Studies on the Complex Formation between Deoxyribonuclease I and Spleen Inhibitor II*

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ABSTRACT: The product of the reaction between deoxyribonuclease I (DNase I) and inhibitor II from calf spleen was studied by Sephadex G-100 chromatography, disc electrophoresis, and ultracentrifugation. Under a number of experimental conditions, in which the proportions of inhibitor and nuclease were varied over a wide range, only one type of complex was found consisting of 1 mole each of DNase I and inhibitor II.

Most of the information concerning the mechanisms of action of proteinaceous inhibitors of DNase I has been derived from studies of the kinetics of nuclease inhibition by protein preparations from testes (Cooper *et al.*, 1950), liver, brain, and lung (Festy and Paoletti, 1963). The different mechanisms that have been suggested from these studies have included binding of inhibitor either to the nuclease (Cooper *et al.*, 1950; Festy and Paoletti, 1963; Berger and May, 1964) or to the DNA substrate (Zalite and Roth, 1964). This author previously presented evidence for the formation of a complex between DNase I and DNase–inhibitor II purified from calf spleen (Lindberg, 1964b, 1966). The present paper describes an investigation of this complex with respect to homogeneity and stoichiometry of formation. The results indicate the presence of only one type of complex (molecular weight between 81,600 and 88,500) from which native DNase could be dissociated.

Materials and Methods

DNase I (Sigma Chemical Co., once crystallized) was purified by chromatography on Sephadex G-100 as described previously (Lindberg, 1967b). *Spleen inhibitor II*, purified by a method described by Lindberg (1967a), was concentrated after chromatography on hydroxylapatite and refiltered before each experiment on a short column (1 × 50 cm) of Sephadex G-100 (equilibrated with 0.5 M potassium phosphate buffer, pH 7.6) to remove aggregates. In most experiments the peak fractions containing native inhibitor II either were used directly or, when necessary, were concentrated by ultrafiltration.

Assay for DNase–Inhibitor Complex. Two samples, equal in volume (5–20 μl), were taken for the analysis. To one of the samples 5–20 μl of 0.5 M HCl was added to obtain a pH of 3.5; the other sample was left untreated. Standard DNA substrate (3 ml) (Lindberg, 1967a) was added to each sample and the amount of DNase activity was measured spectrophotometrically (Lindberg, 1964a). Although enzyme activity usually was observed in both samples, the acidified one was at least ten times more active than the control. This phenomenon resulted from a rapid denaturation of the inhibitor under conditions below pH 4.0, while the DNase remained stable.[1] Assays of DNase and DNase–inhibitor activities, disc electrophoresis, and ultracentrifugation were performed as described previously (Lindberg, 1967a,b).

Results

Stoichiometry of the Complex Formation between DNase and Inhibitor

Chromatography on Sephadex G-100. Results of previous experiments (Lindberg, 1966) suggested the formation of only one type of enzyme–inhibitor complex. However, the possible existence of other types of complexes required a more thorough investigation by chromatography on Sephadex G-100 (see Figure 1), a method which was expected to separate different enzyme–inhibitor complexes from each other and from native inhibitor and enzyme. In expt A and B, respectively, inhibitor[2] (2.0 ml) and DNase I (0.8 mg) were filtered separately on two identical columns (1 × 170 cm) to demonstrate their homogeneity with respect to molecular weight and to determine their positions on the chromatograms. In expt C–G identical amounts of DNase (0.8 mg) were mixed with decreasing amounts of inhibitor (8.0, 4.0, 2.0, 1.0, and 0.5 mg, respectively). In each case the mixtures were passed through columns identical with those used in expt A and B.

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[1] Instead of acidifying the sample, one could add *p*-hydroxymercuribenzoate to denature the inhibitor and unmask the DNase activity.

[2] Where not otherwise stated, this paper deals with DNase–inhibitor II from calf spleen.

