Immunochemistry of Staphylococcal Nuclease. II. Inhibition and Binding Studies with Sequence Fragments*

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ABSTRACT: Peptide fragments of staphylococcal nuclease have been prepared by cyanogen bromide cleavage, by limited tryptic digestion, and by solid-phase synthesis and have been tested for antigenic activity. None of these fragments was able to precipitate with antinuclease antibody. However, one set of peptides representing overlapping sequences from the carboxy-terminal portion and another set from the amino-terminal portion of the enzyme reacted with antibody in two types of immunological systems. The immunoreactive peptides inhibited the turbidimetric assay of the rate of the nuclease-antinuclease precipitin reaction. Three peptides were then labeled with ^{125}I to permit studies of direct binding to antibody and of inhibition of such binding by unlabeled peptides. Antigenic determinants may be localized to the linear sequences 127–149 and 18–47 (possibly 18–26). In addition, however, conformation-dependent determinants or affinity may be inferred from immunoadsorption experiments and from the enhanced binding of radioactively labeled peptides when incorporated into a noncovalent, enzymatically active complex.

For several globular proteins, such as tobacco mosaic virus protein (Benjamini *et al.*, 1968), thyroglobulin (Metzger *et al.*, 1962), lysozyme (Shinka *et al.*, 1962; Arnon and Sela, 1969), and myoglobin (Crumpton and Wilkinson, 1965; Givas *et al.*, 1967), at least some of the antigenic determinants have been demonstrated to be sequential, since antigenically active peptide fragments have been found. Certain peptides from pancreatic ribonuclease have been shown to react with antibody to performic acid oxidized ribonuclease, but not with antibody elicited by the native enzyme (Brown, 1962). As model systems, synthetic polypeptide antigens have been devised with primarily sequential or primarily conformational determinants (Sela *et al.*, 1967; Gill, 1965).

In the preceding paper, antibodies elicited by either native staphylococcal nuclease or by its denatured performic acid oxidized derivative were shown to cross-react with each antigen. This paper reports the activity of a variety of nuclease peptides in inhibiting the reaction of nuclease with antinuclease and in binding directly to the antinuclease antibodies. Certain large nuclease fragments can bind to each other noncovalently to generate an enzymatically active complex whose conformation resembles that of the native enzyme (Taniuchi *et al.*, 1967). The effect of such complex formation on antigenicity has permitted additional inferences about the contribution of conformation to the antigenicity of nuclease.

Materials and Methods

Materials. Highly purified nuclease was prepared as previously described (Morávek *et al.*, 1969).

Cyanogen Bromide Fragments. With the modification described by Taniuchi and Anfinsen (1966) of the method of Gross and Witkop (1962), 200 mg of nuclease (containing 48 μmoles of methionine) was digested with a 100-fold molar excess of cyanogen bromide. All enzymatic activity was lost, and no methionine could be detected upon amino acid analysis of an acid hydrolysate. The digest was applied to a column of Sephadex G-50, and the elution pattern and yields of fragments were similar to those reported by Taniuchi and Anfinsen (1966). The separated peptides were identified by dansyl chloride end-group analysis (Gray, 1967) and amino acid composition (see Figure 1 for sequence). Fragments A and C were isolated together and used as a mixture (molar ratio A–C of 3:1).

Nuclease-T and Fragments P_2 and P_3. Nuclease was subjected to limited trypsin digestion in the presence of deoxythymidine 3′,5′-diphosphate and Ca^{2+}, and sequence fragments P_2 (residues 6–48) and P_3 (residues 49–149) were isolated by the procedures of Taniuchi *et al.* (1967). Each fragment alone was enzymatically inactive, but when mixed they formed the noncovalent complex nuclease-T, with about 8% of the specific activity of native nuclease.

Nuclease Fragments 1–126 and 127–149. These fragments of nuclease, prepared (Taniuchi and Anfinsen, 1969) by limited tryptic digestion of completely trifluoroacetylated nuclease, with preferential cleavage at Arg_{126}, and by subsequent removal of the trifluoroacetyl groups with piperidine, were the generous gifts of Dr. Taniuchi. Both of these fragments are enzymatically inactive, but fragment 1–126 can generate an enzymatically active, noncovalent complex resembling nuclease-T with either P_3 (residues 49–149) or the cyanogen bromide fragment E (residues 99–149) (Taniuchi and Anfinsen, 1969).

Synthetic P_2 (Residues 6–47) Fragments. The sequence corresponding to residues 6–47 was synthesized by the Merrifield solid-phase technique as described (Ontjes and Anfinsen, 1969a,b), and fragments representing residues 33–47, 18–47, and 9–47 were obtained by removal of portions

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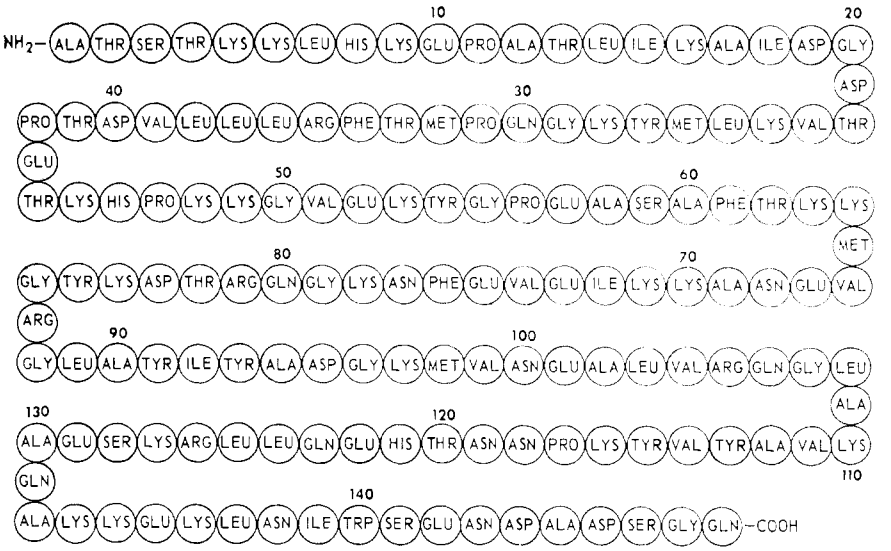

FIGURE 1: Amino acid sequence of staphylococcal nuclease (Cusumano *et al.*, 1968).

of the product during the stepwise synthesis. All peptides were cleaved from the insoluble resin with hydrogen fluoride, deprotected, and partially purified on Sephadex G-25, as previously described.

