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An Immunologic Approach to the Conformational Equilibria of Polypeptides

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ABSTRACT **Antibodies reactive with distinct regions of the staphylococcal nuclease molecule were prepared both by immunization with polypeptide fragments of nuclease and by immunization with intact nuclease followed by fractionation of the antiserum on immunoabsorbent columns bearing the corresponding fragments. Comparisons of the interactions of these antibody preparations with nuclease, by quantitative precipitin assays and enzyme inhibition studies, showed marked differences attributable to the conformation of the immunizing antigens. An interpretive model is proposed in which antibodies fractionated from anti-nuclease serum react effectively only with the "native format determinants" of polypeptide fragments of nuclease. It is postulated that such determinants are generated in polypeptide fragments by spontaneous and reversible folding of the polypeptide chain. The model permits experimental determination of parameters, K_{conf}, for the proposed conformational equilibria.**

Fragment (99–149)* is an enzymatically inactive polypeptide produced by cyanogen bromide digestion of staphylococcal nuclease (1). Although more than half of the sequence of this fragment is folded as α-helix in the native nuclease molecule (2), the fragment has been shown by circular dichroism studies to contain less than 5% α-helix, and has been considered to be devoid of ordered structure (3). When fragment (99–149) is mixed in solution with another inactive fragment of nuclease, fragment (1–126), the two fragments combine to regenerate enzymatic activity and ordered secondary structure (3). It has been postulated that in the folding of polypeptide chains that must occur during this combination, and in the folding of nuclease itself, regions of ordered secondary structure, such as the helices of fragment (99–149), may act as "nucleation" sites (4). We have, therefore, chosen to study conformational equilibria of this region of the nuclease polypeptide chain.

We have recently described the preparation of antibodies specific for an antigenic determinant in region (99–126) of nuclease by sequential immunoabsorption of a goat anti-nuclease serum on columns of Sepharose to which selected polypeptide fragments of nuclease had been covalently attached (5). These antibodies, called anti-$(99\text{–}126)_n$, combine with nuclease to produce an enzymatically inactive, soluble complex (6). It was noted that the ability of these antibodies to bind to the polypeptide fragment (99–149) might be interpreted as indicating either that the antibodies recognized a determinant present in the unfolded form of the polypeptide or, alternatively, that fragment (99–149) can spontaneously and reversibly fold to form the same conformation that is present in the intact, native protein. This paper presents a comparison of the reactions of antibodies prepared against nuclease fragments with the reactions of corresponding antibodies prepared by fractionation of anti-nuclease serum. The results obtained indicate the importance of antigen conformation in the binding of anti-nuclease antibodies, and thus support the second of these interpretations. We wish to propose a general mathematical model based upon this interpretation to describe the interaction of antibodies to a native protein with disordered polypeptide fragments derived from that protein.

* This fragment has been referred to as "Piece E," "CNBr fragment E," (99–149), and nuclease (99–149) interchangeably in previous papers from this laboratory.

MATERIALS AND METHODS

Nuclease was prepared and purified as described (5, 7, 8). Fragments (99–149), (1–126), and (49–149) were prepared by the published methods (1, 9, 10). The synthetic fragment analog (6–43) was kindly provided by Dr. G. Sanchez, who prepared it by the solid-phase synthetic method of Merrifield (11).

Methods for the preparation of goat anti-nuclease serum and its fractionation by immunoabsorption have been described (5). All antibody preparations obtained by fractionation of this serum were designated by the subscript *n*, indicating immunization with intact, *n*ative enzyme. Antisera against fragment (1–126) and fragment (99–149) were prepared in individual goats by the same immunization schedule as that described for nuclease. The precipitable antibody in sera from these goats reached plateaus, as judged by precipitin analysis in gels with homologous antigens, after five immunizations for fragment (1–126) and after seven immunizations for fragment (99–149). Precipitin and fractionation studies were done on pools of sera prepared from blood drawn at 1 and 2 weeks after the seventh injection of each antigen. Antibody preparations obtained by fractionation of sera containing antibodies to fragments were designated by the subscript *r*, indicating the probable *r*andom conformations of the immunizing antigens. Purified antibodies were obtained from each of these sera by immunoabsorption on columns of Sepharose to which the homologous fragments were covalently bound. These columns were prepared and operated as described for nuclease (5). Immunoabsorption of anti-$(1\text{–}126)_r$ on a Sepharose-(1–126) column yielded 5.0 mg of antibody per ml of serum applied, and immunoabsorption of anti-$(99\text{–}149)_r$ yielded 3.5 mg of antibody per ml of serum applied. Each of these purified antibodies consisted of immunoelectrophoretically pure IgG.

