Studies on Acid Deoxyribonuclease

X. Molecular Weight in Denaturing Solvents

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Hog spleen acid DNase, an enzyme which peptide mapping indicates to be formed by two similar or identical subunits, 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 6 M guanidine, with or without added β-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 (deoxyribonucleate 3′-nucleotidohydrolase, EC 3.1.4.6; deoxyribonuclease II) has been the object of extensive investigations during the past 10 years (see Refs. 1 and 2 for review articles).

This enzyme is able to split native, double-stranded DNA according to both a diplotomic and a haplotomic 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 this mixed 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 some complementary nucleotide sequences on the opposite strands; (b) the enzyme has a dimeric structure; the two different types of attack

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 (1, 5), whereas the numbre of arginine and lysine residues of the enzyme molecule (mol wt = 38,000) was 32–34 (6). The second finding was that the sedimentation coefficient of acid DNase (3.4S at zero concentration; 2.8S at 1% concentration) was only 0.8S in the presence of 8 M urea and 0.1 M β-mercaptoethanol (at 1% concentration); in the presence of 4 M urea–0.05 M β-mercaptoethanol two boundaries were seen having S values of 2.6S and 0.8S, respectively (6). Later work in 6 M guanidine showed that the sedimentation coefficient at 1% concentration was 1.75S and 2.1S at pH 5.6 and pH 8.6, respectively; in the presence of added 0.1 M β-mercaptoethanol the sedimentation coefficient was 1.5S (1). The further decrease was not unexpected since the eight half-cystine residues of acid DNase form four disulfide bridges (G. Bernardi, unpublished results cited in Ref. 1).

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Some recent results (7) on proteins in random coil-forming solvents suggest that the S values observed in urea and guanidine are still too high for a completely unfolded polypeptide chain of molecular weight 19,000 which would be present if the native molecule were dissociating into identical subunits. In fact, for a random coil 1.5S corresponds to a molecular weight of *ca.* 150,000, 0.8S to ca. 45,000. The molecular weight of acid DNase in denaturing solvents has, therefore, been investigated in detail.

EXPERIMENTAL PROCEDURE

MATERIALS AND METHODS

Two hog spleen acid DNase preparations, obtained as previously described (8), were used in the present work.

Guanidine hydrochloride (Gu HCl) was purchased from Mann (New York, NY; ultrapure grade) or recrystallized from methanol at $-72°$ from commercial material using the method of Nozaki (9). Other reagents were analytical grade.

pH measurements were made with a glass electrode and were not corrected for the presence of Gu HCl.

Concentrations of acid DNase were determined spectrophotometrically at 280 nm, using an absorptivity of 12.1 (6).

Refractometric measurements were made at 25° in a Brice-Phoenix (Philadelphia, Pa) differential refractometer at 436 nm. The partial refractive index of the protein at constant Gu HCl concentration $(\partial n/\partial C_2)_{T,P,C_3}$, the subscripts 2 and 3 representing the DNase and Gu HCl, respectively, were measured by a modification of the method of Katz (10), as described by Noelken and Timasheff (11).

A partial specific volume ($\bar{V}_2$) of 0.72 g/ml (6) was used for the protein in aqueous solution. The partial specific volume in Gu HCl solutions will be discussed below.

Ultracentrifugal experiments were carried out in a Spinco Model E instrument[3], whose interference optics had been centered on the optical axis according to the procedure of Richards *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 interference cell with sapphire windows and (in some cases) a synthetic cell bottom of FC-43 fluorocar-

bon liquid. A slight thickening of the water fluorocarbon interface was sometimes observed, probably as a consequence of protein denaturation (14). Duplicate runs without fluorocarbon yielded, however, apparently identical plots of ln of fringe displacement vs the square of the rotation radius. Ultracentrifuge plates were analyzed on a Nikon (Tokyo, Japan) Model 6C comparator.

RESULTS AND DISCUSSIONS

The molecular weight of acid DNase was first investigated at low pH, namely, under conditions known to dissociate into subunit proteins such as hemoglobin and β-lactoglobulin. Figure 1 shows the plot of ln of fringe displacement (j) vs r^2 for DNase at pH 5.2, Γ/2 = 0.15 (solid circles), and at pH 2.0 in 0.01 M HCl–0.01 M NaCl (open circles). The apparent molecular weights obtained from the slope of the least-squared lines are 40,800 and 39,900, respectively. It is obvious that the DNase molecule is not dissociated to any extent by the low pH treatment.

