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Antibodies to the Unfolded Form of a Helix-Rich Region in Staphylococcal Nuclease†

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ABSTRACT: Goat antibodies prepared against a polypeptide fragment of staphylococcal nuclease, (99–149), have been fractionated by affinity chromatography to yield a population of antibodies specific for the region of amino acid residues 99–126 of nuclease. These anti-$(99–126)_R$ antibodies do not precipitate with nuclease or with (99–149) and are operationally monospecific, as inferred from sedimentation velocity ultracentrifugation studies, for a distinct antigenic determinant on fragment (99–149). The anti-$(99–126)_R$ antibody populations are heterogeneous with regard to charge but show some restrictions as compared to anti-$(99–149)_R$ in disc gel electrophoresis and isoelectric focusing experiments. Binding data for the interaction of (99–149) and anti-$(99–126)_R$ antibodies, analyzed using a Scatchard plot, suggested that the antibodies were composed of a high affinity population with an average K_{ass} of 1.2×10^7 M^{-1} and a lower affinity population with an average K_{ass} of 1×10^5 M^{-1}. Purification and characterization of this antibody population will facilitate the study of the conformational equilibrium of native nuclease.

Staphylococcal nuclease (nuclease) is an extracellular protein containing 149 amino acids with a molecular weight of 16,900 (Anfinsen *et al.*, 1971). The protein contains three helical regions, two of which occur in the region between amino acid residues 99 and 149 (see Figure 1 of Sachs *et al.* (1972b)) (Arnone *et al.*, 1971). Previous communications from this laboratory (Sachs *et al.*, 1972a–c) have described the preparation and properties of a population of nonprecipitating antibodies isolated from hyperimmune goat anti-nuclease serum. These antibodies, purified by affinity chromatography using nuclease and proteolytic fragments of nuclease covalently bound to Sepharose, are operationally monospecific for the helix-rich region from amino acid residues 99 to 126, bind tightly to nuclease, and inactivate the enzyme. Comparison of the quantitative precipitation reactions of nuclease with antibody prepared to native nuclease and with antibodies prepared directly against the nuclease fragment (99–149)[1] demonstrated the conformational specificity of both antibody populations.[2] Using anti-$(99–126)_N$ antibodies it has been possible to estimate an equilibrium constant describing the conformational equilibrium of (99–149) between its unfolded form and its native format.

Conversely, it would appear feasible and consistent to study the conformational equilibria of native proteins using antibodies directed against the unfolded forms of the protein. As an initial step in such a study we describe the preparation and characteristics of anti-$(99–126)_R$ antibodies purified from antiserum made by immunization with the conformationally disordered nuclease fragment (99–149) (Taniuchi and Anfinsen, 1971; Sachs *et al.*, 1972c).

Materials and Methods

Preparation of Nuclease and Nuclease Fragments. Staphylococcal nuclease was prepared and purified as previously described (Moravek *et al.*, 1969) and then was further purified by affinity chromatography on pdTp-Sepharose (Cuatrecasas *et al.*, 1968). Fragment (99–149) was prepared from the cyanogen bromide digest of nuclease (Taniuchi and Anfinsen, 1966). Fragments (1–126) and (127–149) were prepared by limited trypsin digestion of trifluoroacetylated nuclease (Taniuchi and Anfinsen, 1969).

Goat Anti-(99–149) Serum. Anti-$(99–149)_R$ hyperimmune

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[1] Abbreviation used is: (x–y), a polypeptide fragment of the protein whose amino terminal residue is residue x and whose carboxyl terminal residue is residue y of the protein.

[2] Antibody isolated from hyperimmune sera prepared against native nuclease is indicated by a subscript N (Native). Antibody isolated from hyperimmune sera prepared against (99–149), a disordered species (Taniuchi and Anfinsen, 1971; Sachs *et al.*, 1972c), is indicated by the subscript R (Random). Previous papers in this series (Sachs *et al.*, 1972a–c) have employed lower case n and r to indicate native and random, respectively. These subscripts have now been changed to the upper case in order to maintain consistency with the nomenclature of the literature of protein chemistry (Tanford, 1968, 1970).

serum was produced in goats using fragment (99–149) of staphylococcal nuclease as the immunogen, as previously described (Sachs *et al.*, 1972a).