Quantitative precipitin reactions were performed in duplicate in disposable plastic "Microfuge" tubes (Beckman). Increasing amounts of concentrated solutions of antigen in water were added to 0.1-ml aliquots of the antibody preparation in a volume of buffer (90 mM NaCl–40 mM Tris, pH 8.1) sufficient to bring the total volume of each tube to 0.3 ml. Reactions in the presence of ligands were performed by addition of $CaCl_2$ and thymidine-3′,5′-diphosphate (pdTp) to produce final concentrations of 10 mM in Ca^{++} and 1.0 mM in

pdTp. The tubes were incubated at 25° for 30 min and at 4° for 4 days, with mixing on the first and second days. They were then centrifuged, and the precipitates were washed twice with cold 0.15 M NaCl containing 0.01 M Tris (pH 8.1) and dissolved in 1.0 ml of 1.0 M NaOH. The absorbances of the dissolved precipitates were measured at 280 nm in a Zeiss spectrophotometer and were plotted as a function of the antigen added.

Assays of nuclease activity and the kinetics of nuclease inactivation by antibody were performed on a Gilford model 2000 multiple sample absorption recorder as described (6, 12). For assays of the extent of binding of anti-$(99–126)_n$ to nuclease fragments, aliquots of antibody and of fragment were added to cuvettes containing the standard assay mixture. After stable baselines had been maintained for 5 min, 0.05 μg of nuclease was added to each cuvette and the resultant activities were measured. Half-times of inactivation ($t_{1/2}$) were calculated from semilogarithmic plots of activity against time as described (6).