A sample of DNase was then dissolved in 6 M Gu HCl and dialyzed 2 hr vs 50 ml of solvent (pH 5.4). The sample was then centrifuged at 44,770 rpm for 14 hr (after overspeeding 50% in ω^2 for 2 hr), at which time equilibrium was attained; with overspeeding, other similar runs showed no changes after 12 hr at speed. The analysis of the 14-hr photograph is shown in Fig. 2 and the apparent molecular weight calculated using $\bar{V}_2 = 0.72$ is 36,100, indicating that the DNA is not significantly dissociated by 6 M Gu HCl.

This result was checked by combining a diffusion and sedimentation velocity in the same solvent. A freshly lyophilized sample was dissolved to a concentration of 27 mg/ml and centrifuged at 8000 rpm in a synthetic boundary cell. After 150 min, 15 usable schlieren photographs had been taken, and the rotor was accelerated to 52,640 rpm for velocity measurements. Analysis of photographs was performed on enlarged tracings using the equation $D = (A/H)^2 (1/240 \pi) (1/t) (1/M_x^2)$, where A and H are the area and the height of the peak, t is time in minutes after formation of the synthetic boundary and M_x is the final magnification in the x-direction.

Sedimentation and diffusion coefficients

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[3] Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.
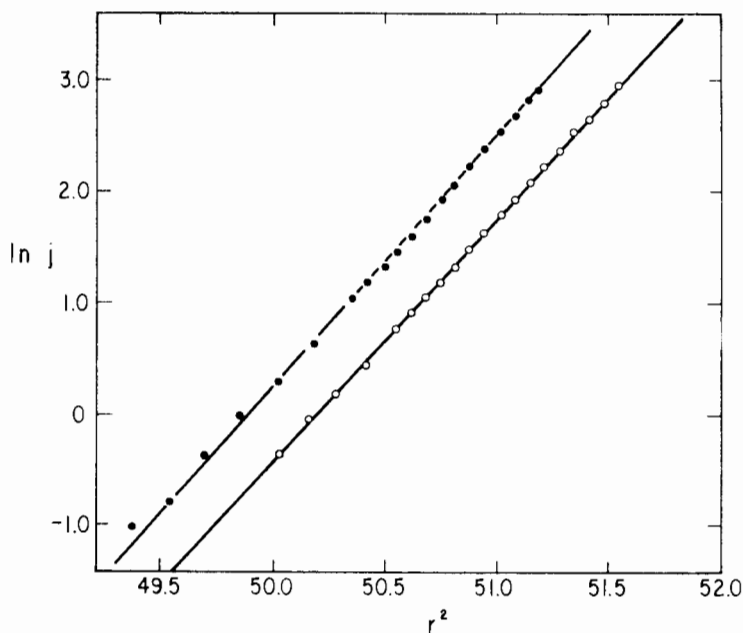
FIG. 1. Logarithm of fringe displacement vs radius squared for acid DNase. ●, 0.15 M Na-acetate buffer, pH 5.2, 20.0°; ○, 0.01 M HCl, 0.01 M NaCl, pH 2.03, 21.3°. 29,500 rpm, 16 hr.
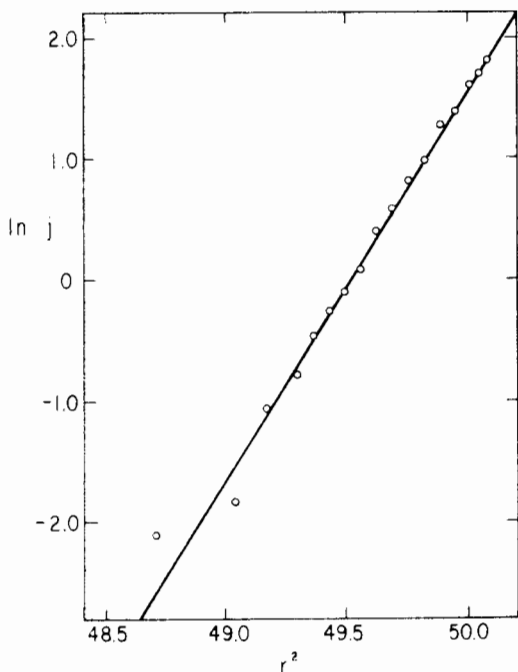

