Studies on Acid Deoxyribonuclease

X. Molecular Weight in Denaturing Solvents

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Hog spleen acid DNAse, an enzyme which peptide mapping indicates is formed in a similar or identical subunit, has been subjected to experiments designed to determine the molecular weight of the monomeric subunit. The molecular weight as determined at low pH and in the presence of 0.1 M guanidine, with or without added 8-mercaptoethanol, was found to be very close to that of the native enzyme molecule. It is concluded that the enzyme molecule cannot be dissociated into subunits under a variety of conditions which are extremely effective with other proteins.

Acid deoxyribonuclease from hog spleen (EC 3.1.4.5: deoxyribonuclease I) has been the object of extensive investigations during the past 10 years (see Refs. 1 and 2 for recent articles).

This enzyme is able to split native, double-stranded DNA according to a biphasic and a hypodiploid mechanism. The first mechanism involves the simultaneous breakage of both DNA strands at the same level, the second one the splitting of one strand at a time. When the native mechanism of action of the enzyme was first recognized (3, 4), two possibilities were considered in order to explain it: (a) the enzyme has only one active site and splits one phosphodiester bond at a time, two such breaks might occur in rapid succession on phosphodiester bonds belonging to two complementary, monomeric sequences on the opposite strands; (b) the enzyme has a dimeric structure; the two different types of attack

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might be due to the fact that both active sites or only one active site are operative according to the complementary sequences found by the enzyme on double-stranded DNA.

Two findings seemed to indicate that the enzyme had indeed a dimeric structure. The first one was that the map of tryptic peptides of acid DNAse showed 17-19 peptides (5, 6), whereas the number of arginine and lysine residues of the enzyme molecule (and the = 35,000) was 32-46 (6). The second finding was that the sedimentation coefficient of acid DNAse (3.4S at zero concentration) was only 0.8S in the presence of 0.1 M NaCl, 0.1 M 8-mercaptoethanol (at 19°C, concentration: in the presence of 0.1 M NaCl uncorrected und mercaptoethanol two hemisates were seen having a value of 0.08S and 0.1 S, respectively, as described elsewhere). Later work on guanidine showed that the sedimentation coefficient at 1°C concentration was 1.58S at 1.8 M guanidine. The sedimentation coefficient was 1.38S; (1). The further decrease was not unexpected since the eight hydrophobic residues of acid DNAse form four disulfide bridges (H. Bernaud, unpublished results cited in Ref. 1). Some recent results on covalent bond formation and S values observed in the still too high for a polypeptide chain of this length, which would be present in a molecule of 65,000 or 45,000. The acid DNAse in denaturing solvents, has been investigated.

EXPERIMENTAL

Materials

Two hog spleen acid DNAse used as previously described were employed. Commercially available from BRL Co., and from Merck Sharp & Dohme (lot No. 110612-14-6) were not identical.

Sedimentation and protein determinations in the cell of RCL were performed at 10°C and 40000 rpm on a Spinco model E ultracentrifuge.

A differential scan of the protein content at 280 and 260 mm wavelength was obtained at 10°C using a Fiske Co., model 2020, spectrophotometer.

A partial specific volume was used for the protein in the partial specific volume of 0.70 discussed below. Ultracentrifugal experiments on a Spinco Model E were run using 8-mm beam column in a cuvette with Cary 14 spectrophotometer.

2 Reference of a companion paper of approval or reference by the U. S. Dept.

in the evaluation of others.
Some recent results (7) on proteins in random coil-forming solvents suggest that the S values observed in acryl and guanidinium are still very high for a completely unfolded polypeptide chain of molecular weight 19,000 which would be present if the native molecule were disintegrating into identical subunits. In fact, for a random coil, S corresponds to a molecular weight of ca. 120,000, 0.88 to ca. 45,000. The molecular weight of acid DNAase in denaturing solvents, therefore, has been investigated in detail.

**EXPERIMENTAL PROCEDURE**

**MATERIALS AND METHODS**

Two long sphero acid DNAase preparations, obtained as previously described (8), were used in the present work.

Guanidinium hydrochloride (1 M) was purchased from Mann (New York, N.Y.); ultrapure grades of arginine and valine were purchased from Noma (9). Other reagents were analytical grade.