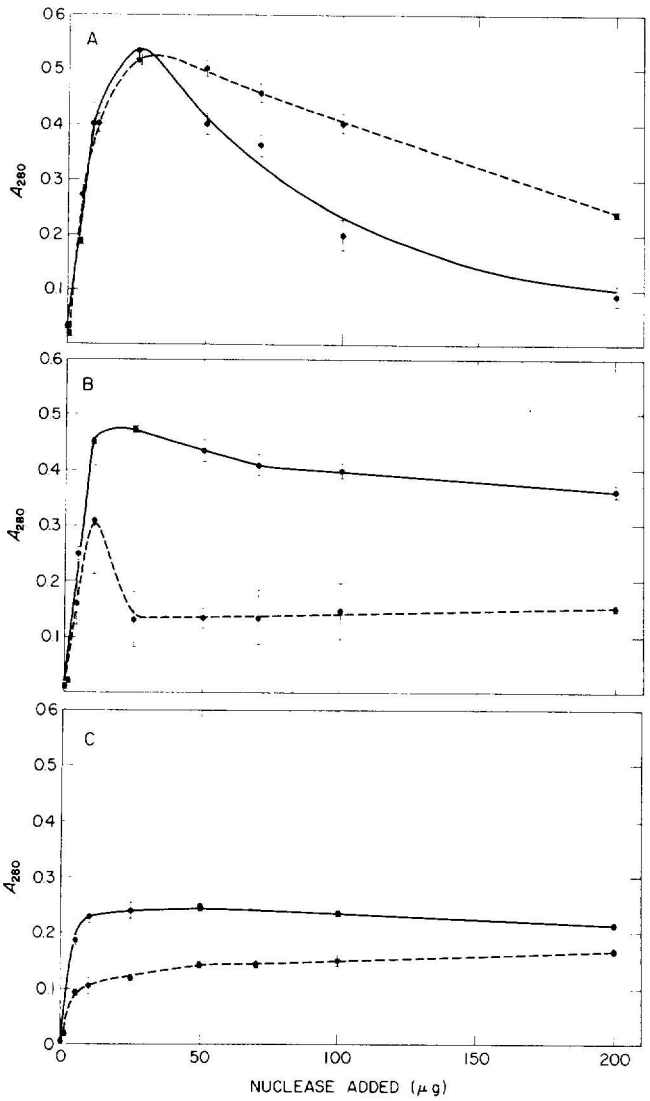

FIG. 1. Quantitative precipitation reactions in the absence (——) and presence (– – –) of the ligands pdTp and Ca^{++}. (*A*) Anti-$(1–149)_n$ diluted 1:2 in saline; (*B*) anti-$(1–126)_r$; (*C*) anti-$(99–149)_r$. Each antibody preparation was reacted with increasing amounts of nuclease.

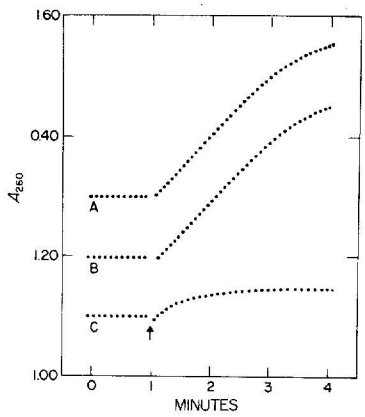

FIG. 2. Conformational specificity of inactivating antibodies. Three simultaneous activity assays are shown as recorded on a Gilford multiple sample absorbance recorder. The cuvette corresponding to the uppermost curve (*A*) received no antibody, the second cuvette (*B*) received 18 μg of anti-$(99–149)_r$, and the third cuvette (*C*) received 6 μg of anti-$(99–149)_n$. At the time indicated by the *arrow*, 0.05 μg of nuclease was added to each cuvette.

RESULTS

Conformational specificity of antibodies

Antisera prepared against fragments (1–126) and (99–149) were each capable of producing precipitin reactions with both the homologous antigen and with nuclease. Fig. 1 shows the results of quantitative precipitin curves of anti-$(1–149)_n$†, anti-$(1–126)_r$, and anti-$(99–149)_r$ against nuclease, in the absence and presence of Ca^{++} and pdTp. The presence of these ligands in large molar excess had little effect on the total precipitable antibody at equivalence for anti-$(1–149)_n$, but produced marked inhibition of precipitation for anti-$(1–126)_r$ and anti-$(99–149)_r$. Since these ligands stabilize the native conformation of nuclease (13, 14), this finding of inhibition supports the hypothesis that many of the antigenic determinants recognized by the antibodies against the fragments are present only in the "unfolded" or "non-native" conformation of nuclease. Similarly, the absence of inhibition of precipitable anti-$(1–149)_n$ by ligands suggests that very little, if any, of these antibodies are directed towards determinants other than those present in the native conformation of nuclease.

Further evidence of the conformational specificity of these antibody populations was obtained from inactivation studies. Fig. 2 shows nuclease assays in the absence of antibody and in the presence of anti-$(99–149)_r$ and anti-$(99–149)_n$. Anti-$(99–149)_n$ led to rapid inactivation of nuclease, with kinetics similar to those that we have previously reported for anti-$(99–126)_n$ (6). However, anti-$(99–149)_r$ produced no perceptible change in nuclease activity, even at a concentration 3.5-times that of anti-$(99–149)_n$. Under the assay conditions nuclease is essentially fully liganded.