Antisera. γ-Globulin (γG) fractions of antisera from individual rabbits were prepared as described in the preceding paper. Specific antinuclease antibody was obtained from γG fractions by purification on a nuclease–Sepharose immunoadsorbent (Omenn *et al.*, 1970b) and was used for all turbidimetric and radioactively labeled antigen binding studies.

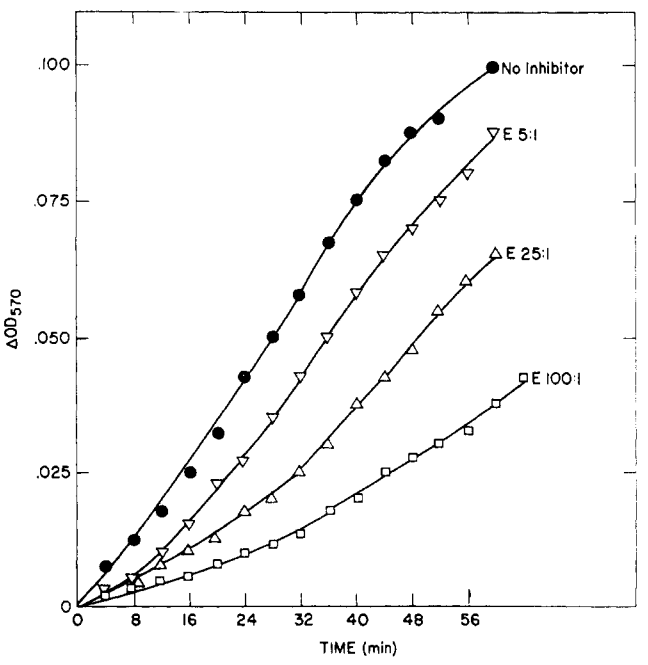

FIGURE: 2 Turbidimetric reaction of nuclease (5 μg) with purified antinuclease (75 μl) at 25° in buffered saline (pH 7.5). Inhibition with increasing concentrations of cyanogen bromide fragment E (residues 99–149).

Methods

Turbidimetric Assay. The rate of the precipitin reaction of nuclease with antinuclease was measured by the turbidity formed as a function of time (Fuchs *et al.*, 1969). The rate of turbidity formation at 25° was followed by a continuous recording of the absorbance at 570 mμ, with a Gilford Model 2000 multiple-sample absorbance recorder equipped with a Beckman DU spectrophotometer. Four samples were reacted and followed simultaneously. Purified antibody (300 μg, 2 μM) was present in 1.0-ml volume of normal saline containing 0.05 M Tris-HCl buffer (pH 7.5) in quartz cuvets. For inhibition studies, the inhibitory peptides were incubated alone with antibody, and absorbance was followed to check for any precipitation before nuclease (5 μg) was added at time zero. The precipitin reaction ordinarily began when nuclease was added to the cuvets.

Radioactive Labeling of Fragments. Trypsin fragments P_3 and P_2, and cyanogen bromide fragment E were labeled with ^{125}I by the iodine monochloride method of McFarlane (1958). P_3, 5.0 mg in 0.5 ml of 0.145 M saline buffered at pH 8.9, was reacted with a solution of 125 μl of ICl (0.06 M) and 50 μCi of [^{125}I]NaI (Radiochemical Centre, Amersham) in saline at pH 9.1. These conditions were calculated to provide about one iodine atom per molecule of P_3. The solution was acidified with 1 drop of glacial acetic acid, applied to a column (0.9 × 50 cm) of Sephadex G-25, and eluted with 0.01 M acetic acid. Fractions of 1.5 ml were collected every 10 min. Absorbance was read at 280 mμ, and 100-μl aliquots were counted for determination of ^{125}I in a Nuclear-Chicago γ scintillation counter. More than 90% of the counts was associated with the protein peak, which gave 3×10^6 cpm/mg. P_2 and fragment E (4 mg of each) were iodinated and separated from [^{125}I]NaI on Sephadex G-25 in exactly the same fashion, giving 5×10^6 and 9×10^6 cpm per mg, respectively.

Fragments P_3 and P_2 were also labeled with [^{14}C]acetic anhydride (New England Nuclear, 0.5 mCi/100 μmoles) in 0.5 M sodium acetate buffer at pH 5.5. These derivatives were acetylated selectively at the α-amino group of the poly-

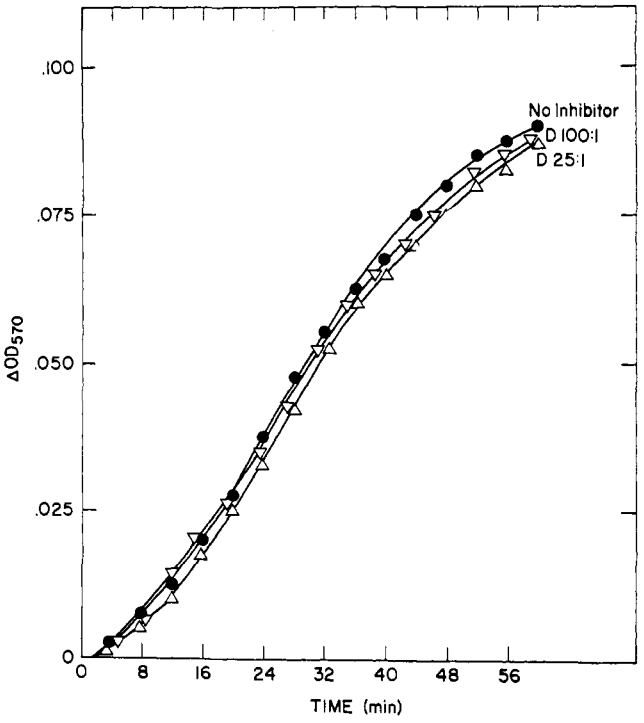

FIGURE 3: Inhibition of nuclease turbidimetric assay with fragment D (residues 66–98) in molar excesses shown.