All measurements were made with a glass electrode and were not corrected for the pressure of HCl.

Concentrations of acid DNAase were determined spectrophotometrically at 260 nm, using an absorbance of 13.5 at M 1.0. Refractometric measurements were made at 25 °C in a Biotronik-Philippines, Per diffraction refractometer at 480 rpm. The partial refractive index of the protein at constant GuHCl concentration at 25 °C was calculated from the refractive indices of the two solutions representing the DNAase and GuHCl, respectively, as measured by a modification of the method of Kato (10), as described by Nishida and Timoshenko (11).

A partial specific volume (v) of 0.72 ml/g was used for the protein in aqueous solution. The partial specific volume of GuHCl solutions will be discussed below.

Ultracentrifugal experiments were carried out in a Spinco Model E instrument, whose interference optics had been centered on the optical train according to the procedure of Richarz et al. (12). Molecular weights were obtained by the high-speed equilibrium method of Yphantis (13) using 3 mm liquid columns in a two-channel interferometer cell with sapphire windows and with some castration of the cell between the angles of the synchrotron source (14) at 0.3 mm wavelength. Ultracentrifugation was performed on an Adatron (1.0 to 1.2) at 1.0.700F, 15,000 rpm.

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The molecular weight of acid DNAase was first investigated at low pH, namely, under conditions known to dissociate into subunit proteins, such as hemoglobin and γ-globulin.

**RESULTS AND DISCUSSIONS**

The molecular weight of acid DNAase was first investigated at low pH, namely, under conditions known to dissociate into subunit proteins, such as hemoglobin and γ-globulin. Figure 1 shows the plot of S in HCl dilution (7) as a function of the pH at 5.3. 2° C. 0.100 M HCl (open circles), and at pH 6.50.0.01 M HCl (solid circles). The apparent molecular weights obtained from the slope of the least-squares line are 40,000 and 28,000, respectively. It is obvious that the DNAase molecule is not dissociated at any extent by the low pH treatment.

A sample of acid DNAase was then dissolved in 6.0 M GuHCl and dialyzed 2 hr at 50 ml of solvent (pH 5.4). The sample was then retested at 44,750 rpm for 14 hr after overconditioning for 2 hr at 4°C. At which time the partial specific volume was measured and the apparent molecular weight calculated using 1404 ml g was measured and 2.0 ml g was calculated, indicating that the DNA is not significantly dissociated by 5 M GuHCl.

This result was checked by combining a sedimentation and diffusion velocity in the same solvent. A freshly lyophilized sample was dissolved to a concentration of 25 mg ml at 15,000 rpm for 1 hr. At 150 min, 5 ml of water was added, and the rotor was accelerated to 32,000 rpm for velocity measurements. Analysis of photographs was performed on enlarged images using the equation D = 3.3.2 (1) cm (1.4 M), where A and B are the area and the height of the peak, t is time in minutes after formation of the synthetic boundary, and f, is the final magnification in the z-direction.

Sedimentation and diffusion coefficients...
for this undialyzed sample were corrected to the viscosity and density of water using data for 6M GuHCl tabulated by Kauzmann and Tanford (15). Values obtained were 1.68 and 1.3 × 10^−4 cm^2 sec/1, respectively, resulting in an apparent molecular weight of 40,000 daltons. Large errors are possible in this type of experiment; nonetheless, the high molecular weight estimate confirms that no dissociation into subunits takes place in 6M GuHCl.

The equilibrium centrifugation experiments were repeated on an enzyme sample dialyzed 40 hr at 25°C against 6M GuHCl 0.3 M a-methoxyethanol (dME). The plot of In j vs z^2 has, in this case, a more rapid upward curvature toward the cell bottom. This is indicative of a high degree of heterogeneity in this sample, which had become noticeably yellow during the prolonged dialysis. If one draws a straight line by eye through a few points near the minima (Fig. 4, upper dashed line), the slope obtained corresponds to material of mol wt around 11,000. Extrapolating this line to the bottom of the cell, the difference in fringe levels solid and dashed line indicates the presence of a component with a mol wt of approximately 45,000. This gives an average mol wt of 45,000 at the cell bottom and suggests that the difference plot to the right of the minima, this heavy line represents material of mol wt 45,000.

