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DETERMINATION OF PROTEIN MOLECULAR WEIGHTS IN DENATURING SOLVENTS USING GLYCERYL-CPG

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SUMMARY

Glyceryl-CPG is controlled-pore glass whose surface has been chemically modified to block its slight negative charge in aqueous solution. In contrast to normal controlled-pore glass, glyceryl-CPG can be used successfully for gel filtration of proteins in a variety of denaturing solvents, *viz.*, 8 *M* urea, 6 *M* guanidine hydrochloride, and 0.1% sodium dodecyl sulphate. It has been found that glass with a mean pore diameter near 35 nm is suitable for the molecular weight range 17,000–100,000. Glyceryl-CPG with a smaller pore diameter is more satisfactory for molecular weights below 30,000. The stable pore size and bed dimensions of controlled-pore glass allows comparison of the conformation of a particular protein in different solvents to be made with the same column.

INTRODUCTION

Previous studies have shown that protein polypeptide molecular weights may be determined by gel filtration over controlled-pore glass (CPG) in the presence of sodium dodecyl sulphate (SDS). Collins and Haller[1] found that a mean pore diameter of 50 nm was suitable for the range 17,000–385,000 but adsorption to porous glass can occur under their conditions[2]. The likelihood of adsorption of protein–SDS complexes can be greatly reduced by using a mixed solvent containing both urea and SDS[2]. The problem of adsorption has prevented the use of traditional denaturants such as 8 *M* urea or 6 *M* guanidine hydrochloride (GuHCl) with CPG. In particular, it would be an advantage to be able to use 6 *M* GuHCl since, after reduction of any existing disulphide bonds, most protein polypeptide chains behave hydrodynamically as randomly coiled linear homopolymers in this solvent[3]. Although 8 *M* urea is commonly used as a denaturing agent, many proteins do not undergo a complete transition to random coils in urea solutions at room temperature[3].

Glyceryl-CPG is controlled-pore glass whose surface has been chemically modified with a hydrophilic non-ionic coating in order to reduce the possibility of adsorption of proteins from aqueous buffers. The aim of the present investigation was to compare the performance of regular-CPG with glyceryl-CPG for the determination of protein polypeptide molecular weights in denaturing solvents.

EXPERIMENTAL

Materials

Glyceryl-CPG, 36.8, 22.7, and 11.8 nm mean pore diameters, 120–200 mesh, and pore volumes of 0.90, 0.93, and 0.71 ml/g, respectively, was kindly supplied by Electro-Nucleonics (Fairfield, N.J., U.S.A.). SDS was BDH (Poole, Great Britain) specially pure grade; GuHCl was Schwarz/Mann (Orangeburg, N.Y., U.S.A.) ultra-pure grade, while all other chemicals were of analytical or equivalent grade. The values assumed for the molecular weights of the dissociated proteins are given in Table I.

TABLE I

MOLECULAR WEIGHTS OF POLYPEPTIDE CHAINS

No.	*Protein*	*Reference*	*Subunit molecular weight*
1	Insulin B chain*	4	3,400
2	High-tyrosine component 0.62	5	7,000
3	Cytochrome *c*	6	11,700
4	Apomyoglobin	6	17,200
5	α-Chymotrypsinogen A	6	25,700
6	Rabbit tropomyosin	7	33,500
7	Ovalbumin	6	43,000
8	Bovine serum albumin	6	68,000
9	Oyster paramyosin	8	97,000

* Prepared as described in ref. 9.

Methods

The denaturing solvents used were: 8 *M* urea-0.15 *M* sodium phosphate buffer pH 7, 6 *M* GuHCl–0.15 *M* sodium phosphate buffer pH 7, 0.1% SDS–0.05 *M* sodium phosphate buffer pH 7, and 6 *M* urea–0.5% SDS–0.05 *M* sodium phosphate buffer pH 7. The proteins were used in the S-carboxymethyl form. Protein–SDS complexes were prepared by incubating approximately 10 mg of protein with 20 mg of SDS in 1 ml of the appropriate buffer at 90° for 10 min. Sucrose, 20% (w/v), was added to all solutions to facilitate the loading of 0.1-ml samples of each.