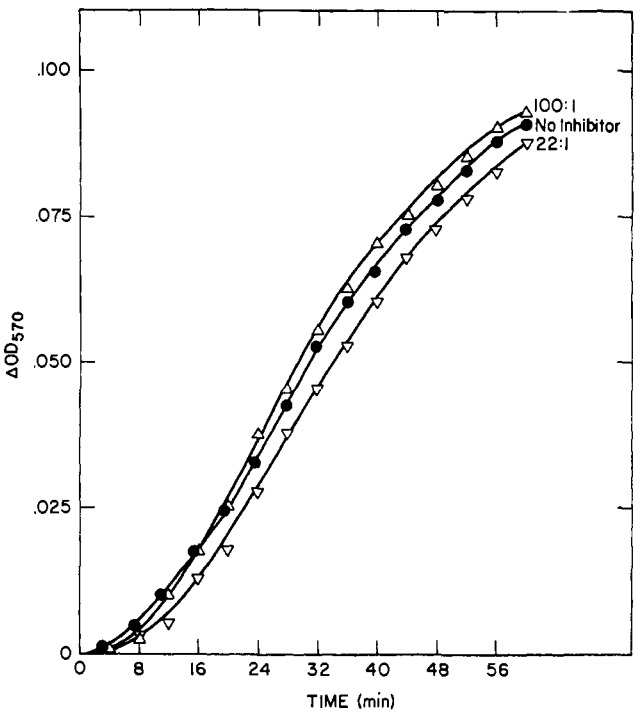

FIGURE 4: Inhibition of nuclease turbidimetric assay with fragment B (residues 27–32) in molar excesses shown.

peptide, with minimal modification of other residues, and were used to confirm studies with the iodinated derivatives.

Binding Experiments. All studies of the direct binding of radioactively labeled peptide fragments and of inhibition by unlabeled peptides were carried out using purified antinuclease antibody 14. Antibody solution (25 μl of 3 mg/ml) was added to a total volume of 0.5 ml of saline buffered with 0.05 M Tris-HCl at pH 7.5. Unlabeled peptide inhibitor was then added at room temperature and incubated at 4° for 24 hr. Finally, the labeled peptide was incubated with the mixture for 24 hr at 4°. Separation of bound from free labeled peptide was achieved by precipitation of antibody globulin with 50 μl of sheep anti-rabbit γ-globulin antiserum (gift of Dr. Saul Rosen). After 2 days at 4°, the precipitate was centrifuged, rinsed once with cold buffer, and resuspended in 1.0 ml of 0.1 N NaOH for measurement of the 280 mμ absorbancy of the precipitate. Dissolved precipitates consistently gave 280-mμ absorbancy of 0.54–0.58 under these conditions. A 500-μl aliquot of the supernatant (total volume 550 μl) and the solution of the entire precipitate were counted, usually for 10 min. Control tubes with normal rabbit IgG (Microbiological Associates, Inc.) in amounts that gave similar amounts of precipitation with sheep antiserum were used in each experiment to correct for nonspecific absorption of labeled peptide to the glass tubes or to the precipitate. Results are expressed as percentage of total counts bound in the precipitate, corrected for background and for nonspecific adsorption.

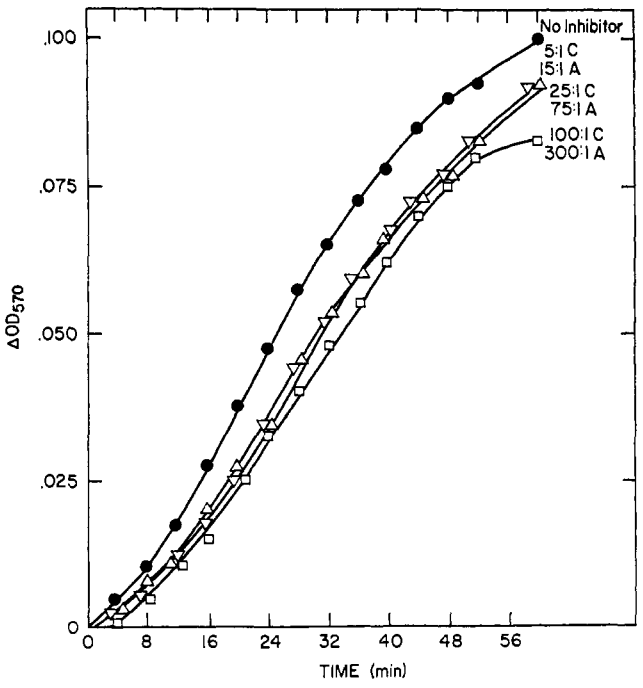

FIGURE 5: Inhibition of nuclease turbidimetric assay by mixture of fragments A (residues 1–26) and C (residues 33–65), in ratio A–C of 3:1, in molar excesses shown.