Preparation of Anti-(99–126)$_R$. Goat hyperimmune serum containing anti-(99–149)$_R$ antibodies was fractionated by affinity chromatography on a column (1 × 5 cm) of nuclease covalently bound to Sepharose. Antibodies were introduced onto these columns in phosphate buffered saline (0.019 M phosphate (pH 7.4)–0.13 M NaCl) and eluted with 0.1 N Sorensen's citrate buffer (pH 2.2) as previously described (Sachs *et al.*, 1972a). Protein which bound to the column was eluted, pooled, adjusted to pH 7.0 with 1 M NaOH, and lyophilized. The dry protein was redissolved in water, dialyzed against 0.15 M NaCl, and applied to a column of Sepharose–(1–126). Protein which failed to bind to this column was labeled as anti-(127–149)$_R$; acid-eluted protein, labeled as anti-(99–126)$_R$, was adjusted to pH 7.0, lyophilized, dialyzed against 0.15 M NaCl, and stored at −15°.

Preparation of ^{14}C-Labeled Fragment 99–149. The α-NH_2 group of (99–149) was preferentially carbamylated using the cyanate method (Stark, 1965). Fragment (99–149), 200 μg, was lyophilized and dissolved in 20 μl of 50% *N*-ethylmorpholine in water (pH 7.0). Approximately 0.3 mg of dry K^{14}CO (New England Nuclear; 7.5 g/Ci) was added, and the solution was incubated for 1 hr at 23°. The reaction was quenched by the addition of 2 mg of Gly-Gly in 1 ml of 40 mM NH_4HCO_3 (pH 7.2).

The carbamylated polypeptide was separated from free cyanate and carbamylated Gly-Gly by gel filtration on a Bio-Gel P-2 column (1 × 70 cm) equilibrated with 40 mM NH_4HCO_3 (pH 7.2). Fractions, 1 ml, were assayed for radioactivity, and the first peak was pooled, lyophilized, and redissolved in water. The specific radioactivity of ^{14}C-labeled (99–149) was 8300 cpm/μg, as determined by quantitating the complementary enzymatic activity of ^{14}C-labeled (99–149) by comparison with a standard (99–149) solution (Taniuchi and Anfinsen, 1966). Based on this enzymatic activity, 60 μg of ^{14}C-labeled (99–149) was recovered.

Polyacrylamide disc gel electrophoresis was performed at pH 8.5 (Davis, 1964) and using highly cross-linked gels in 8 M urea–1% sodium dodecyl sulfate (Swank and Munkres, 1971).

Ouchterlony double immunodiffusion in agar was performed using Immunoplates, pattern B (Hyland Division, Travenol Laboratories, Inc., Costa Mesa, Calif.) (Ouchterlony, 1949). Patterns were examined after 24 hr.

Isoelectric focusing was performed at 4° in 4% polyacrylamide gels containing pH 5–8 Ampholine (Doerr and Chrambach, 1971). A constant voltage of 200 V was applied for 18 hr. Gels were stained with Coomassie Blue and destained according to the procedure of Vesterberg (1971). To determine the pH gradient which was established, a single unstained gel was cleaved transversely into 39 equal slices. Each slice was placed in 1 ml of 0.03 M KCl in a CO_2-free atmosphere, and the pH of the salt solution was determined after 24 hr at 25°.

Antibody Desialation. Sialic acid was removed from antibody preparations by incubation of antibody (75 μg) and neuraminidase (1.5 μg) in 0.04 M sodium acetate–0.06 M NaCl (pH 5.5) for 30 min at 37°. *Clostridium perfringens* neuraminidase (Worthington), purified by affinity chromatography by the method of Cuatrecasas and Illiano (1971), was the gift of L. Corley.

Sedimentation velocity ultracentrifugation studies were performed in double-sector cells using a titanium four-hole ANH rotor using a Spinco Model E ultracentrifuge equipped with scanning optics (Schachman and Edelstein, 1966). Solutions studied included (a) anti-(99–126)$_R$ (2.7×10^{-6} M) and fragment (99–149) (6.0×10^{-6} M), (b) anti-(99–126)$_R$ (2.7×10^{-6} M), and (c) anti-(99–149)$_R$ (2.7×10^{-6} M) and fragment (99–149) (6×10^{-6} M); all solutions contained 0.15 M NaCl and 0.015 M phosphate buffer (pH 7.4). Positions of boundaries were recorded every 8 min after reaching a maximum rotor speed of 40,000 rpm. The temperature was maintained at 26°.