Interaction of nuclease fragments with anti-$(99–126)_n$

Since the fragments (99–149) and (50–149) have no enzymatic activity of their own, the extent of their interaction with

† Although fragment (1–149) is actually intact nuclease rather that a nuclease fragment, we have used the same nomenclature as that used for fragments to describe antibodies obtained by fractionation of serum containing antibody to nuclease on a Sepharose–nuclease immunoabsorbent column.

antibody was measured indirectly by assaying for the free antibody remaining in the equilibrium mixture of antibody and fragment. Fig. 3 shows a semilogarithmic plot of activity as a function of time for the antibody-induced inactivation of 0.05 μg of nuclease after previous incubation of the antibody with several concentrations of fragment (99–149). The values of $t_{1/2}$ for these inactivations increased progressively with increasing concentration of fragment (99–149) in the preincubation mixture. A similar relationship was found for inhibitions with fragment (50–149). These data are summarized in Table 1.

It will be noted in Table 1 that even at concentrations of fragments many times that of the antibody, inhibition of antibody-induced inactivation was incomplete, indicating that at any time only a fraction of the fragment molecules could have been bound to antibody-combining sites. If this fraction correlated with the similarity in conformation between the antigenic determinant in the fragment and in native nuclease, then a manipulation that would increase that similarity might be expected to increase the inhibition caused by the fragment. One such manipulation that could readily be tested was the addition of a complementing nuclease fragment, known from previous studies to combine with the fragment in question to produce activity and physical characteristics suggestive of the ordered structure of native nuclease. Such complementing systems include fragment (6–48) plus fragment (50–149), and fragment (1–126) plus fragment (99–149) (3, 10). A further requirement of the indirect assay system, however, is that the inhibiting antigen must not itself have enzymatic activity, for otherwise a stable baseline in the kinetic assay of nuclease inactivation cannot be obtained. We therefore made use of synthetic (6–43), a synthetic fragment analog that has been shown to bind to fragment (50–149) in solution, yielding fluorescence properties characteristic of native nuclease and similar to those produced by fragment (6–48), but not producing enzymatic activity (Sanchez, G., Chaiken, I., and Anfinsen, C. B.; unpublished). Synthetic (6–43) was added to assay cuvettes containing anti-$(99–126)_n$ and fragment (50–149) and allowed to come to equilibrium before addition of nuclease. Increasing concentrations of the synthetic analog led to decreasing antibody-induced inactivation of nuclease, indicating increasing binding of the antibody to fragment (50–149). The measured half-times of inactivation for three concentrations of synthetic (6–43) with a single concentration of fragment (50–149) are shown in Table 1.

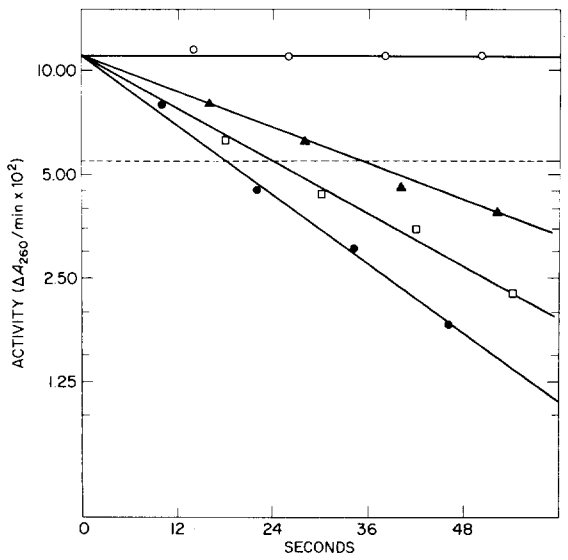

FIG. 3. Inhibition of antibody-induced inactivation. A semilogarithmic plot of activity against time for assays of 0.05 μg of nuclease in the presence of: O——O, no antibody; ●——●, 6 μg of anti-$(99–126)_n$; □——□, 6 μg of anti-$(99–126)_n$ plus 12 μg of fragment (99–149); and ▲——▲, 6 μg of anti-$(99–126)_n$ plus 48 μg of fragment (99–149). The *dotted line* represents one-half of the initial activity.