Preparation and operation of column

The glyceryl-CPG columns were packed and operated in the manner described previously for regular-CPG[2]. Glass wall columns of 0.9 cm diameter were packed to a height of 165 cm with glyceryl-CPG of 36.8 nm pore diameter and to a height of 60 cm with glyceryl-CPG of 22.7 and 11.8 nm. The elution data have been expressed again as a distribution coefficient, K_d, defined as

$$K_d = \frac{V_e - V_0}{V_t - V_0}$$

where V_e is the weight of solvent corresponding to the peak concentration of the eluting solute, V_0 is the weight of solvent in the column external to the glyceryl-CPG matrix,

and V_i is the weight of solvent contained within and without the glyceryl-CPG matrix. Weight rather than volume was used as the measure of elution position in order to obtain sufficient precision[2]. The void volume, V_0, and total intrusion volume, V_i, were measured with Blue Dextran 2000 (Pharmacia, Uppsala, Sweden) and tryptophan, respectively.

RESULTS AND DISCUSSION

Truman *et al.*[10] reported the use of 8 *M* urea with regular-CPG to determine protein molecular weights but their results are not satisfactory because of retardation of their standard proteins. Other studies have shown adsorption of proteins to regular-CPG in 8 *M* urea and this could not be overcome by raising the pH or ionic strength, adding alcohols or Carbowax 6000 or 20M[2]. However, this is not the case if glyceryl-CPG is used with urea and sharp, symmetrical elution peaks are observed with K_d values less than unity. Calibration curves for various protein polypeptides in 8 *M* urea–0.15 *M* phosphate buffer pH 7 are shown in Fig. 1. Most of the points lie on a smooth curve, with paramyosin and tropomyosin being notable exceptions. Other studies have shown that these highly helical muscle proteins are not fully denatured in 8 *M* urea and this presumably accounts for their anomalous behaviour[8,11]. The apparent loss of discrimination below molecular weight 10,000 for the column of 11.8 nm pore diameter was unexpected since the elution profiles for both the high-tyrosine component from wool and the insulin B chain were normal in appearance. Further experiments with a range of polypeptides will be necessary to properly calibrate this region.

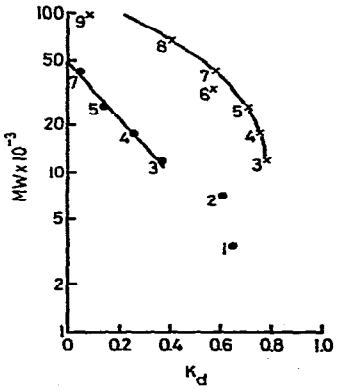

Fig. 1. Semilogarithmic plot of molecular weight *versus* K_d using 8 *M* urea–0.15 *M* phosphate buffer pH 7 as eluant. ×, glyceryl-CPG of 36.8 nm pore diameter; ●, glyceryl-CPG of 11.8 nm pore diameter. The numbers correspond to those given to the proteins listed in Table I.

In recent times 6 *M* GuHCl has replaced 8 *M* urea as the solvent of choice for protein denaturation. Only a few exceptionally stable proteins fail to adopt a conformation close to a random coil under these conditions[3,8]. As with 8 *M* urea, the adsorption of most of the standard proteins in 6 *M* GuHCl is eliminated by the use of glyceryl-CPG rather than regular-CPG. This judgement is based on the sharp,

symmetrical peaks eluting in order of known molecular weight with K_d values less than unity. However, the insulin B chain and a high-tyrosine component from wool still show signs of partial adsorption by way of a trailing edge on their elution profile. Obviously, the molecular weight of an unknown sample cannot be determined if there is any suggestion of adsorption. It is difficult to understand why this problem only applies here to molecular weights below 10,000 unless it is merely fortuitous that the limited range of higher-molecular-weight samples used was free of adsorbing proteins.