The same procedure (lower data points of filled circles) for the enzyme sample dialyzed 48 hr against 6M GuHCl 0.3 M dME, analysis 10 hr, during which a solution had been incubated at 37°C, shows that no characteristic signal is obtained from the fringes, in contrast, only a few hour analysis is not detectable.
The differences in fringe displacement between the solid and dashed lines (shown in the logarithmic plot of Fig. 3) may be reported as in $j$ or $s^2$. This gives an apparent mol wt of 45,000 at the cell bottom. Considering the approximations made, and the sensitivity of the difference plot to the points selected near the menisci, this heavy material undoubtedly represents undegraded protein.

The same procedure was applied to the lower data points of Fig. 3 (triangles and filled circles). This experiment shows that the apparent nonspecific degradation observed is not due to the macroscopization, as it was made on a different GU HC solution of DNase, without dME, which had similarly dialyzed 48 hr, during which time this protein solution had also become yellowish. Comparison of the data taken after 25 and 45 hr of centrifugation shows some movement of the fringes, in contrast to experiments dialyzed only a few hours, where fringe movement is not detectable between 12 and 14 hr of centrifugation. This, in these lengthy experiments, degradation is extensive and continuing. The data of Fig. 3 indicate 40-45% of high molecular weight material in the cell bottom.

A possible explanation might be found in differences in exposure of the solutions to air during dialysis, or to a big period before breakdown of the poly peptide chain becomes measurable, significant. These experiments show, nevertheless, that there is no accumulation of material of half the molecular weight of the native enzyme, which would accompany dissociation to the hypothetical monomer unit.
In the case of 6-aminoimidazole, this value represents a preferential binding of $-0.21 \text{ g}$ of guanidine per gram of protein, or stated in a more usual manner, a preferential binding of water to the extent of $0.28 \text{ g}$ DNA, when concentrations are expressed in molar units. In the limit of zero protein concentration, this value may be converted (using equation 4 of Ref. 11) to molar units, in which case guanidine is preferentially bound to an extent of $0.30 \text{ g}$ per gram DNA. This is comparable with a $\Gamma_1$ of 0.05 for this protein, a fairly large change. The literature indicates that a majority of the proteins so far studied show essentially no change in $\Gamma_1$ in strong Gu HCl solutions, or only a slight (2-3%) decrease (15-18). Considering that the 0.05 value, obtained in a single measurement, is likely to be too low, a value of 0.20 is used for calculations; this is 2.5 times higher than the value of 0.072 in aqueous solutions.

Table I summarizes the molecular weight determinations mentioned in the present paper. Molecular weights were calculated using the two $\Gamma_1$ values.

<table>
<thead>
<tr>
<th>Molecular Weight of Acid DNA</th>
<th>$\Gamma_1 = 0.2$</th>
<th>$\Gamma_1 = 0.7$</th>
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<tbody>
<tr>
<td>5.0 g DNA</td>
<td>8.0 g</td>
<td>5.0 g</td>
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<td>5.0 g DNA</td>
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<tr>
<td>5.0 g DNA</td>
<td>8.0 g</td>
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Note: The values given are for the molar mass of guanidine, which is 121.14 g/mol. The molecular weight of DNA is calculated assuming a molecular weight of 700,000 daltons for each polymer. The values are given in g/mol.

*Values from Ref. 15.

**Values from Ref. 16 and 17.

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In conclusion, all clearly show that an agreement cannot be drawn under a variety of conditions. Therefore, this study is further confirmed by estimations done in duplicate. A problem is that of the agreement with the theoretical values.
Molecular Weight of Acid DNase

In conclusion, all the results obtained clearly show that acid DNase from hog spleen cannot be dissociated into subunits under a variety of conditions which are extremely effective with other proteins. Although these experiments indicate that acid DNase is a single polypeptide chain, the possibility still exists that the enzyme is formed by subunits joined by non-peptide linkages that resist denaturation by guanidine or mercaptoethanol treatment, or that large portions of the polypeptide chain occur in duplicate. A more raising similar problems is that of transferase (19).

References