Binding Studies. The binding of ^{14}C-labeled (99–149) to anti-(99–126)$_R$ was examined using the double antibody precipitation method. A 150-μl solution containing anti-(99–146)$_R$ (8×10^{-7} M), ^{14}C-labeled (99–149) (6×10^{-8}–1.9×10^{-5} M), 40 mM Tris-HCl (pH 8.1), and 90 mM NaCl was incubated at 23°. After 1 hr, a 50-μl solution of 4 mg of rabbit anti-goat γ-globulin per ml of hyperimmune sera (Nutritional Biochemical Co.) and 20 μg of goat γ-globulin (1 mg/ml; Nutritional Biochemical Co.) were added.[3] After 1 hr at 23°, the solutions were placed at 4° for at least 24 hr. The precipitate which formed was sedimented by centrifugation using a Beckman Microfuge (Model 152), and an aliquot of supernatant was assayed for radioactivity. After removal of the remaining supernatant, the precipitate was resuspended in 200 μl of 40 mM Tris-HCl (pH 8.1)–90 mM NaCl at 4° and centrifuged. Following removal of the supernatant, the pellet was dissolved in 50 μl of 1 M NaOH. Aliquots were assayed for radioactivity in 10 ml of Bray's solution (Bray, 1960) using a Nuclear-Chicago Mark II liquid scintillation counter.

The data were analyzed by the method of Scatchard (1949), according to eq 1, in which r represents the number of ^{14}C-labeled (99–149) molecules bound per antibody molecule at a concentration of free ^{14}C-labeled (99–149) equal to c. K is the association constant between antibody and ^{14}C-labeled (99–149), while n equals the maximum number of ^{14}C-labeled (99–149) molecules that can be bound per antibody molecule.

$$r/c = Kn - Kr \quad (1)$$

Results

Fragment (99–149) was purified by gel filtration on Sephadex G-50 and phosphocellulose chromatography to yield a preparation which appeared homogeneous by disc gel electrophoresis in sodium dodecyl sulfate–urea (Figure 1). Fragment 99–149 was used as the immunogen for preparation of anti-(99–149)$_R$. Goat anti-(99–149)$_R$ hyperimmune sera contained approximately 6 mg/ml of anti-(99–149)$_R$ as determined by quantitative precipitation.

Goat anti-(99–149)$_R$ hyperimmune sera (5 ml) was passed onto a Sepharose–nuclease column in phosphate buffered saline and bound antibody was removed by acid elution (Figure 2a). Goat anti-(99–149)$_R$ antibodies were placed on a Sepharose–(1–126) column and fractionated into two populations, anti-(127–149)$_R$ and anti-(99–126)$_R$, using phosphate buffered saline and acid citrate elution, respectively (Figure 2b). A summary of the purification procedure is given in Table I. It would appear that all but 11% of the anti-(99–149)$_R$ antibody populations can bind to native nuclease. Of the antibodies which do bind to nuclease, approximately 50% have specificity for the region (99–126) and approximately 50% have specificity for 127–149. In analogous experiments, anti-(99–149)$_R$ antibodies applied to a Sepharose–(127–149) column also were fractionated into an unbound (anti-(99–126)$_R$) and bound

[3] The ratio of rabbit anti-goat γ-globulin to goat immunoglobulin represented conditions of antibody excess, as determined by quantitative precipitin curves.

TABLE I: Selective Fractionation of Anti-(99–149) Antibodies.

Adsorbing Antigen (Sepharose-Bound)	Antibody Preparation Fractionated	Specific γG Applied (mg)	γG Bound and Eluted		% of Ab Recovered[a]	γG Unbound		% of Ab Recovered
			mg	Specificity		mg	Specificity	
Nuclease (1–149)	Anti-(99–149)$_R$ (whole serum)	30[b]	25	Anti-(99–149)$_R$	87	3.7[c]	Anti-(99–149)$_R$	13
Nuclease fragment 1–126	Anti-(99–149)$_R$ (purified)	12.8	3.9	Anti-(99–126)$_R$	51	3.7	Anti-(127–149)$_R$	49

[a] It appears that low-affinity antibodies do not bind to these Sepharose–polypeptide columns; of the antibody population that does bind, preliminary results indicate that a small (presumably high affinity) antibody population may be eluted with 6 M guanidine-HCl but does not elute using acid citrate buffer. [b] Determined by quantitative precipitation using 99–149. [c] Capable of binding to Sepharose–(99–149).

