MONOMERIC BEHAVIOR OF THE SMALL SUBUNIT OF RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE[1]

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SUMMARY: The molecular weights of alkylated small subunits of ribulose-1,5-bisphosphate carboxylase of pea and spinach were determined from gel filtration data in the presence of 6M guanidinium chloride as 12,800 and 13,500, respectively. In the presence of 0.1 M sodium phosphate (pH 12.0) these molecules chromatograph at the same position as chymotrypsinogen (M=25,700) on Sephadex G-75. The intrinsic viscosity of the small subunit of spinach ribulose bisphosphate carboxylase, measured in this solvent, was $[\eta] = 30$ cm^3/gm, while the intrinsic viscosity of chymotrypsinogen measured in this solvent was $[\eta] = 2.4$ cm^3/gm. These data rule out a globular dimeric model for the sructure of the small subunit in 0.1 M sodium phosphate (pH 12.0).

INTRODUCTION: Gel filtration of ribulose-1,5-bisphosphate carboxylase (E.C.1.1.4.39) from tobacco showed that at alkaline pH the smaller subunit of the enzyme behaves like a particle whose molecular weight M = 24,000 (1). In the presence of sodium dodecyl sulfate, however, the small subunit appeared to have M = 12,000 (1,2). Thus it was proposed that the small subunit is dimeric in alkaline media and is split into monomeric subunits by addition of the detergent (1,3,4). Some results we present here confirm these observations. Additional viscosity data support a different conclusion, however: the small subunit is probably an asymmetric monomer in alkaline solutions.

MATERIALS AND METHODS: Ultra-Pure guanidinium chloride (Schwarz/Mann) and reagent grade chemicals were used throughout. Alkylation and gel filtration of proteins in guanidinium chloride were carried out exactly as described (5). Ribulose bisphosphate carboxylase was purified from pea and spinach leaves as described (6), except that, after the gel filtration step, the protein was concentrated, dialysed, and sedimented through sucrose gradients. Purity of routine carboxylase preparations was judged from staining patterns of polyacrylamide gels after electrophoresis in the presence of SDS by the method of Chua and Bennoun (7) using the slab gel apparatus described by Studier (8). Although numerous minor contaminants were detected, the protein was 95% pure carboxylase. Small subunit for viscosity studies was prepared by dialysing spinach carboxylase against 0.01 M Tris-HCl (pH 8.0) to remove ammonium sulfate, using small pore dialysis tubing (Arthur Thomas Co. #3787-H47). The dialysis bag was then transferred to 0.1 M sodium phosphate (pH 12.0) for two hours at 4°C. The contents of the bag were applied to a Sephadex G-75 column (2.4 x 60 cm) equilibrated in the same buffer and eluted at room temperature. The

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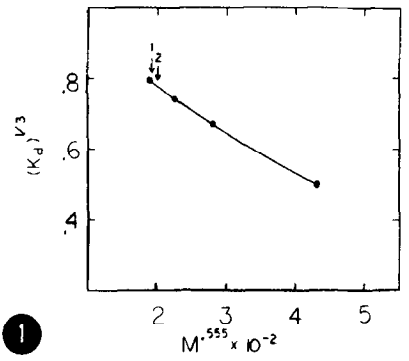
small subunit peak was concentrated by ultrafiltration, using an Amicon cell and a UM-2 filter at pressures up to 70 p.s.i., re-chromatographed, re-concentrated, and forced through Millipore filters to remove any dust. Electrophoresis of this material showed a single band in SDS-polyacrylamide gels. The protein concentration was determined by the biuret method.

RESULTS AND DISCUSSION: When disulfide links are broken, and sulfhydryl groups blocked, proteins in 6M guanidinium chloride behave as random coils, resulting in a rigorous correlation between Stokes' radius and molecular weight (5). It has been demonstrated that a large number of polypeptides between 2,000 and 80,000 daltons fit a nearly straight linear standard curve, similar to that shown in Figure 1, in gel filtration on Bio-Gel A5M(5). Data summarized in Figure 1 indicate a molecular weight for pea small subunit, M = 12,800 ± 300; and for spinach small subunit, M $\cong$ 13,500. The estimation of molecular weight for pea small subunit closely corresponds with that made from amino acid composition data (4). These estimates improve upon those made by SDS-polyacrylamide gel electrophoresis, which is usually unreliable for small proteins (9-14).

In 0.1 M sodium phosphate (pH 12.0), the intrinsic viscosity $[\eta]$ of spinach small subunit is 30 cm^3/gm, as determined by extrapolation of the data to the ordinate in Figure 2A. The corresponding value for chymotrypsinogen in this solvent, by contrast, is 2.4 cm^3/gm (Figure 2B). This value is about the same as that obtained at neutral pH (ref. 15).

The data in Table I show the elution behavior of several proteins on the Sephadex G-75 column in 0.1 M sodium phosphate (pH 12.0). There is no reliable correlation between elution position and molecular weight in this solvent; nor is such a correlation expected (15-17). It is confirmed, however, that small subunit co-chromatographs with chymotrypsinogen (M = 25,700).

