quarters and one-quarter of the collagen molecule. Values for the number-average molecular weight for the small fragment (TC^B), the larger fragment (TC^A), and the intact molecule (TC) were 70,000, 202,000, and 298,000, respectively. Calculations for molecular length and rigidity from viscosity measurements were consistent with a rigid rod structure for two fragments, one, one-quarter, and the other, three-, quarters of the intact molecule. Optical rotatory dispersion measurements indicated that helical content was preserved in the fragments. The fragments heat denatured more readily than collagen; at acid pH T_m values were 32 and 29° for TC^A and TC^B, respectively, as compared with 36° for TC (calf skin). Tadpole collagen at acid pH gave T_m values of 23.2, 24.5, and 29°, respectively, for TC^B, TC^A, and TC; at neutral pH the values were 1–2° higher. TC^B and TC^A differed significantly from each other in the content of 15 of the amino acids; only glycine, glutamic acid, and aspartic acid showed less than 10% difference. The total imino acid percentage was higher in TC^B than in either TC^A or TC, yet the denaturation temperature was lower. Other points characteristic of the fragments as compared to native collagen were increased solubility at neutral pH and greater susceptibility to degradation by trypsin. A hypothesis on the mechanism of physiologic resorption of collagen based on these findings is proposed.

An enzyme isolated from the medium of cultures of living tadpole tissues cleaves the native collagen molecule into two fragments in a highly specific manner at physiologic pH and at a temperature below that of substrate denaturation (Gross and Lapiere, 1962; Lapiere and Gross, 1963; Nagai *et al.*, 1966; Gross and Nagai, 1965; Kang *et al.*, 1966). The enzyme has been purified and partially characterized (Nagai *et al.*, 1966). Electron microscope studies of segment long spacings (SLS)[1] indicated that the molecule is severed one-quarter of the distance from the "B" end (Gross and Nagai, 1965). There is no further attack on the two intact fragments, and preliminary analyses of the terminal amino acids indicate that no more than three peptide bonds per collagen molecule are broken (Nagai *et al.*, 1964), probably one in each polypeptide chain. Kang *et al.* (1966) have reported the amino acid analysis and molecular weights of the separated polypeptide chains obtained by denaturation of the reaction products and isolation by chromatography. In this paper we describe the separation of the undenatured reaction products into the large (TC^A) and small (TC^B) fragments, and measurements of their size, shape, molecular weight, amino acid composition, and conformational stability.

Experimental Procedures

The tadpole enzyme was prepared and purified as described by Nagai *et al.* (1966) with the exception that the starch block electrophoresis and DEAE-cellulose chromatography steps were omitted, since it was found that the characteristic enzyme action was unchanged by further purification and that the more

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[1] Abbreviations used: SLS, segment long spacing; TC^A and TC^B, large and small fragments, respectively, of TC (the intact molecule); TCA, trichloroacetic acid; ATP, adenosine triphosphate; PTA, phosphotungstic acid.