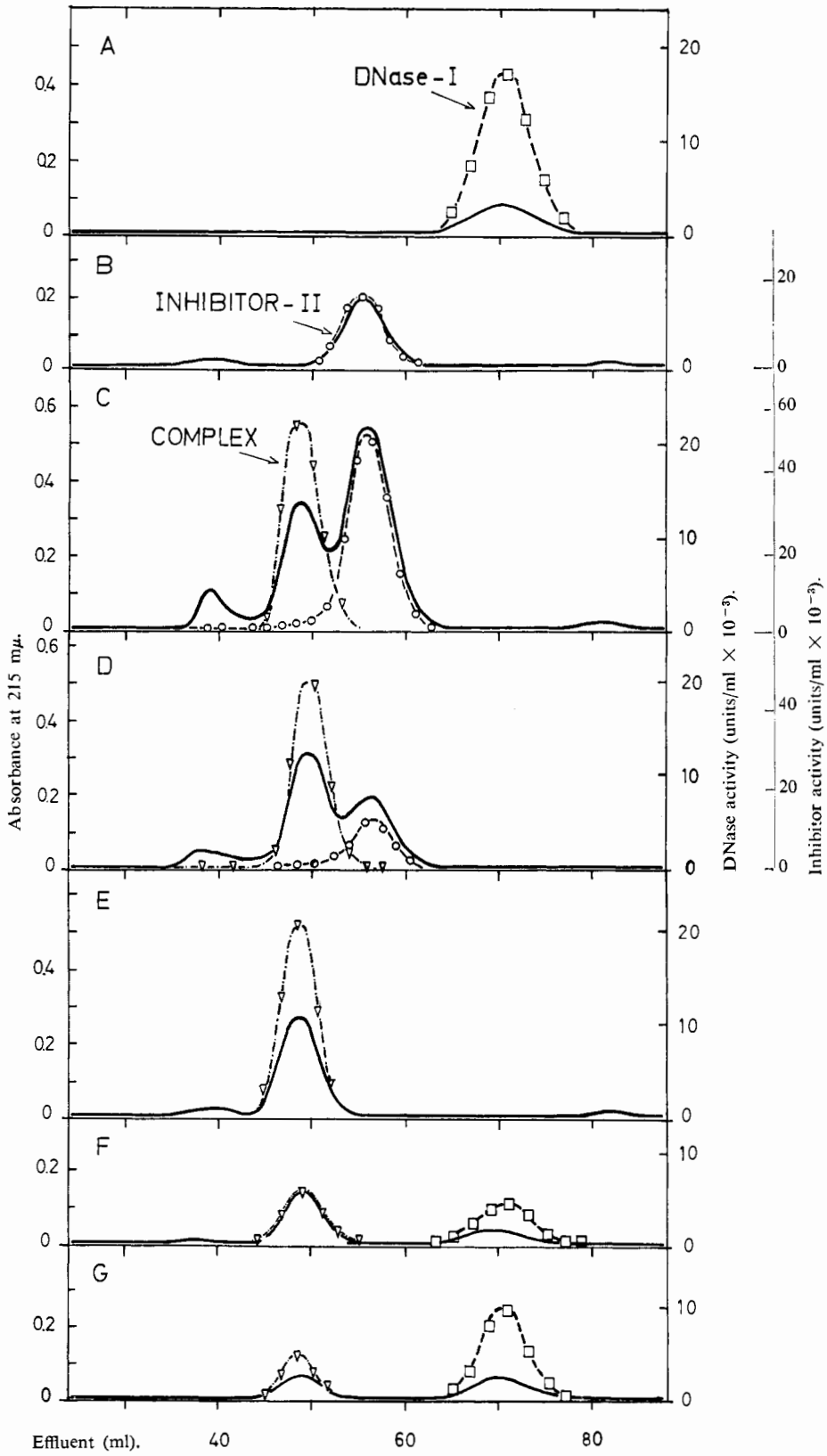

FIGURE 1: Sephadex (G-100) chromatography of DNase I, inhibitor II, and mixtures containing the two proteins in different proportions. The amount of protein used in the different experiments is given in the text. Absorbance at 215 mμ (solid line ———) was measured after a 20-fold dilution with water using a similarly diluted blank of the elution buffer (0.5 M potassium phosphate buffer, pH 7.6). Each chromatogram was analyzed for DNase activity (open squares, —□—□—□—), inhibitor activity (open circles, —O—O—O—), and for the presence of the DNase–inhibitor complex, in this figure represented as DNase activity, which was measured on samples of the fractions after adjustment of the pH to 3.5 with HCl (open triangles, —·—▽—·—▽—·—).

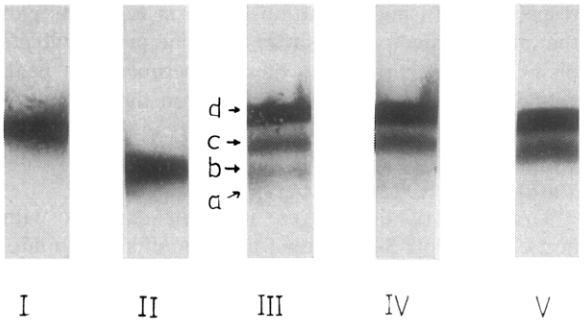

FIGURE 2: Disc electrophoretic analyses of (I) inhibitor II, (II) DNase I, and (III–V) mixtures of the DNase and the inhibitor in the proportions given in the text. The discontinuous buffer system of Ornstein (1964) and Davis (1964) was used. In this system electrophoresis and molecular sieving of protein molecules occur at pH 10.3.