Results

Inhibition of Nuclease–Antinuclease Reaction by Fragments of Linear Sequence. The cyanogen bromide digest of nuclease, a mixture of five peptide fragments, gave no precipitation with antinuclease antibody either in agar immunodiffusion or in the turbidometric assay. However, both the cyanogen bromide digest and certain of the isolated fragments were

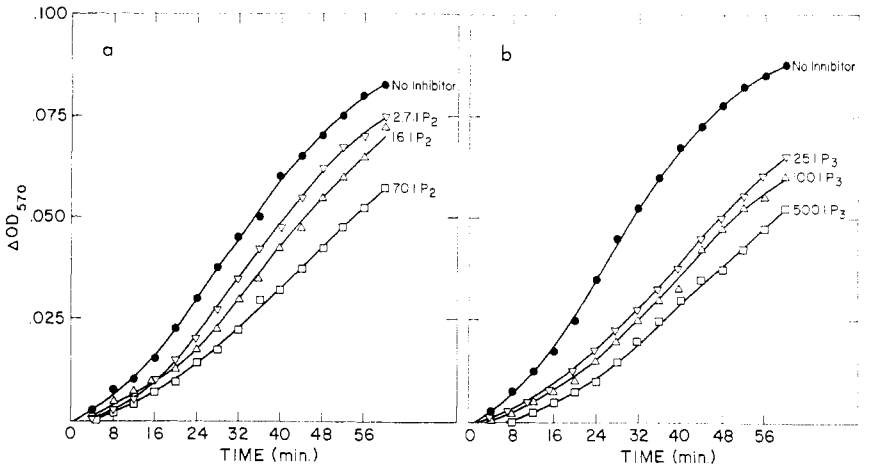

FIGURE 6: (a) Inhibition of turbidimetric assay of nuclease by tryptic fragment P_2 (residues 6–48). (b) Inhibition of turbidimetric assay of nuclease by tryptic fragment P_3 (residues 49–149).

demonstrated to inhibit the development of turbidity in the nuclease–antinuclease reaction. Figures 2–5 show typical results of such turbidimetric assays for fragment E (residues 99–149), fragment D (residues 66–98), fragment B (residues 27–32), and the combined fragments A + C (residues 1–26 and 33–65). Fragments D and B, even in very large excess, were incapable of inhibiting the reaction. Fragment E and the mixture of A + C were effective inhibitors, with extent of inhibition increasing with excess of peptide. These fragments are derived from quite distant portions of the primary structure of nuclease (Figure 1).

The fragments derived from limited trypsin digestion, P_2 and P_3, also failed to precipitate with antinuclease antibody, but were each effective inhibitors (Figure 6). Fragment P_2 and cyanogen bromide fragment E were additive in their inhibition (Figure 7). Testing the additive effects of P_2 and P_3 was complicated by the fact that these fragments associate noncovalently to form the enzymically active complex nuclease-T. As shown in Figure 8, incubation of P_2 with antibody or of P_3 with antibody, followed by addition of the complementing fragment, led to precipitation in a manner quantitatively similar to that of nuclease-T. Nuclease-T developed only 40% as much turbidity as nuclease under identical conditions (Figure 9), but its maximal rate of

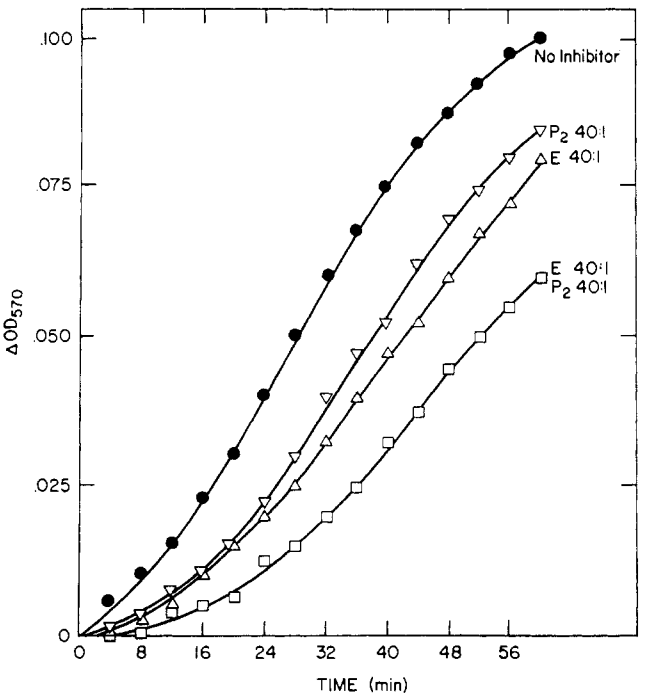

FIGURE 7: Inhibition of nuclease turbidimetric assay (●—●) by given molar excess of P_2 (▽—▽), of fragment E (△—△), and of P_2 and E together (□—□).

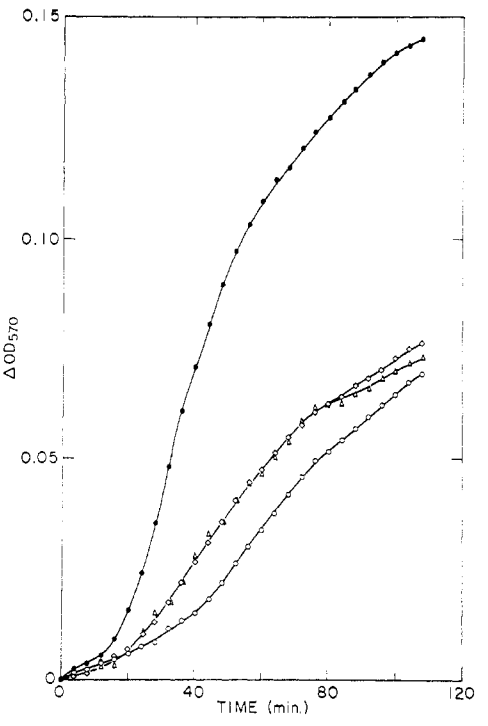

FIGURE 8: Turbidometric assays of nuclease (●—●) and nuclease-T (○—○); P_3 was added to a solution in which P_2 plus antibody had given a zero base line for 20 min (△—△), and P_2 was added to a solution in which P_3 plus antibody had given a zero base line for 20 min (□—□). All concentrations are 0.3 nmole of nuclease or derivative in total volume 1.0 ml. These concentrations give maximal rates for turbidity development in each system.

