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Biochimica et Biophysica Acta 1464 (2000) 199–206

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Molecular weight determination of membrane proteins by sedimentation equilibrium at the sucrose or Nycodenz-adjusted density of the hydrated detergent micelle[1]

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Received 6 September 1999; received in revised form 9 December 1999; accepted 22 December 1999

Abstract

The determination of the molecular weight of a membrane protein by sedimentation equilibrium is complicated by the fact that these proteins interact with detergents and form complexes of unknown density. These effects become marginal when running sedimentation equilibrium at gravitational transparency, i.e., at the density corresponding to that of the hydrated detergent micelles. Dodecyl-maltoside and octyl-glucoside are commonly used for dissolving membrane proteins. The density of micelles thereof was measured in sucrose or Nycodenz. Both proved to be about 50% lower than those of the corresponding non-hydrated micelles. Several membrane proteins were centrifuged at sedimentation equilibrium in sucrose- and in Nycodenz-enriched solutions of various densities. Their molecular weights were then calculated by using the resulting slope value at the density of the hydrated detergent micelles, i.e. at gravitational transparency, and the partial specific volume corrected for a 50% hydration of the membrane protein. The molecular weights of all measured membrane proteins, i.e. of photosystem II complex, reaction center of *Rhodobacter sphaeroides* R26, spinach photosystem II reaction center (core complex), bacteriorhodopsin, OmpF-porin and rhodopsin from Bovine retina corresponded within ±15% to those reported previously, indicating a general applicability of this approach.

Keywords: Mass determination; Sedimentation equilibrium; Detergent micelle; Membrane protein

Abbreviations: AU, analytical ultracentrifugation; CMC, critical micelle concentration; HM, hydrated micelle; SS, single sector; DS, double sector; MP, membrane protein; SE, sedimentation equilibrium; SV, sedimentation velocity; OG, octyl-glucoside (*n*-octyl-α-D-glucopyranoside); octyl-POE, octyl-tetraoxyethylene ($C_8E_{4,5}$); DDM, dodecylmaltoside (lauryl-D-maltoside); Suc, sucrose; Nyc, Nycodenz; PBS, phosphate buffered saline; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, Tris-(hydoxymethyl)-amino-methane; FC-43, perfluorotributylamine $(C_4F_9)_3N$

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[1] Dedication: In memory of our late friend Martin Zulauf, who crashed with his 'Ultralight' on June 17, 1995 in France. He published more than 50 papers on detergents and their interactions with proteins. It was always a pleasure to work with him and we hope that this modest contribution will be in his sense.

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0005-2736/00/$ – see front matter
PII: S0005-2736(99)00254-0

1. Introduction

Membrane proteins (MP) are generally isolated in association with detergents. Determination of the molecular mass of the protein in the protein-detergent complex can be accomplished by analytical ultracentrifugation (AU) via sedimentation equilibrium (SE) - without prior knowledge of detergent binding - provided SE is carried out at gravitational transparency, i.e. at conditions where the density of the solution equals that of the detergent micelles ($\rho_{solution} = \rho_{hydrated\ micelle\ (HM)}$) [1]. As Ralston [2] pointed out, '*If SE experiments are performed in solutions where the density is the same as that of the bound detergent, the detergent becomes effectively transparent to the gravitational field*'.

Conditions for gravitational transparency had already been achieved for specific proteins. Reynolds et al. [1] extrapolated the state of gravitational transparency from the densities obtained by SE at increasing concentrations of D_2O. Suarez et al. [3] further modified this approach by combining $D_2^{18}O$ with dodecylmaltoside (lauryl-D-maltoside) (DDM), a detergent with a density close to that of the solvent. Rosenbusch et al. [4] introduced a different approach: Instead of adjusting the density of the solvent to that of the detergent micelle, a detergent with a density close to water: octyl-tetraoxylethylene ($C_8E_{4,5}$) (octyl-POE) was used [5]. However, both ways of achieving gravitational transparency have their problems: Many detergents have densities exceeding that of D_2O and, in turn, the density of bound detergent cannot be compensated for. Adding the protein dissolved in buffer and detergent to D_2O lowers the solvent density, and dialysis of small volumes is not trivial. The use of $D_2^{18}O$ in combination with detergents whose micelle density is higher than that of D_2O would make sense, though costs might be a limiting factor. The application of octyl-POE was successful in the case of porin and some other MP's. Other proteins may, however, undergo denaturation by octyl-POE.