The interpretation of these data depends on a knowledge of the structure of chymotrypsinogen, which is used as a standard. X-ray crystallography shows that the molecules are nearly spherical; four of them occupy a unit cell with crystallographic axes a = 52, b= 63.9, c = 77.1, the space group $P2_12_12_1$ (18,19). The intrinsic viscosity at neutral pH is $[\eta]$ = 2.5 cm^3/gm (15). Equations 1 and 2



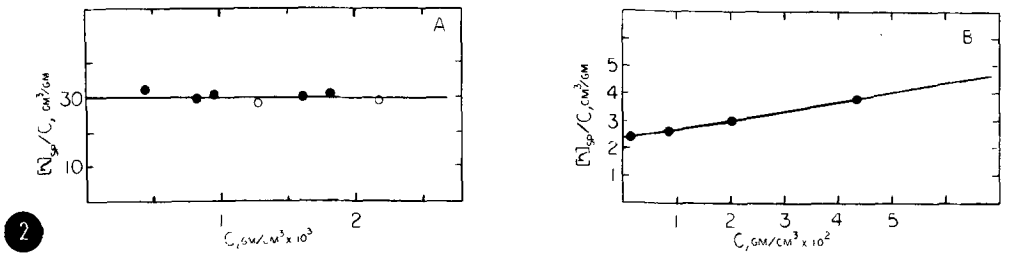


Figure 1. Gel Filtration of Proteins in 6M Guanidinium Chloride (pH 5.0)
Proteins(from Calbiochem) and highly purified ribulose bisphosphate carboxylase from pea and spinach leaves were reduced, alkylated and chromatographed exactly as in (5). $K_d = (V_e - V_o)/(V_i - V_o)$, symbols as in Table I, except V_i = elution of phenylalanine peak used as marker. Solid line with filled circles: values for standards and large subunit; Arrow 1: average of values for pea small subunit ($K_d^{1/3} = .7957$, .7908, and .7864; Arrow 2: value for spinach small subunit ($K_d^{1/3} = .7680$).

Figure 2. Intrinsic Viscosity Determinations in 0.1 M Sodium Phosphate (pH 12.0)
Each point represents the average of five measurements, at each protein concentration, of the flow time t of the solution through an Ostwald viscometer at 25° C ± .1 C°. The relative viscosity, η/η_o, is determined from $(\eta/\eta_o) = (\rho/\rho_o)/(t/t_o)$, where ρ is the density of the sample solution (calculated from solvent density ρ_o and the partial specific volume, which is .71 cm^3/gm for chymotrypsinogen (15) and assumed to be .74 cm^3/gm for small subunit), and t_o is the flow time for solvent alone through the viscometer. The specific viscosity, $[\eta]_{sp} = (\eta/\eta_o)-1$, is divided by the protein concentration C and plotted as a function of C in the figure. The intrinsic viscosity is the value to which the data are extrapolated on the ordinate. A) Small subunit values: filled and open circles represent two independently isolated samples of small subunit; B)Chymotrypsinogen values: note difference in magnitude of the scale on the abscissa compared to (A): this reflects the much higher concentration of chymotrypsinogen needed to obtain equivalent flow times.

permit the calculation of R_e, the Stokes radius, from $[\eta]$, the intrinsic viscosity:

$$[\eta] = \nu N V_h/M \qquad \text{(Equation 1)}$$

$$V_h = 4/3\ (\pi R_e^3) \qquad \text{(Equation 2)}$$

(ν = Simha's shape factor = 2.5 for spheres, and higher for asymmetric particles;

Table I. Chromatography of Proteins on Sephadex G-75 in 0.1 M Sodium Phosphate (pH 12.0)

Protein	M, Molecular Weight	K_{av} *
cytochrome c	12,400	.32
lysozyme	14,000	.52
myoglobin	17,000	.23
α-chymotrypsinogen	25,700	.23
large subunit	55,000	.01
small subunit		.24

V_h = hydrodynamic volume; N = Avogadro's number; M = molecular weight). The Stokes radius of chymotrypsinogen at neutral pH, calculated in this way, is R_e = 21 Å. It is apparent that this value agrees with the X-ray data. From the data in Figure 2B, the Stokes radius of chymotrypsinogen at pH 12.0, calculated in the same way, comes out to be essentially the same as the value at neutral pH. In addition, the elution parameter (K_{av} =.23, Table I) for this protein on Sephadex G-75 at pH 12.0 can be used in conjuction with published data on the behavior of globular proteins on Sephadex G-75 to estimate R_e at pH 12.0 (16), assuming negligible effects of pH on the structure of Sephadex. The result is R_e = 23 Å. Thus, evidently chymotrypsinogen has roughly the same size and shape in crystals, neutral buffers, and 0.1 M sodium phosphate (pH 12.0) (see 15,18).