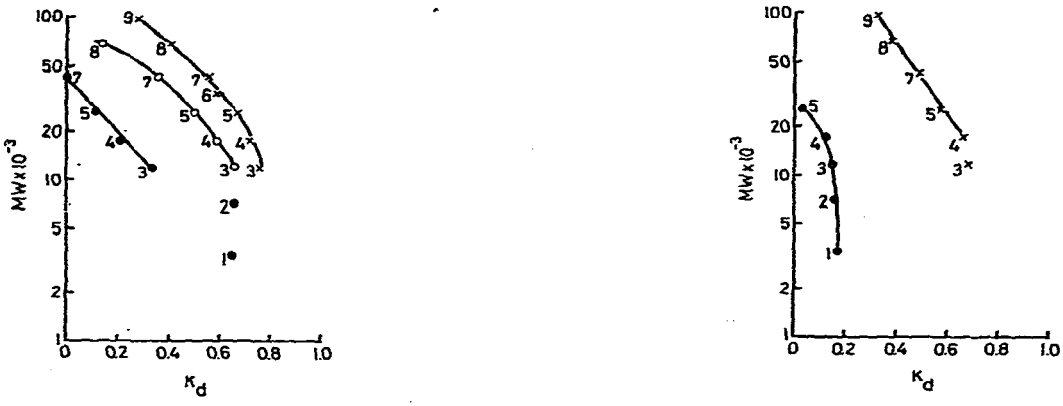

Fig. 2. Semilogarithmic plot of molecular weight *versus* K_d using 6 *M* GuHCl–0.15 *M* phosphate buffer pH 7 as eluant. ×, glyceryl-CPG of 36.8 nm pore diameter; ○, glyceryl-CPG of 22.7 nm pore diameter; ●, glyceryl-CPG of 11.8 nm pore diameter. The numbers correspond to those given to the proteins listed in Table I.

Fig. 3. Semilogarithmic plot of molecular weight versus K_d using 0.1 % SDS–0.05 M phosphate buffer pH 7 as eluant. ×, glyceryl-CPG of 36.8 nm pore diameter; ●, glyceryl-CPG of 11.8 nm pore diameter. The numbers correspond to those given to the proteins listed in Table I.

The calibration curves for protein polypeptides in 6 *M* GuHCl–0.15 *M* phosphate buffer pH 7 using glyceryl-CPG of three mean pore diameters are shown in Fig. 2. Within normal experimental error for column chromatographic studies, the points lie on a series of smooth curves shifting to lower K_d values with decreasing pore size. The curve for the glass of 11.8 nm pore diameter has not been extended below molecular weight 10,000 because of uncertainty in this region arising from partial adsorption. Tropomyosin and paramyosin now show the expected effective hydrodynamic size for their respective molecular weights (the paramyosin was denatured in 8 *M* GuHCl before application to the column following the studies of Woods[8]). Using the column of 36.8 nm mean pore diameter with 6 *M* GuHCl, results have been obtained for the molecular weights of several purified low-sulphur protein components from wool which agree well with those obtained in the analytical ultracentrifuge. These wool components give anomalous high-molecular-weight values[2] when determined as protein–SDS complexes on regular-CPG.

Although there are problems with adsorption of proteins to regular-CPG using simple buffered solutions of SDS[2], this is not the case for glyceryl-CPG. Results for various standard proteins denatured in 0.1 % SDS–0.05 *M* phosphate buffer pH 7 are shown in Fig. 3. The semilogarithmic plot is linear for the glass of 36.8 nm

pore diameter in the molecular weight range 17,000–100,000. However, the results with the glass of 11.8 nm pore diameter show little discrimination for molecular weights below 17,000. Even though there was some improvement in this region using 6 *M* urea–0.5% SDS, the plot was still non-linear and furthermore the resolution was not as good as that observed previously with regular-CPG of 12.3 nm pore diameter.