FIG. 2. Equilibrium plot of DNase in 6 M guanidine hydrochloride, pH 5.4. Time of dialysis 2 hr, 44,770 rpm. photograph at 14 hr.

for this undialyzed sample were corrected to the viscosity and density of water using data for 6 M Gu HCl tabulated by Kawahara and Tanford (15). Values obtained were 1.6S and 4.3×10^{-7} cm²/sec, respectively, resulting in an apparent molecular weight of 49,900 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 6 M Gu HCl.

The equilibrium centrifugation experiments were repeated on an enzyme sample dialyzed 40 hr at 25° against 6 M Gu HCl-0.1 M β-mercaptoethanol (βME). The plot of ln j vs r^2 has, in this case, a pronounced 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 meniscus (Fig. 3, upper dashed line), the slope obtained corresponds to material of mol wt around 14,000. Extrapolating this line to the bottom of the cell,
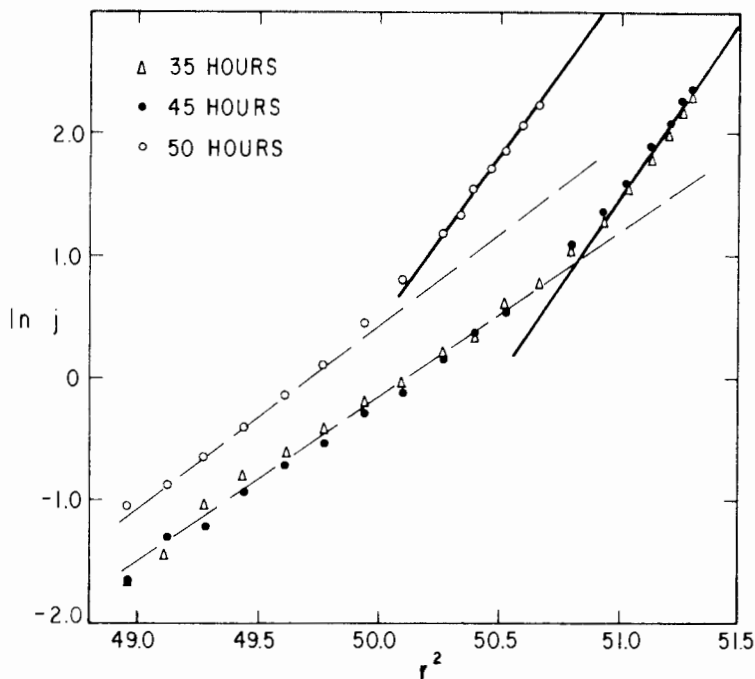
FIG. 3. Equilibrium plots of DNase in 6 M Gu HCl. ○, sample with 0.1 M β-mercapto-ethanol, 45-hr dialysis, photograph taken after 50 hr at 50,740 rpm. Lower points, same solvent without βME, 48-hr dialysis. Photographs taken after 35 hr (△) and 45 hr (●) at 50,740 rpm, 25°.

the difference in fringe displacement between the solid and dashed lines (shown in the logarithmic plot of Fig. 3) may be replotted as $\ln j$ vs r^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 meniscus, 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 mercaptoethanol, as it was made on a different Gu HCl solution of DNase, without βME, which had similarly dialyzed 48 hr, during which time this protein solution had also become yellowish. Comparison of the data taken after 35 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. Obviously, 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 lag period before breakdown of the polypeptide chain becomes measurably 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.

Sedimentation-diffusion experiments were performed on these same solutions (with and without βME) after 5–6 days of additional storage at 4°. These yielded the following data: with βME, $s_{20,w} = 0.9S$, $D_{20,w} = 3.3 \times 10^{-7}$; without βME, $s_{20,w} = 1.0S$, $D_{20,w} = 4.3 \times 10^{-7}$. Combined, these give mol wt of 38,100 and 33,100, respectively,

close to the weight averages of the low and high values of ca 14,000 and 45,000 obtained from the equilibrium runs of Fig. 3.