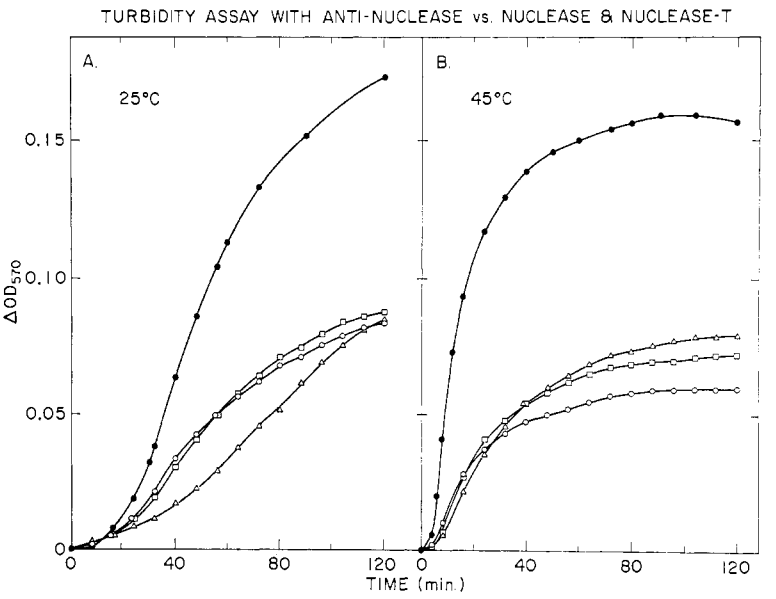

FIGURE 9: Turbidimetric assays of nuclease (●—●) and of three concentrations of nuclease-T at and near its equivalence point at 25° (A) and at 45° (B).

TABLE I: Binding of ^{125}I-P_3 and of ^{125}I-Fragment E.[a]

Amt ^{125}I-P_3 Added		^{125}I-P_3 Bound in Ppt		Amt ^{125}I-E Added		^{125}I-E Bound in Ppt	
μg	μμmoles	Net % cpm[b]	μμmoles[c]	μg	μμmoles	Net % cpm	μμmoles[c]
0.2	20	45.3	9	0.2	40	16.4%	6
0.4	40	35.0	14	0.4	80	10.1	8
0.8	80	33.2	26	1	200	6.8	14
2	200	32.1	64	3	600	4.6	28
4	400	24.9	96	10	2000	3.0	60

[a] All tubes contained 25 μl of purified antinuclease solution (75 μg), giving a final antibody concentration of 1 μM in 0.5-ml volume, pH 7.5 buffered saline. [b] Control experiments gave 5–9% of counts in the precipitate, counted in glass tubes with normal rabbit IgG, without significant dependence on amount of labeled antigen added in the range studied. Tests of time of incubation of labeled P_3 with the antibody solution from 10 min to 24 hr gave no significant difference, but solutions were routinely allowed to equilibrate overnight before addition of the sheep anti-rabbit γ-globulin antiserum. [c] Calculated from net per cent counts per minute in the antigen–antibody precipitate.

development of turbidity was attained with exactly the same amount added (0.3 nmole) as by nuclease. From agar immunodiffusion studies of nuclease-T, Taniuchi *et al.* (1967) estimated qualitatively that nuclease-T precipitated less well than nuclease. Figure 9 shows the turbidity formed in the reaction of nuclease-T with antibody, both at 25° and at 45°. At the latter temperature, nuclease-T in free solution would be fully dissociated into the nonprecipitating P_2 and P_3 fragments. The observation that nuclease-T still precipitates at this temperature suggests that antibody may stabilize the nuclease-T complex, as does the nucleotide ligand, deoxythymidine 3′,5′-diphosphate (Taniuchi *et al.*, 1967).

Control experiments with lysozyme or bovine serum albumin showed that large excesses of these unrelated protein molecules had no effect on the turbidimetric assay.

Antibody Binding of Radioactively Labeled Peptides Derived from the C-Terminal Portion of the Nuclease Sequence. ^{125}I-P_3 (residues 49–149) was bound directly by antinuclease antibody, with inhibition of binding by excess labeled or unlabeled P_3. Increasing quantities of ^{125}I-P_3, added to a fixed amount of antibody, gave a progressively smaller percentage of total counts bound (Table I). In a large number of experiments, the exact percentage varied somewhat with the batch of purified antibody and with dilution of labeled peptide to the desired concentration. Parallel experiments with [^{14}C]acetylated P_3 confirmed the specificity of binding and the cross-reactivity with unlabeled P_3.

^{125}I-Fragment E (residues 99–149) also was bound by the antinuclease antibody, and the percentage of labeled antigen bound decreased with increasing amount of antigen. Over the range from 0.2 to 10 μg, the net fraction bound fell from 16.4 to 3.0% (Table I). The antibody solution was capable of binding a higher percentage of labeled P_3 than of labeled fragment E at comparable molar concentrations

TABLE II: Inhibition of Binding of Labeled Fragments P_3 and E.

Inhibitor			Inhibitor		
Amt (μg)	Molar Ratio to ^{125}I-P_3 (2 μg)	Per Cent Inhibition	Amt (μg)	Molar Ratio to ^{125}I-E_3 (0.2 μg)	Per Cent Inhibition
Unlabeled P_3 (49–149)			Unlabeled fragment E (99–149)		
1	0.5:1	20	0.1	0.5:1	10
2	1:1	37	0.25	1.2:1	16
4	2:1	46	0.5	2.5:1	30
6	3:1	59			
Fragment E (99–149)			Fragment P_3 (49–149)		
60	60:1	8–25%[a]	0.2	0.5:1	28
			0.5	1.2:1	40
Fragment 127–149			1.0	2.5:1	51
1	2:1	13	Fragment 127–149		
5	10:1	25	0.2	2:1	23
16	30:1	36	10:1	10:1	31
80	150:1	34	5	50:1	40
			16	150:1	63
			80	750:1	73

[a] Inhibition of ^{125}I-P_3 by fragment E gave inconsistent results, for unexplained reasons, so a range of values is listed at a high ratio of inhibitor to labeled P_3.