TABLE 1. *Interaction of anti-$(99–126)_n$ with nuclease fragments**

Fragment(s) added	Concentration of fragment(s) (μM)	$t_{1/2}$	Concentration of free *Ab* sites (nM)	Concentration of bound *Ab* sites (nM)
0	0	18.0†	76	0
(99–149)	0.6	20.0	68	8.0
(99–149)	2.0	24.0	57	19
(99–149)	2.6	27.0	51	25
(99–149)	6.5	33.0	42	34
(50–149)	0	19.6†	76	0
(50–149)	2.4	27.0	55	21
(50–149)	4.7	39.5	38	38
(50–149) + Syn (6–43)	2.4 + 0.5	44.0	34	42
(50–149) + Syn (6–43)	2.4 + 1.0	86.0	17	59
(50–149) + Syn (6–43)	2.4 + 1.9	226.0	6.5	69

* Each incubation mixture contained 10 μl of an antibody solution containing about 0.8 mg/ml, producing a total concentration of antibody combining sites of 76 nM.

† Inhibitions by fragment (99–149) and by fragment (50–149) were performed with different preparations of anti-$(99–126)_n$. Small differences in antibody concentration or antibody denaturation probably account for the different control values for $t_{1/2}$ in the two sets of data.

DISCUSSION

Our choice of the subscripts *n* and *r* to describe antibody populations obtained by immunization with intact protein and protein fragments, respectively, was based on the presumption that the corresponding *n*ative and *r*andom‡ conformations might be distinguished by the antibodies produced. Our experimental data indicate this indeed to be the case. Inactivation studies showed almost no overlap in the specificities of anti-$(99–149)_n$ and anti-$(99–149)_r$. Precipitation data in the presence and absence of ligands showed marked differences in the two categories of antibody populations, also attributable to differences in conformation of the corresponding antigens. Detection of "disordered" determinants, therefore, seems an unlikely explanation for the binding

‡ The term *random* is used to describe the array of possible *non*-native conformations that a polypeptide fragment in solution can presumably assume.

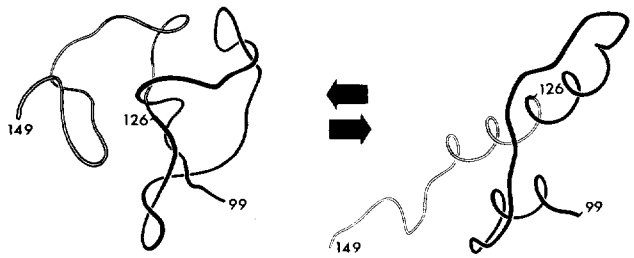

FIG. 4. Artist's representation of the postulated spontaneous, reversible folding of the nuclease fragment (99–149) in solution. The "native format," represented on the *right*, corresponds to the conformation of this portion of the molecule in intact, native nuclease, based on the x-ray crystallographic structure (2).

of antibodies from anti-$(1–149)_n$ to polypeptide fragments of nuclease.

We wish to propose a simple model, involving two simultaneous equilibria, to interpret our results. Polypeptide fragments of nuclease are presumed to exist in solution in a conformational equilibrium between a variety of disordered or random conformations (P_r) and the native conformation (P_n) assumed by the corresponding amino-acid sequence in the native protein. The antigenic determinant of P_n, all the components of which can be generated by a limited length of the polypeptide chain, is called a "native format determinant§." The proposed equilibrium is illustrated schematically for the fragment (99–149) in Fig. 4. One can describe the equilibrium formally as:

$$[P_r] \rightleftarrows [P_n]; \qquad K_{conf} = [P_n]/[P_r] \qquad [1]$$

in which it is assumed that $[P_r]$ refers to the sum of the concentrations of all disordered conformations, and K_{conf} is an over-all constant defined by this conformational equilibrium at the antigenic site. Antibodies to the native protein are presumed to react effectively only with the form P_n, according to the equilibrium:

$$Ab + P_n \rightleftarrows AbP_n; \qquad K_{assoc} = ([AbP_n]/[Ab][P_n]) \qquad [2]$$