In all five experiments the enzyme–inhibitor complex appeared in the same position on the chromatogram despite alteration of the relative proportions of inhibitor and enzyme in the initial mixture. In expt C and D (molar excess of inhibitor) the inhibitor peak appeared immediately behind that of the complex. In expt F and G (molar excess enzyme) the inhibitor peak was missing and a peak appeared behind that of the complex in the position expected for DNase. Finally, in expt E, in which approximately equimolar amounts of inhibitor and enzyme had been mixed, only the complex peak could be seen on the chromatogram. The small peak emerging in the chromatograms with the void volume (around 40 ml) probably represented aggregated material present in the inhibitor preparation (*cf.* expt B). No measurable DNase activity was found in this material and the size of the peak was a function of the absolute amount of inhibitor added during the start of the experiment.

Disc Electrophoresis on Polyacrylamide Gel. Complex formation also was analyzed by disc electrophoresis on polyacrylamide gels. In these experiments fresh mixtures of DNase and inhibitor were added in different proportions to a series of electrophoresis tubes and analyzed in the same electrophoretic run. Figure 2 shows the stained polyacrylamide gels (I–V) obtained in such an experiment. Numbers I and II demonstrate the patterns obtained with 30 μg of inhibitor and 20 μg of DNase, respectively. The patterns of tubes III–V were obtained with mixtures of 20 μg of DNase with 30, 40, and 50 μg of inhibitor, respectively. The protein bands on the gels are lettered a–d.

The interpretation of this experiment is more difficult than the interpretation of the Sephadex G-100 experiment for the following reasons. (1) No analysis of enzyme or inhibitor activity could be performed; therefore the interpretation rests completely on the appearance of the protein bands. (2) At the high pH values used during the electrophoresis secondary changes, such as minor dissociation of the complex, may have occurred. Nevertheless, it was clear that in all three experiments (III–V), in which a mixture of enzyme and inhibitor were analyzed, only one new band (band d) was formed. This band probably represents the enzyme–inhibitor complex. Band c corresponds in position to the inhibitor band and increases from expt III to V (increasing amounts of inhibitor added). The reverse is true for band b, which corresponds in position to DNase. Band a apparently arises from DNase (*cf.* expt II).

Ultracentrifugal Analyses. Approach to sedimentation equilibrium studies, performed according to Ehrenberg's (1957) modification of the method of Archibald, were used to determine the molecular weight of the isolated enzyme–inhibitor complex. For this purpose three different mixtures of enzyme and inhibitor were chromatographed on Sephadex G-100, and the purified complex was analyzed directly in the ultracentrifuge. Mixture A contained equimolar amounts of enzyme and inhibitor, mixture B an excess of enzyme, and mixture C an excess of inhibitor. The proportions are given in Table I, together with the molecular weights

TABLE I: The Molecular Weight of the DNase–Inhibitor Complex.[a]

Expt	Reaction Mixture Filtered through Sephadex G-100 DNase I (mg)	Inhibitor II (mg)	Mol Wt of Isolated Complex
A	5	10	81,600
B	14	7	82,700
C	3.5	14	88,500

[a] DNase I and inhibitor II were mixed in different proportions and the resulting complex was isolated by filtration on a column of Sephadex G-100 in 0.5 M potassium phosphate buffer, pH 7.6. Before ultracentrifugation the samples were equilibrated with 0.1 M potassium phosphate buffer, pH 7.6, on Sephadex G-25.

obtained for the three different complexes. The expected molecular weight for a 1:1 addition compound between DNase (molecular weight 31,000) and inhibitor (molecular weight 60,000) is 91,000. The experimentally determined results approached this value but were somewhat lower in all three cases. This observation may have been an indication of possible dissociation of the enzyme–inhibitor complex during the ultracentrifugal run.

