Analytical Ultracentrifugation of Proteins in Solutions of Triton X-100 Using Absorption Optics

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Analytical ultracentrifugation studies on proteins in solutions of the nonionic detergent Triton X-100, by means of a UV scanning system, can be conveniently performed by the following procedure: (1) Scanning of the cells is done at a wavelength of 246 nm. (2) The sedimentation of the detergent is prevented by appropriate adjustment of the solvent density. (3) The reference cell is filled with water. Reliable molecular weight determinations can be performed at detergent concentrations up to 0.4% (w/w) in cells of pathlength d = 12 mm and at correspondingly higher concentrations for d = 3 mm. Approx. 10 μg of protein are sufficient for a determination. The method should be useful for studies of membrane proteins.

Introduction

Nonionic detergents are indispensible tools in the study of membrane proteins [1–3]. Among this class of detergents, the polyoxyethylene derivative Triton X-100 seems to be especially useful, since it is both very effective as a solubilizing agent and very mild with respect to the preservation of the functional properties of the proteins [1, 3, 4]. However, in many biophysical investigations on Triton-solubilized membrane proteins, the strong UV absorbance of the detergent (which contains an aromatic ring) is a serious drawback. In particular, the UV absorbance has hindered studies on the self-association of membrane proteins in solutions of Triton X-100, since the most powerful method available, analytical ultracentrifugation using a UV scanner, apparently was thought to be non-applicable (at least at detergent concentrations > 0.05%). This is unfortunate, as the self-association of membrane proteins in solutions of nonionic detergents is thought to reflect that in their native membranes [2, 4, 5].

We have looked for a way of performing analytical ultracentrifugation experiments in solutions of Triton X-100, using the UV scanning equipment of the Spinco model E ultracentrifuge. We have found that such experiments can be conveniently performed if scanning of the cells is done at a wavelength of approx. 246 nm and if sedimentation of the detergent is prevented by appropriate adjustment of the solvent density. In the present paper, the feasibility and reliability of the method is demonstrated by sedimentation equilibrium experiments on a homogeneous protein complex, tetrameric glucose dehydrogenase from Bacillus megaterium [6]. Application of the method to the study of the self-association of a membrane protein, band 3 protein from human erythrocyte membranes, has already been reported [7].

Materials and Methods

Glucose dehydrogenase from Bacillus megaterium was a kind gift from Dr. E. Maurer, Stuttgart. For the purification and storage of the enzyme see [6, 8] and references cited therein. Before use, it was dialyzed against 100 mM sodium phosphate (pH 6.5), 500 mM NaCl. The enzyme is tetrameric and fully active in this solvent [6, 8, 9]. For the ultracentrifuge experiments, the samples were diluted by the same buffer plus a volume of analogous buffer made up in D_2O such that the final D_2O concentration of the samples was 59% (v/v) (see below). Protein concentration was determined photometrically [9].

Triton X-100 ("peroxide-free") was purchased from Boehringer Mannheim. D_2O (99.7%) was a kind gift from the Karlsruhe Nuclear Research Centre. Solutions were freshly prepared before use.

Analytical ultracentrifugation was performed in a Beckman Spinco model E ultracentrifuge equipped with UV scanning system, monochromator, multiplexer and temperature control unit. Epon double sector cells of pathlength d = 12 mm and single sector cells of d = 3 mm (4°) were used in combination with an An-G Ti rotor. Sample volume was 150 μl (d = 12 mm) or 75 μl (d = 3 mm). The reference sector/cell was filled with 170/85 μl H_2O. Rotor temperature was 10 °C. In the sedimentation equilibrium runs, rotor speed was 15,000 rpm, which resulted in c(r)-profiles of the meniscus depletion type. Sedimentation equilibrium was reached after approx. 24 h. Absorbance-versus-protein concentration data were obtained, at 40,000 rpm, from the difference of the absorbance values near the meniscus before and after the protein had moved out of this region. Measurements of solvent density were

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made using a DMA O2 C digital densitometer (Anton Paar, Graz).

Results

The UV absorbance spectrum of Triton X-100 shows a pronounced maximum around 276 nm, the wavelength range in which standard ultracentrifugation experiments on protein solutions by means of absorption optics are performed. At 280 nm, the absorbance $A^{1\%}_{1cm}$ of Triton X-100 is approx. 20. In the range of Triton concentrations typically used for studies on membrane proteins, 0.3–0.5%, ultracentrifugation experiments will therefore not be feasible even with cells of pathlength d = 3 mm. However, around 246 nm, the absorption minimum of Triton X-100, the absorbance of the detergent is lower by a factor of approx. 10, whereas the absorbance of a typical protein is reduced only by a factor of approx. 2. At that wavelength, which is within the operating range of the UV scanning equipment of the Spinco model E analytical ultracentrifuge, ultracentrifuge experiments on protein dissolved in Triton X-100 would therefore seem feasible.