Since the antigenic determinant of P_n is, by definition, identical to the corresponding antigenic determinant of nuclease, the association constant for this interaction is assumed to be equal to the experimentally determined association constant for the reaction of these antibodies with nuclease (6). Eliminating $[P_n]$ from Eqs. **1** and **2**, we have:

$$[AbP_n]/[P_r] = K_{conf}K_{assoc}[Ab] \qquad [3]$$

or,

$$K_{conf} = ([AbP_n]/K_{assoc}[P_r][Ab]) \qquad [4]$$

For those conformational equilibria for which Eq. **1** lies far to the left (i.e., low K_{conf}), $[P_r]$ is an adequate approximation to total polypeptide $[P_t]$, so that:

$$[AbP_n]/[P_t] = K_{conf}K_{assoc}[Ab] \qquad [5]$$

§ The term "native format determinant" is intended to designate a subclass of the more general category, "conformational determinants" (23), the latter including those determinants involving distant portions of the intact protein, the juxtaposition of which could only occur through the tertiary folding of the polypeptide chain.

Thus, for a particular antibody concentration, only a fraction of $[P_t]$ would be expected to be bound to antibody, that fraction being the product of the antibody concentration, the conformational equilibrium constant, and the association constant for the reaction with antibody. This would account for our finding that, even with large molar excess of the polypeptide fragments (99–149) and (50–149), the inhibition of antibody was incomplete.

Eq. **4** provides an expression for K_{conf} of a polypeptide fragment of low K_{conf}. Since

$$[P_t] = [P_r] + [P_n] + [AbP_n] \qquad [6]$$

a more general expression for K_{conf} in terms of $[P_t]$ can be obtained by combining Eqs. **1**, **2**, and **3** to give:

$$K_{conf} = \frac{[AbP_n]}{K_{assoc}[Ab][P_t - (AbP_n)] - [AbP_n]} \qquad [7]$$

For low K_{conf} the term $[AbP_n]$ would be small, and this expression would reduce to that of Eq. **4**.

The association constant for the reaction of anti-$(99–126)_n$ with nuclease in the concentration range used for the fragment inhibitions has been previously determined as $8.3 \times 10^8\ M^{-1}$ (6). With this value for K_{assoc} and the experimentally determined half-times from Table 1, one can calculate values for the K_{conf} of the polypeptide fragments (99–149) and (50–149) by Eq. **7**, as presented in Table 2. The final column of this table shows values for the percentage of each unbound fragment in the native format conformation, as derived from the corresponding values of K_{conf}. These values indicate that the folded fraction would be present in much too low a concentration to be measured by physical techniques. This is consistent with the findings of Taniuchi and Anfinsen (9) that physical evidence of appreciable tertiary structure does not arise until the polypeptide chain is almost complete.

Also shown in Table 2 are the values obtained for K_{conf} of fragment (50–149) in the presence of synthetic fragment (6–43). Since the synthetic analog was used at much less than

TABLE 2. K_{conf} *of nuclease fragments*

Fragment(s)	Concentration of fragment(s) (μM)	K_{conf} ($\times 10^4$)	% free P_t as P_n
(99-149)	0.6	2.20	0.022
(99-149)	2.0	2.02	0.020
(99-149)	2.6	2.29	0.023
(99-149)	7.8	1.47	0.015
(99-149)	6.5	1.51	0.015
		Avg. 2.0	
(50-149)	2.4	2.0	0.020
(50-149)	4.7	2.6	0.026
		Avg. 2.3	
(50-149) + Syn (6-43)	2.4 + 0.5	6.5	0.065
(5μ-149) + Syn (6-43)	2.4 + 1.0	18	0.180
(50-149) + Syn (6-43)	2.4 + 1.9	57	0.560

molar equivalence and since it had not been purified from the other incorrect sequences undoubtedly present, one cannot quantitate the fraction of fragment (50–149) that might be expected to be "folded" by the interaction between the two peptides. Nevertheless it is apparent from this table that increasing amounts of the synthetic analog led to increasing derived values of K_{conf}. This same analog has been shown by fluorescence measurements to induce an environment around tryptophan 140 closely resembling that of native nuclease (ref. 9 and Sanchez, G., Chaiken, I., and Anfinsen, C. B.; unpublished). The simplest interpretation of the increases in K_{conf} induced by synthetic (6–43) is thus that K_{conf} reflects the degree of "nativeness" of the polypeptide in solution, in accordance with our model.