(anti-(127–149)$_R$) population with a ratio of 1:1 (Figure 2c).

The ability of these antibody fractions to form precipitates in the presence of antigen was evaluated using (99–149) and antibody preparations at various stages of purification. Anti-(99–149)$_R$ hyperimmune serum and anti-(99–149)$_R$ antibodies prepared by immunoadsorption to nuclease–Sepharose both formed precipitin bands (Figure 3). However, neither anti-(99–126)$_R$ nor anti-(127–149)$_R$ formed precipitin bands with (99–149), although antibody concentrations exceeded that estimated for each species in the original hyperimmune sera. When anti-(99–126)$_R$ and anti-(127–149)$_R$ were mixed, a precipitin band was formed against (99–149). Possible reasons for the failure of the highly fractionated antibodies to form precipitates will be discussed below.

Sedimentation Velocity Studies. The molecular size of the soluble anti-(99–126)$_R$–(99–149) complex was examined by sedimentation velocity ultracentrifugation. Sedimentation velocity studies of a solution containing anti-(99–126)$_R$ and (99–149) demonstrated that after 24 min, 84% of the original optical density could be detected in the cell. A single boundary sedimenting at $s_{20,w}$ of 7.4 S was observed (Figure 4). These results were compared to a $s_{20,w}$ of 7.0 S determined for anti-(99–126)$_R$. However, when a precipitating antibody population, anti-(99–149)$_R$, was mixed with (99–149), only 65% of the optical density remained unsedimented after 24 min; the major observed boundary sedimented at 10.0 S. These results suggested that anti-(99–126)$_R$ is operationally monospecific; that is, soluble complex formation involves the binding of one antibody per antigen.

Disc Gel Electrophoresis and Isoelectric Focusing. Further characterization of anti-(99–126)$_R$ immunoglobulin demonstrated heterogeneity of the antibody population. Figure 5 shows the results of disc gel electrophoresis of the products of successive purification of the anti-(99–126)$_R$. Anti-(99–126)$_R$ immunoglobulin contains at least four discrete bands, but does show restriction in the number of bands observed as compared to those in the anti-(99–149)$_R$ fraction. Isoelectric focusing performed between pH 5 and 8 demonstrated at least seven bands in preparations of anti-(99–126)$_R$ (Figure 6) as compared to ten bands observed in gels of anti-(99–149)$_R$. The estimated isoelectric points for the antibody subpopulations varied between 5.5 and 6.5. Incubation of anti-(99–126)$_R$ with neuraminidase significantly modified the apparent isoelectric points of the antibody subpopulations but did not reduce the heterogeneity observed by isoelectric focusing. These results would support an argument for subpopulations in the anti-(99–126)$_R$ antibody fraction.

Determination of K_{ass} of Anti-(99–126)$_R$ and (99–149). The K_{ass} of anti-(99–126)$_R$ and (99–149) was estimated under equilibrium conditions using ^{14}C-labeled (99–149) as antigen. Because the antibody–antigen complexes were soluble, the quantity of radioactive antigen bound to anti-(99–126)$_R$ was determined by precipitation of goat antibody with rabbit anti-goat γ-globulin hyperimmune sera. The results of these experiments are given in Figure 7 in the form of a Scatchard plot. These data represent the results of three independent experiments. It would appear that approximately 15–20% of the antibody population has an average K_{ass} of 1.2×10^7 M^{-1}; a value of 1×10^5 M^{-1} was obtained for the K_{ass} of 80–85% of the antibody population.

Discussion

Recent data from this laboratory (Sachs *et al.*, 1972c) have demonstrated that it is possible to prepare conformationally specific antibody populations directed against either the native antigen using native nuclease or the disordered antigen using fragments of nuclease. Under the conditions employed, the former inhibited the enzymatic activity of nuclease while the latter did not. The conformational equilibrium constant (K_{conf}) defined for the transition between the native and

FIGURE 1: Gel electrophoresis of fragment (99–149). Fragment (99–149) (20 μg) was electrophoresed in sodium dodecyl sulfate gels containing 8 M urea.