(Table I). The greater binding of fragment P_3 may reflect greater affinity of binding, or a larger number of binding sites for P_3, or both.

Inhibition of Binding of ^{125}I-P_3 and ^{125}I-Fragment E. As shown in Table II, binding of ^{125}I-P_3 or of ^{125}I-fragment E could be inhibited by unlabeled peptides P_3, fragment E, and the fragment corresponding to residues 127–149. The results are given as percentage inhibition, compared with the averaged values for net binding of either 2 μg of ^{125}I-P_3 or 0.2 μg of ^{125}I-fragment E in the respective experiments. Synthetic hexapeptides corresponding to residues 83–88 and 134–139 gave no inhibition. Fragments D and A + C from cyanogen bromide cleavage gave no inhibition, even in high excess.

Fragment P_3 inhibited the binding of labeled fragment E better than did unlabeled fragment E, suggesting that the antibody had a higher affinity for P_3. The overlapping carboxyl-terminal fragment, $P_{127-149}$, gave marked inhibition with both labeled antigens, with greater inhibition of the binding of fragment E than of P_3 at the excesses used in both systems.

TABLE III: Binding of Labeled P_2 (Residues 6–48) to Antinuclease.[a]

^{125}I-P_2 (Native) Bound		Amt of Labeled Peptide Added		^{125}I-6-47 (Synthetic) Bound	
Net % cpm	$\mu\mu$-moles[b]	μg	$\mu\mu$moles	Net % cpm	$\mu\mu$-moles[b]
39.9	8	0.1	20	20.0	4
34.3	14	0.2	40	16.8	7
24.5	20	0.4	80	13.8	11
20.2	32	0.8	160	13.4	21
15.6	62	2	400	12.3	48
12.2	98	4	800	8.5	68

[a] All tubes contained 25 μl of purified antinuclease antibody solution (75 μg), giving a final antibody concentration of 1 μM in 0.5-ml volume pH 7.5, buffered saline. [b] Calculated from net percentage counts per minute in the antigen–antibody precipitate.

TABLE IV: Inhibition of Labeled P_2 Binding with Synthetic Fragments.

Inhibitor	Molar Ratio to ^{125}I-P_2 (0.2 μg)	Per Cent Inhibition	Inhibitor	Molar Ratio to ^{125}I-6-47 (0.2 μg)	Per Cent Inhibition
6–47	5:1	49	6–47	2:1	61
	50:1	73		5:1	77
9–47	5:1	49		10:1	93
	50:1	78	9–47	2:1	34
18–47	7:1	48		5:1	80
	70:1	58		10:1	89
33–47	14:1	1	18–47	7:1	17
	35:1	0		14:1	53
	140:1	3		70:1	66
			33–37	14:1	0
				35:1	0
				140:1	0

Binding of Radioactively Labeled Peptides Derived from the N-Terminal Portion of Nuclease. The peptide P_2 derived from native nuclease (residues 6–48) and the synthetic peptide product corresponding to residues 6–47 were labeled with ^{125}I at the single tyrosine residue (position 27). Binding to antinuclease antibody was readily demonstrated, with greater specific binding of the native fragment than of the synthetic product at all concentrations (Table III). As in the binding of labeled P_3, higher excesses of either labeled or unlabeled antigens reduced the percentage bound in these systems.

Inhibition of Binding of ^{125}I-P_2 and of ^{125}I-6–47 (Synthetic). Labeled peptide (0.2 μg of each) was used in the studies of inhibition of binding by analogs available from synthetic work (Ontjes and Anfinsen, 1969b). As shown in Table IV, unlabeled sequences corresponding to residues 6–47 and 9–47 were equally effective in inhibiting binding, when tested against either the native or synthetically labeled antigen. Fragment 18–47 was somewhat less effective, suggesting a decrease in binding affinity, but continued recognition of the antigenic determinant. The fragment containing only residues 33–47 did not inhibit at all, even at greater excess.

Binding of Radioactively Labeled Complexes of P_2 and P_3. Neither P_2 nor P_3 inhibited the binding of the labeled comple-

TABLE V: Enhancement of Binding of Labeled P_3 by P_2 Fragments.

Peptide	Molar Ratio to ^{125}I-P_3 (2 μg)	Net % cpm Bound
None		29.6
6–47	1:1	27.9
	2.5:1	44.1
	10:1	65.1
9–47	1:1	28.6
	2.5:1	42.1
	10:1	60.6
18–47	1.4:1	23.4
	3.5:1	32.3
	14:1	42.7
33–47	7:1	27.8
	28:1	25.7

mentary fragment. Instead, a marked enhancement of binding was observed. The enzymatically active complex, nuclease-T, is formed by noncovalent interaction of P_2 and P_3, usually carried out at concentrations of about 1 mg/ml (Taniuchi and Anfinsen, 1968). In the binding experiments (Table V), labeled P_3 (2 μg/0.5 ml) was added to a solution in which 1 to 10 μg of P_2 or synthetic fragments had been equilibrated with 75 μg of antinuclease IgG. As suggested from the turbidometric assay of nuclease-T binding to antibody at elevated temperature (Figure 9b), antibody may act to stabilize any nuclease-T formed and thus greatly favor the combination of P_2 and P_3 even at these low concentrations. Complementary experiments with the addition of unlabeled P_3 to solutions of labeled P_2 have demonstrated enhancement of binding of P_2, presumably also by formation of nuclease-T with higher affinity.