Table II contains sedimentation velocity and diffusion data for the isolated enzyme–inhibitor complex. Sedimentation experiments were performed in 0.1 M potas-

TABLE II: Sedimentation and Diffusion of the Isolated DNase–Inhibitor Complex.[a]

Sedimentation		Diffusion	
Protein Concn (mg/ml)	$s_{20,w}$ ($\times 10^{-13}$ sec)	Protein Concn (mg/ml)	$D_{20,w}$ ($\times 10^{-7}$ cm²/sec), 5227 rpm
7.50	4.62	9.28	5.0
4.80	4.75		
1.90	4.87		
0.00	4.94		

[a] The experimental conditions and the calculations of the $s_{20,w}$ and $D_{20,w}$ were identical with those employed in the characterization of spleen inhibitor II (Lindberg, 1966b).

sium phosphate buffer, pH 7.6, at three different protein concentrations. When the values were extrapolated to zero concentration a $s_{20,w}$ value of 4.95×10^{-13} sec was obtained. The concentration dependence of $s_{20,w}$ seen here is approximately the same as that seen earlier with the free inhibitor (Lindberg, 1967a). No such concentration dependence was observed with DNase (Lindberg, 1967b).

Figure 3 depicts the Schlieren patterns from one of the sedimentation experiments with the enzyme–inhibitor complex. A single symmetric peak was seen at all time points and thus this experiment does not reveal any impurities in the DNase–inhibitor complex.

Stability of Enzyme–Inhibitor Complex. A number of experimental approaches failed to achieve a reversible dissociation of the enzyme–inhibitor complex. Instead only irreversible dissociation, accompanied by the appearance of DNase activity, was accomplished. Nevertheless, conditions resulting in irreversible dissociation were also of interest, since they gave information about the nature of the forces holding DNase and inhibitor together. A brief description of these experiments is given below.

The stability of the complex was studied under a variety of conditions by filtration through Sephadex G-100. When the complex was filtered in 0.1 M potassium phosphate buffers of pH 5.8, 6.5, and 9.5 complete stability was observed. However, at pH 10.5 and 11.3, respectively (using a solution of 0.1 M K_2HPO_4 with the pH adjusted with NaOH), partial and complete dissociation occurred. In these experiments a peak containing enzyme activity appeared on the chromatogram in the position of DNase, whereas the inhibitor was eluted in an aggregated, inactive form with the void volume.

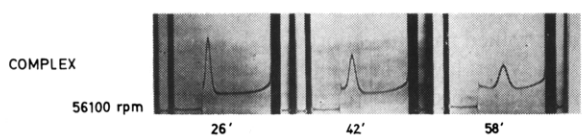

FIGURE 3: Sedimentation of the isolated DNase–inhibitor complex in 0.1 M potassium phosphate buffer, pH 7.6. The protein concentration was 7.5 mg/ml.

Treatment of the enzyme–inhibitor complex with acetic acid to pH 5 led to the formation of a precipitate. If this material was immediately dissolved at pH 7.6, the enzyme–inhibitor complex could be recovered intact. However, if the precipitate remained at pH 5 for extended periods (1–2 hr), or if the pH was lowered to approximately 4, DNase was solubilized from the complex, and the inhibitor (never recovered in an active form) apparently remained in the precipitate.

The effect of high salt concentrations on the enzyme–inhibitor complex was investigated. The complex was not dissociated during gel filtration at pH 6.0 or 7.6 in 0.75 M potassium phosphate buffer. In concentrations of the potassium phosphate buffer greater than 0.8 M the complex was precipitated, but still no apparent dissociation occurred. Similarly, a precipitate containing stable complex was formed on precipitation with ammonium sulfate at pH 5.8, 7.0, and 7.6.

An increase in the dielectric constant of the medium did not lead to dissociation of the complex. Thus the inclusion of a high concentration of glycine (1.5 M) during chromatography on Sephadex G-100 in 0.1 M potassium phosphate buffer, pH 6.0, 7.6, and 9.6, resulted in the recovery of an unchanged complex.

Two final experiments demonstrating the irreversible dissociation of the enzyme–inhibitor complex by urea and by exposure to an alkaline pH are illustrated in Figure 4. The complex (5 mg) was dialyzed for 6 hr against either 3 M urea in 0.05 M potassium phosphate buffer (pH 7.6) or 0.04 M NaOH in 0.1 M K_2HPO_4, pH 11.3. Each sample was filtered immediately through a column of Sephadex G-100 (1 × 50 cm), equilibrated with 0.1 M potassium phosphate buffer, pH 7.6. Fractions of 1–2 ml were collected every 30 min and assayed for the presence of DNase, inhibitor, and complex. In both experiments only two peaks were recovered. The first peak coincided with the void volume and contained aggregated inactive inhibitor. The second peak contained DNase activity and was located on the chromatogram in the position expected for the enzyme.