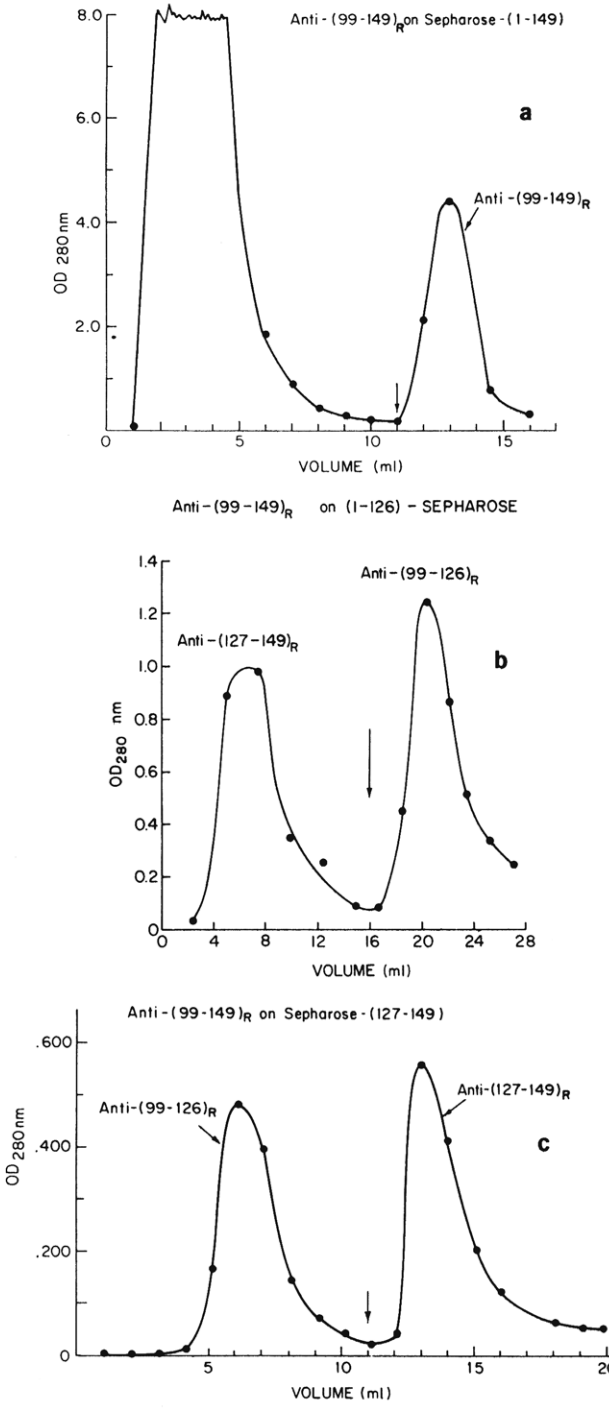

FIGURE 2: Purification of anti-$(99–126)_R$. (a) Anti-$(99–149)_R$ hyperimmune sera (5 ml) was passed onto a Sepharose–nuclease column in phosphate buffered saline. Protein capable of binding to nuclease was then eluted with Sorensen's citrate buffer (pH 2.2) and labeled as anti-$(99–149)_R$. (b) Anti-$(99–149)_R$ was further fractionated on a Sepharose–(1–126) column. The bound fraction, anti-$(99–126)_R$, was eluted with citrate buffer (pH 2.2). (c) Fractionation of anti-$(99–149)_R$ on a Sepharose–(127–149) column. The quantity of the unbound antibody anti-$(99–126)_R$, and the bound antibody, anti-$(127–149)_R$, were approximately equivalent. All studies were performed at 23°.

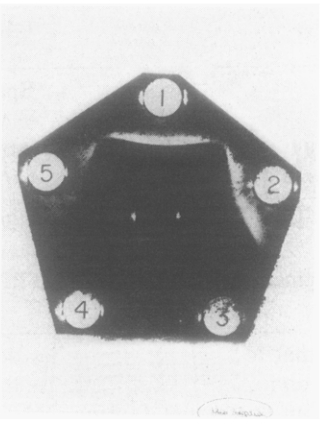

FIGURE 3: Ouchterlony double diffusion: (1) anti-$(99–149)_R$ hyperimmune sera (6 mg/ml); (2) anti-$(99–149)_R$ (3.2 mg/ml); (3) anti-$(99–126)_R$ (2.5 mg/ml); (4) anti-$(127–149)_R$ (2.5 mg/ml); (5) anti-$(99–126)_R$ + anti-$(127–149)_R$ (5 mg/ml); (O) center well—fragment 99–149 (0.1 mg/ml). A precipitin band was observed between the center well and wells 1, 2, and 5.

random forms of fragment (99–149) has been estimated as 2×10^{-4}. This would suggest that when free in solution about one out of 5000 molecules of (99–149) exists in its native format. From these results and the fluorescence, circular dichroism, and viscosity studies of (99–149) (Taniuchi and Anfinsen, 1971) it would appear that the predominant species of 99–149 used for immunization in the present study was the disordered forms of (99–149). This conclusion is based on the assumption that the conformational equilibrium of (99–149) is not affected by Freund's adjuvant.