The overall interaction of antibody with polypeptides can, of course, be satisfactorily described by an effective association constant:

$$K' = ([AbP]/[Ab][P]) \quad [8]$$

without reference to the conformational equilibrium of the fragment P (15). However, an implication of our model apparent from Eq. **4** is that the effective association constant that one would measure for such systems is the simple product of the two component equilibria, i.e.,

$$K' = K_{conf}K_{assoc} \quad [9]$$

What has previously been considered as "lower affinity" compared to the native protein for antibody binding to peptide fragments and derivatives of apomyoglobin (16, 17), lysozyme (15, 18), ribonuclease (19), and nuclease (20) may therefore result from lower effective concentration of the appropriate antigenic determinant (i.e., lower K_{conf}) rather than from an actual decrease in the binding energy of the interaction. Conversely, the binding of antibodies prepared against native proteins, such as β-galactosidase (21), to incomplete, ribosome-bound protein fragments, need not imply that the bound fragments are actively "folded" but only that they are undergoing the same type of conformational equilibrium as we have postulated for fragments in solution.

In addition to our data, which correlate K_{conf} with an independent measure of protein conformation, there are other lines of evidence to support this interpretation[¶]. Schechter *et al.* have shown that antibodies to the helical polypeptide (Tyr-Ala-Glu) are capable of "inducing" increased helicity in the oligopeptide $(Tyr\text{-}Ala\text{-}Glu)_{13}$ (22). Eq. **3** explains this finding in terms of the stabilization of the helical conformation P_n by antibody through a shift of equilibrium. Alternatively, in these studies as well as in our own, one might postulate the binding of antibodies to polypeptides that are less than fully folded, followed by further folding of the antigens after binding. In this case, however, all bound antigen would eventually achieve the native format and one would expect to measure a single k_{off} for the dissociation of the antibody–antigen complex. Operationally, then, this model would be indistinguishable from that which we have proposed for the stabilization of the spontaneously folded form P_n, in which the definition of P_n includes all forms of P that are sufficiently folded to bind effectively to antibody. It would, in addition, have much less heuristic value than our model since an active folding process could not be adequately quantitated.

The model also predicts that the half-time of dissociation of a polypeptide fragment from antibody to a native format determinant should be similar to that for the dissociation of the native protein, rather than the much smaller half-time that would be anticipated if K' actually described the binding. This may explain the tight binding that has been observed during the immunoabsorption of antibodies against nuclease on columns bearing the polypeptide fragments (6).

Since antibodies such as anti-$(99\text{–}126)_n$ presumably react with some antigenic determinant within the region of amino acids (99–126), the K_{conf} determined by using these antibodies is theoretically only relevant to those residues directly involved in the determinant. However, since the folding of proteins seems to be a cooperative phenomenon, it seems probable that the measured K_{conf} may be a parameter for folding of the entire fragment. The proposed model thus provides a new and generally applicable parameter for the conformation of a polypeptide fragment of a protein. This parameter may be of particular usefulness in studies of conformational equilibria in which the proportion of native conformation is too small to be measured by physical means, the fragments of nuclease being examples of such equilibria.

[¶] The extended plateaus in the region of antigen excess of the precipitin curves produced by anti-$(1\text{–}126)_r$ and anti-$(99\text{–}149)_r$ with nuclease are similar to those that have been reported for the precipitin reactions between antibodies against apomyoglobin and large proteolytic fragments of apomyoglobin (16). These plateaus may also offer evidence for the conformational equilibria of the corresponding antigens, a hypothesis that will be further elaborated in a future communication.

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