The micelles of Triton X-100 have a particle weight of approx. 90,000 Dalton [1] and a partial specific volume of approx. 0.91 ml/g [2]. In normal buffers, their absorbance will thus contribute in a complex way to the absorbance-versus-radius profiles $A(r)$ obtained from ultracentrifuge experiments. However, the sedimentation of the micelles can be prevented by adjustment of the solvent density, by addition of D_2O, to the point where it matches the buoyant density of the detergent. This will reduce the contribution of protein-free detergent micelles to $A(r)$ to a constant background. In addition, it will cancel the contribution of protein-bound detergent to the apparent molecular weight of the protein [2, 5, 10]. We have found that the solvent density necessary to prevent sedimentation of Triton X-100 depends on buffer composition and temperature. For each experimental condition, the content of D_2O needed for exact density adjustment was therefore determined from ultracentrifuge runs on Triton/buffer mixtures with different D_2O concentrations.

The dependency of ΔA, the contribution of the protein to total absorbance $A(r)$, on protein concentration c is shown in Fig. 1. The dependency is linear, in accord with the law of Lambert-Beer. Thus it is not affected by stray light, despite the high background due to the absorbance of the detergent. A

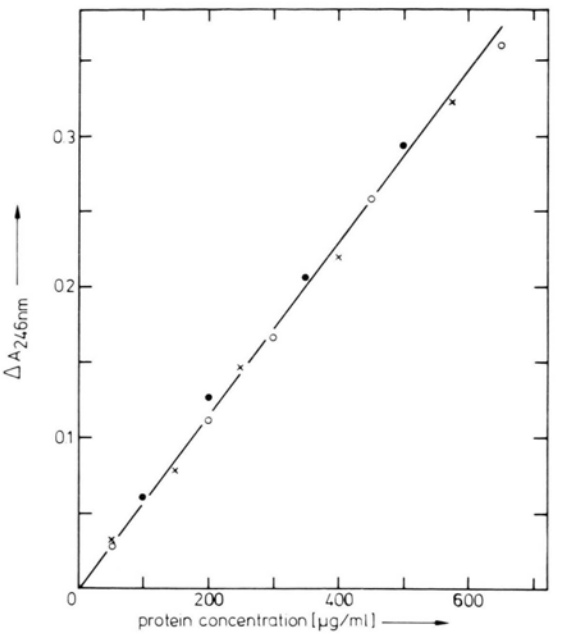

Fig. 1. Contribution ΔA of glucose dehydrogenase to total absorbance at 246 nm *versus* protein concentration. The concentration of Triton X-100 was 0.3%, and the pathlength 12 mm. The different symbols refer to different ultracentrifuge runs.

filter effect caused by the shape of the detergent's absorbance spectrum and its high maximum absorbance may contribute to this behaviour. It should be noted that the data did not follow Lambert-Beer's law if the reference cell contained, instead of water, Triton X-100 at the same concentration as the sample cell.

Plots of ln c *versus* r^2 derived from sedimentation equilibrium experiments on glucose dehydrogenase are shown in Fig. 2. The experiments were performed in cells of pathlength d = 12 mm, at concentrations of Triton X-100 between 0 and 0.4% and at initial protein concentrations between 75 and 225 µg/ml (which, in the range of r which can be evaluated reliably, led to local protein concentrations $c(r)$ of up to 600 µg/ml). The initial protein concentration of 75 µg/ml was the lowest one for which reliable data could be collected; it corresponds to 11 µg protein per cell[a]. All plots yielded straight

[a] As a soluble protein, glucose dehydrogenase will probably bind at most a few molecules of Triton X-100 per polypeptide chain [2]. The large amount of Triton X-100 bound by intrinsic membrane proteins will increase the absorbance of the protein-detergent complex and thus enable its detection at lower protein concentration [13].