To complement the studies of anti-$(99–126)_N$, we have purified and characterized anti-$(99–126)_R$ with the intention of investigating the interaction of anti-$(99–126)_R$ with a postulated unfolded form of native nuclease. For these reasons, the fractions of anti-$(99–126)_R$ capable of binding to nuclease were selected. The failure of about 10% of the antibody population to the polypeptide to bind to nuclease is considered to be due to steric factors present in whole nuclease but not in the fragment (99–149); however, this hypothesis must be considered tentative in the absence of additional data. This population has been produced by immunization with (99–149) but purified by its capability of binding to native nuclease and (1–126) with a high affinity. Anti-$(99–126)_R$ preparations isolated from anti-$(99–149)_R$ using either Sepharose–(1–126) or Sepharose–(127–149) appeared identical in amount and specificity. This implies that there are few, if any, antibodies whose antigenic determinants overlap both fragments.

The failure of the highly fractionated antibody populations to form precipitates in the presence of (99–149) may indicate that the antigen is so small as to comprise only a distinct antigenic determinant (monospecificity).[4] According to the lattice theory a lattice of antigen and antibody is formed due to the presence of multiple antigenic determinants on the antigen and the presence of antibodies with specificities for these determinants (Marrack *et al.*, 1951). If, however, the antigen contains but one antigenic determinant, the largest complex which may develop for IgG antibodies would include one antibody and two antigens. If the antigen contains two antigenic determinants, soluble antigen–antibody ring and short-chain complexes may be formed, while only if the antigen contains three or more antigenic determinants can a precipitating lattice be formed (Sachs, 1974). Our results indicate that anti-$(99–126)_R$ individually do not precipitate with (99–149), but, when mixed, do form a precipitate with (99–149).

[4] As in previous papers (Sachs *et al.*, 1972a) our definition of *distinct antigenic determinant* is an operational one based on the demonstration that only one molecule of antibody can bind to a molecule of nuclease at one time. Such distinct determinants could arise from single or overlapping antigenic determinants.

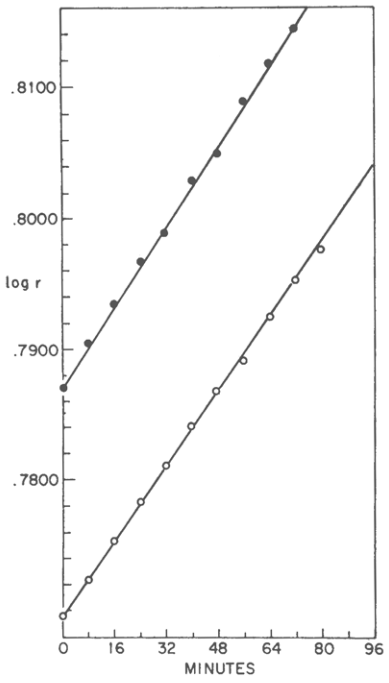

FIGURE 4: Sedimentation velocity ultracentrifugation of purified antibodies: (A) double-sector cell contained anti-(99–126)$_R$ (0.4 mg/ml; 2.7 $\times$ 10^{-6} M) in phosphate buffered saline (○); (B) double-sector cell contained anti-(99–126)$_R$ (0.4 mg/ml; 2.7 $\times$ 10^{-6} M), fragment 99–149 (6 $\times$ 10^{-6} M) in phosphate buffered saline (●). The reference cell in each experiment contained phosphate buffered saline. The ANH four-holed titanium rotor was employed. Temperature, 26°; 40,000 rpm. Observed sedimentation coefficients were corrected to $s_{20,w}$ using values of $\bar{v}$ of 0.74 ml/g and $\eta_{buffer}/\eta_{H_2O}$ of 1.016.