Chromatography over glyceryl-CPG also provides a simple means for comparison of the Stokes radius, R_s, of a protein in a range of denaturing solvents. Other physico-chemical methods which can be used for this kind of study, for example, viscosity or ultracentrifugation, are more tedious and generally require greater amounts of sample. Similar empirical column procedures using cross-linked agaroses are less reliable because of possible uncontrolled changes in the effective pore radius of the support in different solvents. It has not been the purpose of this study to pursue this interesting area, but from the results shown in Figs. 1 and 2 it can be seen that there is little difference in the hydrodynamic size of individual proteins in 8 *M* urea and 6 *M* GuHCl. This is consistent with the results of Davison[12] for 6% cross-linked agarose. Paramyosin is the most striking exception, with a large decrease in effective radius in going from 8 *M* urea to 6 *M* GuHCl.

It is of interest to compare the size of proteins denatured in 6 *M* GuHCl with their respective protein–SDS complexes when considering possible models for the conformation of these complexes. Fish *et al.*[13] have made a study of this type for protein polypeptides in the molecular weight range 12,000–177,000 using agarose. It can be seen from Figs. 2 and 3 that, at the higher molecular weights, the effective hydrodynamic radii in 6 *M* GuHCl and 0.1% SDS are very similar. However, as the molecular weight decreases, the radii in SDS gradually increase relative to those in GuHCl. Moreover, for molecular weights below 20,000, this difference is greatly accentuated because the size of the protein–SDS complex appears to remain constant even for a polypeptide as small as the insulin B chain. The results for molecular weights 12,000 and above confirm those of Fish *et al.*[13] but the extension here to protein molecular weights below 12,000 casts some doubt on their hypothesis regarding the Stokes radii of protein–SDS complexes below molecular weight 15,000. They proposed that the break in the relation between R_s and molecular weight at low molecular weight is possibly explained by the fact that a rodlike particle begins to approximate a sphere when the length approaches the diameter in magnitude. This, of course, is based on their proposal that the intrinsic viscosity data for protein–SDS complexes are consistent with a prolate ellipsoid model for such complexes[14]. However, it can be seen from Fig. 3 that polypeptide–SDS complexes show no significant decrease in size as the molecular weight falls from 15,000 to 3,500 and the Stokes radius, judged from the K_d value, is comparable to a protein of molecular weight 20,000 in the random coil form in 6 *M* GuHCl. This behaviour is reminiscent of the inclusion of tracer dyes into SDS micelles[15] and could be explained if small polypeptides were encased in an SDS shell of fairly constant size until the molecular weight is greater than 15,000. The fact that it was found possible to discriminate between small protein–SDS complexes on regular-CPG in the presence of urea[2] suggests that urea can alter the extent of SDS binding or at least the conformation of such complexes. Nevertheless, in the molecular weight range 15,000–50,000 and with the glyceryl-CPG of 36.8 nm pore diameter used in this study, there was no effect on elution position when urea was added. At higher molecular weights, there was an increase in the size of the protein–SDS complexes with urea,

especially for paramyosin, whose Stokes radius apparently increased to the value found in urea alone.

CONCLUSIONS

Gel filtration over glyceryl-CPG is a suitable method for determining the molecular weights of protein polypeptide chains with a number of denaturing agents. The results presented indicate that adsorption of proteins to glyceryl-CPG is greatly reduced when compared to regular-CPG, so that urea, GuHCl, or SDS can be used for polypeptides with molecular weights greater than 17,000. For smaller proteins, gel filtration over regular-CPG in urea–SDS is the preferred method[2].

Molecular weight determination of polypeptide chains by gel filtration in 6 *M* GuHCl over agarose[16] provides the best empirical chromatographic procedure available at present. However, glyceryl-CPG is a very convenient support medium for use with 6 *M* GuHCl except that retardation due to partial adsorption can occur with some proteins and the resolving power of glass is less than for agarose due to the lower intrusion volume of the former. Nevertheless, the speed of measurement and the bed stability of glyceryl-CPG more than compensates for these disadvantages, making chromatography in 6 *M* GuHCl a more attractive method than it has been previously.

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