Discussion

The mechanism of action of the two proteins from calf spleen inhibiting DNase I involves a direct interaction between the enzyme and the inhibitor proteins. It was reported earlier (Lindberg, 1966) that this interaction resulted in the formation of a stable complex, which could be isolated by filtration through Sephadex G-100. In the case of inhibitor II a 1:1 stoichiometry was indicated.

In the present paper the interaction between inhibitor II and DNase was studied further. Both the filtration

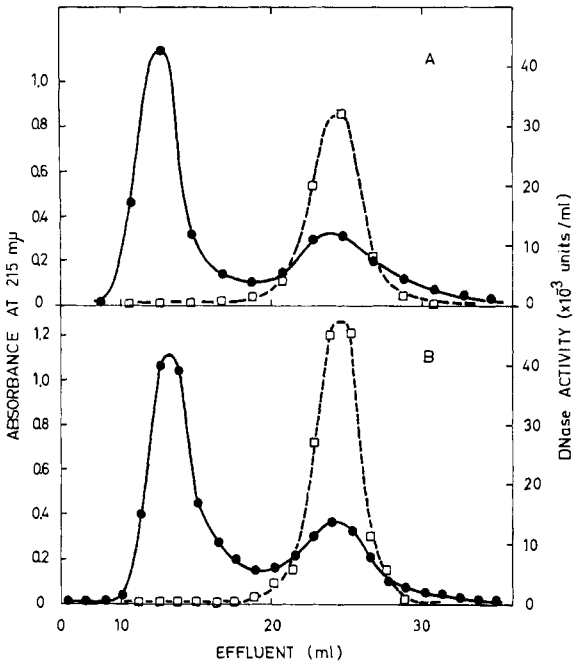

FIGURE 4: Separation on Sephadex G-100 of the components of the DNase–inhibitor complex after dissociation in: (A) 3 M urea in 0.05 M potassium phosphate buffer, pH 7.6, and (B) 0.04 M NaOH in 0.1 M K_2HPO_4, pH 11.3. The solid lines (——) represent absorbance at 215 mμ and the open squares (—□—□—□—) depict the DNase activity of the fractions.

experiments on Sephadex G-100 and the analyses by disc electrophoresis demonstrated that only one type of complex could be isolated from mixtures of native inhibitor and DNase I, regardless of the relative proportions of the reacting proteins.

The molecular weight determinations in the ultracentrifuge indicated that 1 mole of inhibitor II was bound to 1 mole of the enzyme under the ionic conditions used. The chromatography of the complex on Sephadex G-100 and the ultracentrifugations were performed at *high* ionic strength (KPO_4 buffer, pH 7.6; 0.5 and 0.1 M, respectively). Therefore one cannot be sure that other stoichiometries do not exist under other ionic conditions. In this connection it should be mentioned that a few experiments have indicated that aggregated inhibitor material complexes with DNase to some extent. However, on a weight basis the aggregated inhibitor bound much less enzyme, and in general the size of the aggregates was inversely related to the efficiency of inhibition.

The molecular weight of the complex as measured by the Archibald method is somewhat lower than expected for a 1:1 stoichiometry. This might indicate that the complex preparation is heterogeneous, and interspersed with impurities of lower molecular weight. Such impurities may result either from an incomplete separation of the complex and the inhibitor (or impurities of the same size as that of the inhibitor) during chromatography on Sephadex or from dissociation of the complex during the ultracentrifugation, or both. Moreover, some of the deviations between the values were due to the large experimental error of the method which has been estimated to be as much as 3.5% (Ehrenberg, 1957). Under certain circumstances the error of the method can be even larger, as was reported by La Bar (1966a,b).

The complex is dissociated either by extremes of pH or by the addition of a disruptive agent such as urea. In all attempts at disruption no active inhibitor can be recovered. In general the complex is stable under conditions in which the inhibitor protein itself is stable. When the complex is exposed to conditions which are known to inactivate the inhibitor, irreversible dissociation occurs.

Glycine protects the inhibitor under otherwise inactivating conditions (Lindberg, 1967a). Combination of glycine with a phosphate buffer of high ionic strength gives still better stabilization. Similarly glycine (1.5 M) prevents the dissociation of the complex by urea (6 M).

The results of these experiments suggest that the primary event in the dissociation process is a disruption of the native configuration of the inhibitor protein that secondarily leads to disruption of the complex and finally rapid aggregation of the denatured inhibitor molecules. After this series of events, functional reassociation of the inhibitor with the enzyme becomes exceedingly difficult.

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