The enhancement of binding of labeled P_3 by the apparent generation of nuclease-T served as a sensitive assay for the ability of synthetic 6–47 sequence fragments to complex with P_3. As shown in Table V, 6–47 and 9–47 were about equally effective, 18–47 effective but clearly less so, and 33–47 altogether ineffective in enhancing the binding of ^{125}I-P_3. Exactly the same pattern of results was obtained with [^{14}C]acetyl-P_3, with enhancement of binding from 5% to a maximum of 40–45%. Native P_2 gave such a maximal effect at P_2–P_3 ratio of 1:4, while the synthetic 6–47 required at least a 3:1 excess. These results corroborate enzymatic, affinity chromatographic, and fluorescence studies on the binding to fragment P_3 of synthetic fragments representing residues 6–47, 9–47, 18–47, and 33–47 of the nuclease sequence (Ontjes and Anfinsen, 1969b).

Antigenicity of Nuclease Fragment 1-126. The large fragment, P_{1-126}, produced by limited tryptic digestion of trifluoroacetylnuclease, also is incapable of precipitating with antinuclease antibody (Taniuchi and Anfinsen, 1969). However, at a molar ratio of 1:3, P_{1-126} inhibited the binding of native P_2-^{125}I by 16% and at a ratio of 1:1 P_{1-126} inhibited binding by 33%.

This fragment, like P_2, is able to interact noncovalently with P_3 to generate a complex similar to nuclease-T. It was,

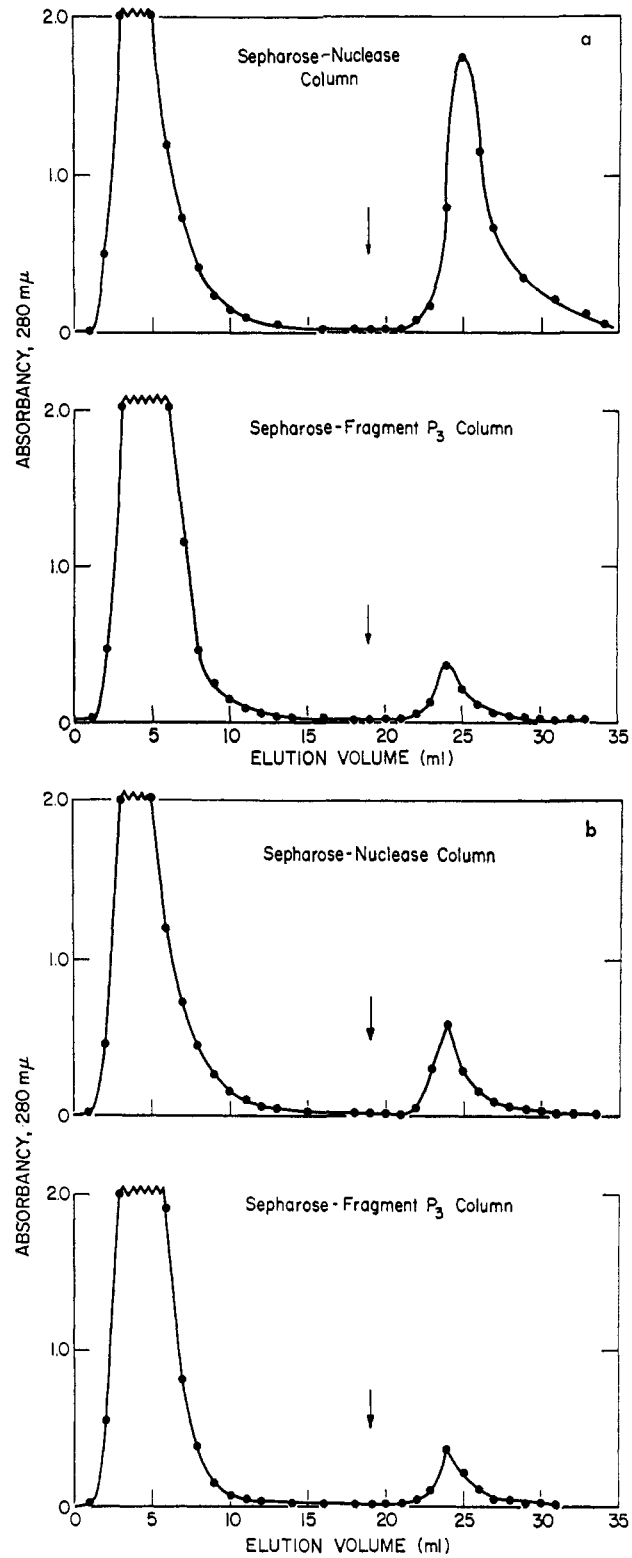

FIGURE 10: (a) Immunoadsorption of antinuclease γG14 on nuclease–Sepharose (top) and on P_3–Sepharose (bottom). A 1.0-ml sample containing about 17 mg of protein was applied to each column (0.9 × 5 cm) at 4°. (b) Immunoadsorption of antiperformic acid oxidized nuclease γG15 on nuclease–Sepharose (top) and on P_3–Sepharose (bottom). A 1.0-ml sample containing about 20 mg of protein was applied to each column as above. Adsorption on nuclease–Sepharose removed all antibody reacting with performic acid oxidized nuclease.

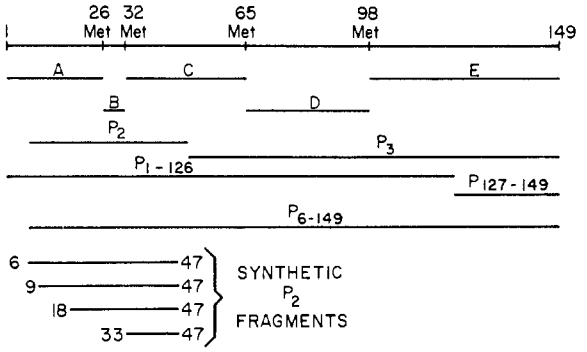

FIGURE 11: Line diagrams of the linear sequence fragments tested in these studies of antigenicity of staphylococcal nuclease (drawn to scale).

therefore, of interest to test its ability to enhance binding of ^{125}I-P_3 to antibody. At ratios of 1:5 and 1:1 compared with ^{125}I-P_3, P_{1-126} and native P_2 both enhanced binding to the same extent.