Equilibrium experiments were also run with 6 M Gu HCl at low pH (pH 1.6). When dialyzed for short times or at low temperature (see Table I), molecular weights of 41,900 and 39,400 were obtained, and no curvature was seen either near the meniscus or at the cell bottom.

Since preferential solvation of DNase in guanidine solutions may have a serious effect on the partial specific volume and thus on the molecular weight estimation, this point was checked.

The refractive index increment of DNase, dialyzed against 6 M Gu HCl, was measured using the dialyzate as the reference liquid, and this value combined with the increment given when the reference is prepared at exactly the same molar concentration in Gu HCl (11, 12). A single determination yielded a value for the term $(\partial G/\partial C_2)T, _{\mu_1, \mu_3}$ of -0.21. This parameter can be used to estimate the change in $\bar{V}_2$ due to preferential solvation.

In the case of 6 M guanidine, this value represents a preferential binding of -0.21 g of guanidine per gram of protein, or stated in a more usual manner, a preferential binding of water to the extent of 0.208 g/g DNase, 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 molal units, in which case guanidine is preferentially bound to an extent of 0.36 g per gram DNase. This is compatible with a $\bar{V}_2$ of 0.68 for this protein, a fairly large change. The literature indicates that a majority of the proteins so far studied show essentially no change in $\bar{V}_2$ in strong Gu HCl solutions, or only a slight (2–3%) decrease (15–18). Considering that the 0.68 value, obtained in a single measurement, is likely to be too low, a value of 0.70 is used for calculations; this is 3% lower than the value of 0.72 in aqueous solutions.

Table I summarizes the molecular weight determinations mentioned in the present paper. Molecular weights were calculated

TABLE I
APPARENT MOLECULAR WEIGHT OF ACID DNASE

Solvent	Time dialyzed (20–25°)	Method	Mol wt × 10⁻³	
			$\bar{V} = 0.72$	$\bar{V} = 0.70$
pH 5.2, 0.15 M NaAc, 0.01 M EDTA[a]	...	S-D	38	
pH 5.2, 0.15 M Na Acetate	72 hr	Equil.	40.8	
pH 2.03, 0.01 M HCl, 0.01 M NaCl	8 hr	Equil.	39.9	
6 M GuHCl, pH 5.40	2 hr	Equil.	36.1	32.2
6 M GuHCl, pH 5.40	0	S-D	49.9	41.8
6 M GuHCl, pH 5.40 + 0.1 M βME	40	Equil.	45.0[b]	40.1[b]
			14.6[c]	13.0[c]
6 M GuHCl, pH 5.40	48	Equil.	45.1[b]	40.2[b]
			13.5[c]	12.0[c]
6 M GuHCl, pH 5.40	48	S-D	33.1	27.9
6 M GuHCl, pH 5.40 + 0.1 M βME	40	S-D	38.1	31.9
6 M GuHCl, pH 1.6	5	Equil.	41.9[d]	37.4
6 M GuHCl + 0.1 M βME	16 hr (4°)	Equil.	39.4[d]	35.1

[a] Values from Ref. (5).

[b] Values at bottom of cell obtained by subtracting displacement due to light material in upper portion. See text.

[c] Value in upper portion of cell.

[d] Runs done in Yphantis 6-place centerpiece at 1.1, 0.8, and 0.4 mg/ml; no dependence upon concentration was found.

using both a $\bar{V}_2 = 0.72$ cm³/g and a $\bar{V}_2 = 0.70$ cm³/g value.

Finally, it should be mentioned that sedimentation equilibrium experiments in 0.1 M acetate buffer containing 0.1% of highly purified sodium dodecylsulfate gave a higher molecular weight than in guanidine; values ranging from 47,350 to 41,400 daltons could be estimated according to whether the partial specific volume was assumed to be equal to 0.72 cm³/g or to 0.68 cm³/g, respectively.

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 chains, the possibility still exists that the enzyme is formed by subunits joined by nonpeptide linkages that resist disruption by guanidine or urea and β-mercaptoethanol treatment, or that large portions of the polypeptide chain occur in duplicate. A case raising similar problems is that of transferrin (19).

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