These results are consistent with the lattice theory, if one assumes that the region (99–126) or the region (127–149) on the (99–149) fragment represents one or two operationally distinct antigenic determinants. In turn, since the ultracentrifugation data independently suggest that the sequence 99–126 contains a distinct antigenic determinant, these results support the lattice theory. In addition, the preparation of a monospecific nonprecipitating antibody permits examination of antigen–antibody interaction using physical methods and simplifies the mathematical analyses describing the interaction of antibody and antigen.

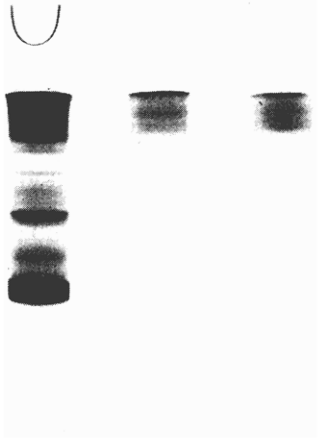

FIGURE 5: Disc gel electrophoresis of antibody preparations: (A) anti-(99–149)$_R$ hyperimmune sera (700 μg); (B) anti-(99–149)$_R$ (20 μg); (C) anti-(99–126)$_R$ (20 μg). Preparations of anti-(99–149)$_R$ demonstrated approximately seven bands while anti-(99–126)$_R$ demonstrated three or four protein bands.

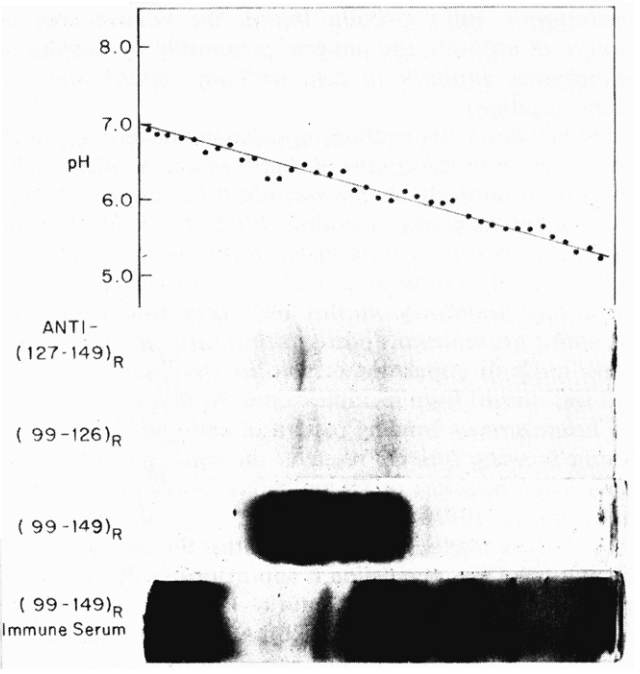

FIGURE 6: Polyacrylamide gel electrofocusing of anti-(99–149)$_R$ serum (700 μg), anti-(99–149)$_R$ (150 μg), anti-(99–126)$_R$ (50 μg), and anti-(127–149)$_R$ (50 μg). The isoelectric point of each band may be estimated by comparison to the pH of identical regions of an unstained gel.

The K_{ass} was measured using a double antibody technique and radiolabeled antigen. Carbamylation with [^{14}C]cyanate represented a convenient method of labeling (99–149) preferentially at the α-NH_2 terminus (Stark, 1965) while minimizing derivatization of the amino acid side chains and the addition of bulky modifying groups. We assume that the binding affinity of anti-(99–126)$_R$ to (99–149) and ^{14}C-labeled (99–149) are identical. If the affinity of anti-(99–126)$_R$ for (99–149) is decreased by selective carbamylation, the K_{ass} obtained in our experiments would be systematically low. Therefore our range of 10^7–10^5 M^{-1} for the K_{ass} must represent a lower limit. The double antibody technique presumes that the addition of