Fractionation of Antibody Preparations on P_3–Sepharose Immunoadsorbents. γ-Globulin preparations of antisera elicited in rabbits by native nuclease (γG14) and by the performic acid oxidized nuclease (γG15) (Omenn *et al.*, 1970a,b) were subjected to immunoadsorption on columns on nuclease–Sepharose and of P_3–Sepharose (Omenn *et al.*, 1970b). A much higher proportion of total γ-globulin was bound to the nuclease–Sepharose column in the fractionation of γG14 than in the fractionation of γG15 (Figure 10). On the P_3–Sepharose column, however, both γ-globulins appeared to yield about the same quantity of bound antibody, recognizing the sequence fragment, P_3. The fact that less γG15 adsorbed to nuclease–Sepharose may be due to a lack of conformational determinants or a decrease in affinity in the immunogenicity of the performic acid oxidized nuclease. The abundant evidence that nuclease-T was bound with much greater affinity than either P_2 or P_3 alone provides further support for the contention that conformation either offers additional determinants or increased affinity.

Discussion

Fragments of the sequence of staphylococcal nuclease that are immunoreactive but which do not precipitate with antinuclease antibody have been prepared. Studies on the inhibition of a turbidometric assay of the reaction between nuclease and antinuclease, of direct binding of radioactively labeled peptides to antibody, and of the inhibition of such binding by other unlabeled peptides have all provided evidence that two major sequential determinants may be distinguished in nuclease. The overlapping sequences of all peptides tested, both native and synthetic, are drawn to scale in Figure 11.

One linear antigenic determinant may be assigned to the C-terminal portion, probably within the sequence 127–149, corresponding to the peptide which was effective in inhibiting the direct binding of both fragments 99–149 and 49–149. Apparently the remainder of the sequence, present in fragment 1–126, lacks the minimum of two distinguishable antigenic determinants necessary for precipitating with antibody.

Within the N-terminal portion of nuclease, fragment P_2 (6–48) appears to contain the same sequential determinant that is present in the larger, overlapping 1–126 fragment. The synthetic 9–47 sequence was as antigenic as 6–48, and 18–47, though bound less well, still carried the determinant of the larger sequences. Further shortening of the sequence to 33–47 led to loss of all immunoreactivity. Thus, the sequential determinant of the N-terminal portion of nuclease may depend upon the sequence 18–33. In the cyanogen bromide peptide mixture, A + C (see Methods), it is probably peptide A that is responsible for the inhibition of turbidity formation, since it contains the 1–26 overlapping sequence.

We must emphasize that the data also strongly implicate a role of conformation in the antigenicity of nuclease. Nuclease-T was bound more avidly than either of its two components, P_2 or P_3. The large fragment 1–126, which lacks any ordered conformation but possesses 85% of the linear sequence of nuclease, binds to antinuclease antibody but does not precipitate. In general, the binding capacity of antibody for native nuclease is at least an order of magnitude greater than its capacity for nuclease fragments. From the turbidimetric and quantitative precipitin assays, 75 μg of antibody 14 would be at equivalence with about 2 μg of nuclease. In the direct binding studies, 0.2 μg of either P_2 or P_3 was sufficient to bring the antigen–antibody binding curves to regions of less than 50% binding. Finally, when antinuclease γG is passed over an affinity column containing the large fragment P_3 (67% of the total sequence), the quantity of γG bound is only 10% of the quantity binding to a comparable column containing native nuclease.

The finding that nuclease-T is more avidly bound than its complementing fragments has provided an additional assay for detection of the binding of P_2 to P_3. The relative abilities of several synthetic P_2 analogs to enhance the binding of labeled native P_3 to antibody corroborates conclusions from other studies on the properties of these analogs (Ontjes and Anfinsen, 1969a, b).

Specific regions of the linear sequence of other well-characterized proteins have been assigned as antigenic determinants. In myoglobin, the C-terminal pentapeptide or heptapeptide (148–153 or 146–153) inhibited the precipitation of myoglobin with antimyoglobin (Crumpton and Wilkinson, 1965) and served to elute antibody adsorbed to a myoglobin immunoadsorbent (Givas *et al.*, 1967). In pancreatic ribonuclease A, Brown (1962) has shown the C-terminal sequence 105–124, as well as the sequence 38–61, to be capable of inhibiting the reaction of performic acid oxidized ribonuclease with its antiserum. In tobacco mosaic virus protein, a decapeptide corresponding to the sequence 103–112 appears to have the full antigenic activity of the protein subunit, and as few as three residues at the C-terminal end of this decapeptide may be recognized by antibody if bound to a hydrophobic octanoyl group (Benjamini *et al.*, 1969). In lysozyme, a disulfide-linked peptide, comprising residues 57–83 and 91–107, was immunoreactive (Shinka *et al.*, 1962) and served to adsorb specific antibodies when coupled to bromoacetylcellulose as an immunoadsorbent (Arnon, 1968). The antigenic determinant may be localized to the 64–83 "loop" (Arnon and Sela, 1969). Another disulfide-linked peptide of lysozyme, comprising residues 1–27 and 122–129, has also proved to be antigenic (Fujio *et al.*, 1968).

As in the results with staphylococcal nuclease, these antigenic sequence fragments account for only a part of the anti-

genicity of the intact protein. Nuclease differs from many other globular proteins by its relatively low helix content and by its lack of disulfide structure. Perhaps because of its relatively high flexibility in solution it is especially susceptible to antibody recognition of both conformational and linear determinants.

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