The addition of densifiers such as sucrose (Suc) or Nycodenz (Nyc) [6] to MP's dissolved in detergents should offer an alternative for achieving gravitational transparency. We have therefore sought to establish a procedure to reliably estimate molecular masses of MP's via the use of densifiers for achieving gravitational transparency. The results of this approach are presented in this communication.

2. Materials and methods

2.1. Chemicals

n-Octyl-α-D-glucopyranoside (OG: Serva GmbH, D-6900 Heidelberg, Germany); *n*-α-D-maltopyranoside (DDM: Fluka AG, CH-9470 Buchs, Switzerland); octyl-tetraoxyethylene (octyl-POE: Alexis Corporation, San Diego, CA 92121-4727); Nycodenz (Nyc: Nyegaard, Oslo, Norway), Perfluorotributylamine (FC43: 3M Company, St. Paul, MN, USA).

The buffers used were phosphate buffered saline (PBS) (20 mM sodium phosphate, 100 mM NaCl, pH 7.4); MES (10 mM MES [2-(*N*-morpholino)ethanesulfonic acid], 100 mM NaCl, pH 6.5), and Tris (10 mM Tris-HCl, [Tris-(hydroxymethyl)-aminomethane], 100 mM NaCl, pH 7.5).

2.2. Ultracentrifugation

Sedimentation velocity (SV) and SE runs recorded with absorption optics were carried out with a Beckman XLA analytical ultracentrifuge (AUC) and those with schlieren and interference optics with the Beckman model E AUC, both at 20°C. Aqueous stock solutions of concentrated Suc or Nyc were used. Their densities were determined by a DMA 02C digital density-meter (A. Paar AG, Austria). Suc and Nyc stock solutions were then used for density adjustment in SE. The absence of electrolyte and detergent in these dense stock solutions was compensated for by adding more detergent or electrolyte to the protein/detergent/buffer solution, thus yielding the desired final concentration of detergent and salt after mixing. Similar mixing and filling techniques have been described in detail by Lustig et al. [7].

All measurements were carried out in thin cells to minimize the interference of the strongly refracting Suc or Nyc. SE runs with absorption (abs) optics were usually performed in 4 mm double sector (DS) cells.

In general, the DS cells were filled up to 2 mm filling height above the FC-43 bottom fluid level. The two sectors (with and without protein) were

adjusted to about the same filling height to assure comparable refraction conditions during centrifugation.

SE runs with schlieren optics were carried out in 3 mm single sector (SS) cells, because the schlieren pattern exhibits a higher contrast than DS cells. The slope $d\ln c/dr^2$, where c is the solute concentration at radial distance r, was determined by using the Lamm equation [8] in combination with a floating baseline computer program essentially as described [9].

2.3. Calculation of molecular masses of MP's

The condition for gravitational transparency in SE of MP's is met by setting the solution density ρ equal to ρ_{HM}, the density of the hydrated detergent micelle. If transparency is attained by use of D_2O or $D_2^{18}O$, without addition of densifier, the molecular mass of the protein M_p is expressed ([1]; eq. 3 of ref. [3]) as:

$$M(1-\bar{\nu}_p\rho_{HM}) = \frac{2RT}{\omega^2}\left(\frac{d\ln c_p}{dr^2}\right)$$

where $\bar{\nu}_p$ is the protein's partial specific volume, R the universal gas constant, T the absolute temperature, ω the angular velocity, c_p the protein concentration and r the distance from the axis of rotation (radial distance).