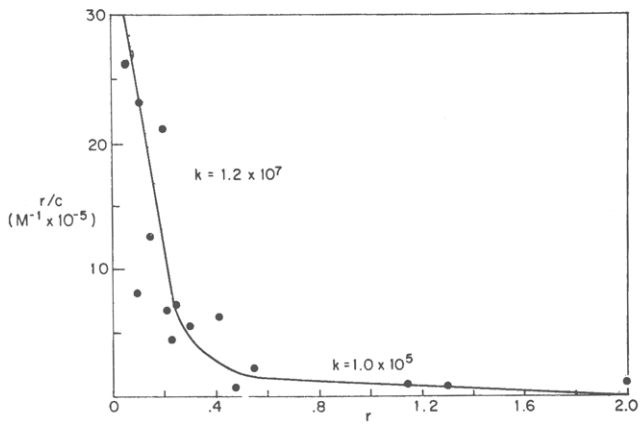

FIGURE 7: Scatchard plot r/c *vs.* r of the binding of (99–149) to anti-(99–126)$_R$. The incubation mixture consisted of anti-(99–126)$_R$ (8 $\times$ 10^{-7} M) and ^{14}C-labeled 99–149 (5 $\times$ 10^{-9}–1.9 $\times$ 10^{-5} M) in 40 mM Tris-HCl (pH 8.1)–90 mM NaCl. After 1 hr of incubation 250 μg of rabbit anti-goat γ-globulin (~4 mg of immunoglobulin/ml of hyperimmune serum) and 10 μg of goat γ-globulin (1 mg/ml) were added. The precipitate was harvested after 24 hr, and assayed for ^{14}C activity after thorough washing.

heterologous anti-γ-globulin freezes the relative concentrations of antibody and antigen, presumably by binding of heterologous antibody to goat antibody—bound and unbound to antigen.

The anti-$(99–126)_R$ antibody population obtained appeared heterogeneous by the criteria of charge as well as affinity. The diversity of antibody charge was noted by disc gel electrophoresis and isoelectric focusing. Whether individual bands observed by isoelectric focusing are homogeneous with regard to the association constant is not known. It is difficult at present to state definitively whether the charge heterogeneity of the antibodies represents post-synthetic variation of a monoclonal antibody population or whether the antibody population was derived from multiple clones. As might be expected, the heterogeneous banding pattern of anti-$(99–126)_R$ by isoelectric focusing does not resemble the regular pattern of the monoclonal myeloma or E9 antibodies (Awdeh *et al.*, 1970; Askonas *et al.*, 1970).

From these results, it may be noted that the average K_{ass} of the anti-$(99–126)_R$ population is approximately two orders of magnitude lower than the anti-$(99–126)_R$ population previously studied (Sachs *et al.*, 1972b). Although it is difficult to generalize on the basis of our experience with the antisera prepared in only several goats, it appears that the antibodies to the nuclease fragment have a lower affinity than antibodies prepared against native nuclease. These differences may be consistent with the concept that fragment (99–149) passes through multiple, predominantly disordered, three-dimensional conformations defined by the thermodynamics of the solute–solvent interaction. Effective concentration of any one structure might be considerably lower than the total antigen concentration. Therefore, the average K_{ass} which was measured, representing the affinity of multiple antibody species for multiple antigen structures, may in fact be the result of a series of higher affinity antibody–antigen interactions, each at lower concentrations. However, such an hypothesis can only be tested when other factors known to effect antibody affinity, including immunization schedule, duration of immunization, simultaneous and incidental exposure to non-cross-reacting antigens, intrinsic genetic factors of the host, and differences in the immunogenicity of the antigen, are more rigorously controlled (for review, see Werblin and Siskind (1972)).

A similar study from Sela's laboratory (Maron *et al.*, 1971) has involved the purification and comparison of antibodies from anti-lysozyme serum specific for region 64–83 and anti-(64–83)-poly-DL-alanine specific for region 64–83. The specificity and electrophoretic similarities of these antibody populations are presumably due to the presence of a disulfide bond in the 64–83 loop peptide immunogen which stabilizes a three-dimensional structure resembling the native format of the region 64–83. In contrast, it would appear that (99–149) shows minimal conformational similarity to the region (99–149) in native nuclease.

The isolation of the antibody population anti-$(99–126)_R$ now permits direct comparison of the binding properties of anti-$(99–126)_R$ and anti-$(99–126)_N$. The preparation and comparison of antibodies to antigens with identical primary structure but major differences in tertiary structure affords the opportunity for systematic investigation of the role of antigen conformation in determining antibody specificity. In an approach analogous to the study of conformational equilibria of disorganized polypeptides using antibodies directed against the native protein, we are employing anti-$(99–126)_R$ in the study of the conformational equilibria of native nuclease and the effect of ligands on these equilibria.

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