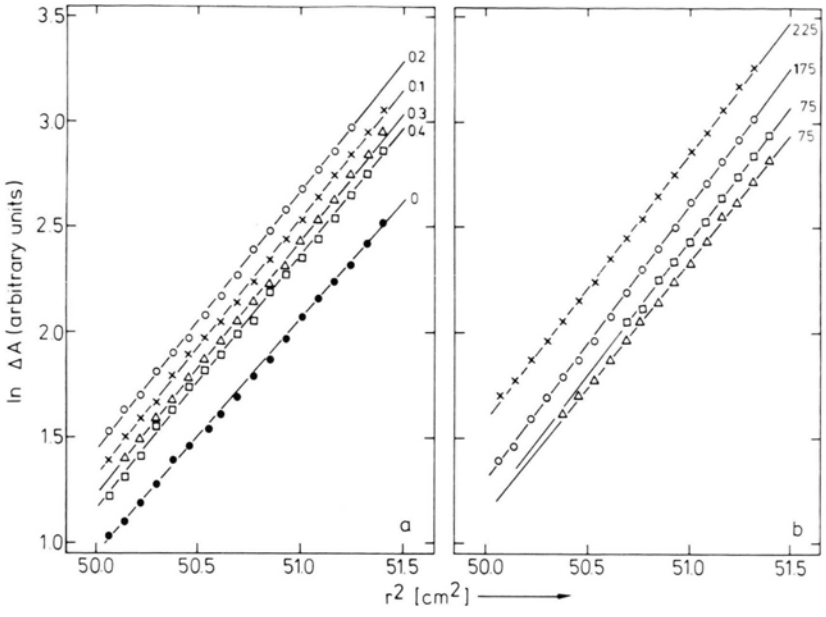

Fig. 2. Plots of ln ΔA *versus* r^2 from sedimentation equilibrium runs on glucose dehydrogenase (a) at an initial protein concentration of 120–160 μg/ml and different concentrations of Triton X-100 (indicated in % (w/w)), and (b) at a Triton concentration of 0.3% (w/w) and different initial protein concentrations (in μg/ml). Cells of pathlength d = 12 mm were used.

lines. From the slopes of the straight lines obtained in the absence of Triton X-100, we found $M_r(1-\bar{v}\varrho_o)$ = 21,500 ± 300 (n = 5). Together with ϱ_o = 1.092 g/ml and $\bar{v}$ = 0.741 ml/g (as derived from the amino acid composition of glucose dehydrogenase [8]) this yields M_r = 112,700 Dalton. If $\bar{v}$ is corrected for the influence of temperature according to [11], which results in $\bar{v}$ = 0.734 ml/g, we obtain M_r = 108,300 Dalton. Both values agree well with the molecular weight derived from amino acid sequence analysis (4 × 28,350 = 113,400 Dalton). The slopes of the straight lines found in the presence of Triton X-100 consistently exceeded the average value found without detergent by approx. 10% and yielded $M_r(1-\bar{v}\varrho_o)$ = 23,800 ± 600 (n = 9). The same behaviour was observed when, at initial protein concentrations of 300–600 μg/ml, Triton concentrations between 0.4 and 1.0% were used in cells of pathlength d = 3 mm (data not shown). The differences between the data from Triton-free and Triton-containing samples can, however, be fully explained by a change in the partial specific volume of the enzyme: a change to $\bar{v}$ = 0.724 ml/g, which would yield the correct M_r, is within the range observed when additional components (*e.g.* ligands) were added to a protein solution [11, 12]. A 12% increase in $M_r(1-\bar{v}\varrho_o)$ has also been found for monomeric AI protein from serum high density lipoprotein when the protein was transferred into solutions of the nonionic detergent Lubrol WX [10]. The data of Fig. 2 thus demonstrate the reliability of our method.

Discussion

Analytical ultracentrifuge studies by means of a UV scanning system on proteins dissolved in Triton X-100 have already been described by others [4, 13]. However, in these studies the concentration of free Triton X-100 (monomeric plus micellar) was only 0.015–0.04%, and the problem of sedimentation of the detergent micelles was not considered. The present paper describes a practical approach to the study of the sedimentation properties of proteins at much higher concentrations of Triton X-100, as necessary for many biochemical studies. It shows that, despite the strong UV absorbance of the detergent, reliable measurements can be made with the commercial version of the UV scanner of the Spinco model E analytical ultracentrifuge and commercial cells. Detergent concentrations of up to 0.4% can be used in cells of pathlength d = 12 mm, and correspondingly higher concentrations at d = 3 mm. Thus, the whole range of detergent concentrations used in studies on membrane proteins can be covered. Approx. 10 μg of protein are sufficient for a reliable determination of

M_r. The evidence presented in this paper is based on sedimentation equilibrium experiments; it is, however, obvious that it can be applied also to sedimentation velocity runs.

According to Fig. 2, Triton X-100 does not affect the state of association of glucose dehydrogenase. It seems worth mentioning that this finding adds additional support to the assumption that nonionic detergents do not significantly perturb protein-protein interactions [1, 2, 4].

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