Since the small subunit of carboxylase co-chromatographs with chymotrypsinogen, it must have the same Stokes radius, $R_e \cong 22$ Å. The relationship between this fact and the molecular weight of small subunit in this alkaline solvent depends, however, on the assumed conformation of the small subunit. A number of possible conformations are evaluated here:

1) Random coil. Using equations 1 and 2, and the intrinsic viscosity of small subunit, a value of R_e can be calculated for a randomly coiled small subunit monomer or dimer (i.e., 13,500 or 27,000 daltons). In the monomeric case, R_e = 41 Å, and in the dimeric case, R_e = 51 Å. Since in fact $R_e \cong 22$ Å, the random coil hypothesis must be rejected.

2) Rigid rod. The intrinsic viscosity alone can be used to calculate ν, the shape factor, which can then be related to the axial ratio a/b of an equivalent prolate

* $K_{av}=(V_e-V_o)/(V_t-V_o)$; V_e=elution volume of peak; V_o= void volume of column; V_t= bed volume of column. Most values are the average of two or more determinations.

ellipsoid of revolution by the equations of Simha (see 15,17 for formulae). Axial ratios a/b calculated in this way exceed 40, and lead to incredible conformations for such a small protein, whether it be monomeric or dimeric.

3) Globular structures. Globular proteins, whether monomeric or oligomeric, typically have intrinsic viscosities from 2.5 to 5 cm^3/gm (20). Since small subunit has an intrinsic viscosity close to 30 cm^3/gm, it cannot be globular.

4) Expanded, partially unfolded structures. Both R_e and $[\eta]$ can be used in equations 1 and 2, assuming first M = 27,000 (dimeric model), to calculate a shape factor, ν, which then yields an axial ratio a/b = 14. This corresponds to an ellipsoid as long as 314 Å and as wide as 21 Å. Two α-helical rods stuck end to end, or two highly stretched chains, tightly bound at points along their length, could be imagined to fill such a space; but these seem extremely unlikely. On the other hand, if we assume M = 13,500 (monomeric model), and repeat the calculation, we obtain an axial ratio a/b = 8, corresponding with a particle as long as 200 Å and as wide as 25 Å. A partial expansion of a globular small subunit monomer, due to electrostatic forces induced by high pH, could easily result in such a structure (15,17). This unfolded small subunit model is consistent with the fact that, upon dialysis to lower pH, the molecule aggregates irreversibly. Accordingly, we favor the concept that small subunit is monomeric in alkaline media.

A globular dimeric structure in this solvent cannot be reconciled with the viscosity data, and must be rejected. A globular monomeric structure is inconsistent with the gel filtration data, and also can be dismissed. However, at pH 9.0, there is some evidence for self-association of small subunit after alkylation with p-mercuribenzoate (21). Whether this reflects a feature of holoenzyme structure or represents partial denaturation of small subunit at the lower pH is unclear.

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REFERENCES

1. Kawashima, N., and Wildman, S.G.(1970) Annu. Rev. Plant Physiol. 21, 325-358.
2. Kawashima, N., and Wildman, S.G.(1970) Biochem. Biophys. Res. Commun. 41,1463-'68.
3. Ellis, R.J.(1973) Comm. Plant Sci. 4, 29-38.
4. Haslett, B.G., Yarwood, A., Evans,I.M., and Boulter, D.(1976) Biochim. Biophys. Acta 420, 122-132.

5. Fish, W.W., Mann, K.G., and Tanford, C. (1969) J. Biol. Chem. 244, 4989-4994.
6. Roy, H., Patterson,R.J.,and Jagendorf,A.T.(1976)Arch. Biochem. Biophys. 172, 64-73.
7. Chua,N-H., and Bennoun, P.(1975) Proc. Nat. Acad. Sci. USA 72, 2175-2179.
8. Studier, F.M.(1973) J. Mol. Biol. 79, 237-248.
9. Siegel, M.I., Wishnick, M., and Lane, M.D. (1972) In The Enzymes, VI, P.D. Boyer, Ed., pp. 169-197.
10. Sugiyama, T., and Akazawa, T.(1970) J. Biochem. 68, 821-831.
11. Kung, S.D., Sakano, K., and Wildman, S.G. (1974) Biochim. Biophys. Acta 365, 138-147.
12. Gray, J.C., and Kekwick, R.G.O. (1974) Eur. J. Biochem. 44, 491-503.
13. Fish, W.W., Reynolds, J.A., and Tanford, C.(1970) J. Biol. Chem. 244, 5177-5188.
14. Neville, D.M.(1971) J. Biol. Chem. 246, 6328-6334,
15. Tanford, C.(1968) Adv. Protein Chem. 23, 121-282.
16. Siegel, L.M., and Monty, K.J.(1966) Biochim. Biophys. Acta 112, 346-362.
17. Tanford, C. (1961) Physical Chemistry of Macromolecules, Wiley, New York.
18. Hess, G.P., and Rupley, J.A.(1971) Annu. Rev. Biochem. 40, 1013-1044.
19. Freer, S.T., Krant,J., Robertus, J.D., Wright, H.T., and Xuong, Ng.H.(1970) Biochemistry 9, 1997-2009.
20. Schachman, H.K. (1963) Cold Spring Harbor Symp. Quant. Biol. 28, 409-430.
21. Nishimura, M., Takabe, T., Sugiyama, T., and Akazawa, T.(1973) J. Biochem. 74, 945-954.