The molecular weights were calculated using the above formula. The molecular masses were determined using a floating baseline computer program that adjusts the baseline absorbance to obtain the best linear fit of the slope $\ln A$ versus r^2, where A is absorbance and r the radial distance. Since gravitational transparency was attained via densifiers (Nyc or Suc) the specific state of hydration of the MP had to be considered. In the absence of specific information on the hydration of individual MP's, we used $\bar{\nu}_p = 0.757$ cm^3/g. The reciprocal of the latter value, 1.32 g/cm^3, derives from the average between 1.27 g/cm^3, the density of fully hydrated proteins in Nyc, Suc or Metrizamide, as determined by Rickwood [20] and 1.37 g/cm^3 the estimated density for non-hydrated proteins [2].

3. Results

In the following, we present a number of MP-detergent systems which have been of interest during recent years. Detailed information about the molecular weights of the MP subunits can be found in the

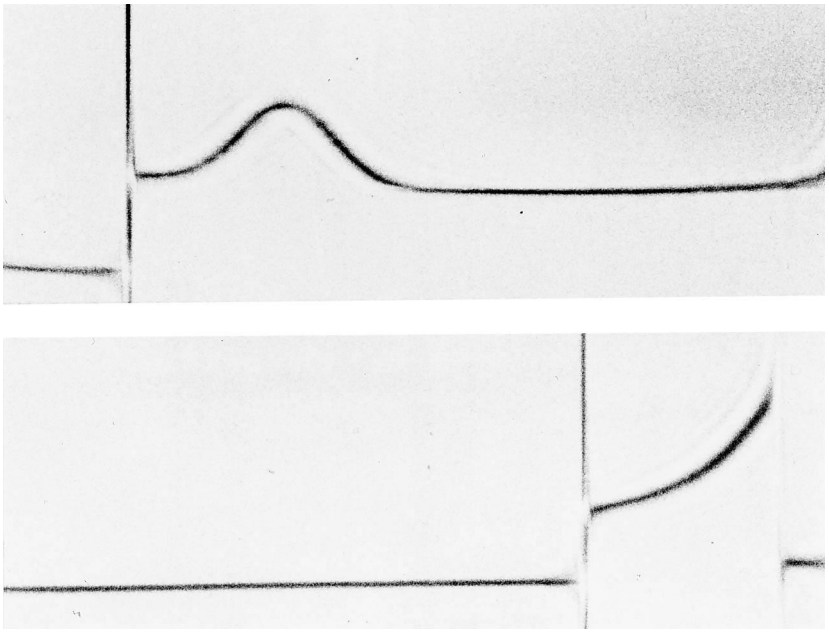

Fig. 1. A: SV of detergent-only micelles used as a purity test: typical schlieren pattern of 0.3% dodecyl maltoside (DDM) in PBS, $\rho_{solution} = 1.003$ g/cm^3 at 56 000 rpm, 22°C, recorded after 63 min at top speed. $S_{20obs} = 3.0$. B: SE of 0.3% DDM in PBS/Nyc in a 3 mm SS cell. $\rho_{solution} = 1.043$ g/cm^3, photographed with schlieren optics at 22 000 rpm, 22°C. Slope $d\ln c/dr^2 = 0.61$.

Table 1
Experimentally determined densities of hydrated detergent micelles

Detergent (%)	Solution	Optics	ρ (g/cm^3) at intersection	rpm	ρ reported for non-hydrated micelles
DDM0.3	Tris/Nyc	sch	1.110	20000	1.21–1.23 [5,10]
DDM0.3	PBS/Suc	sch	1.116	24000	
DDM0.3	PBS/Suc	int	1.124	34000	
OG1.5	PBS/Suc	sch	1.082	24000	1.15–1.16 [11,12]
OG1.5	PBS/Nyc	sch	1.086	32000	
OG1.5	PBS/Nyc	sch	1.082	24000	

sch = schlieren; int = interference

cited literature. Since the molecular weights of these MP's could not be determined by conventional UC methods, we aimed at measuring them via density adjustment by common densifiers such as Suc or Nyc. Our experimental molecular weights of the MP's are summarized (Table 2: experimental Mw in kDa).

3.1. Determination of the density of hydrated detergent micelles ρ_{HM} (detergent only)

The density (ρ_{HM}) of protein-free hydrated detergent micelles was determined in Suc or in Nyc-enriched buffer by SE using schlieren or interference optics. The buffer corresponded to that used for the molecular weight determination of the respective MP. SE runs were performed at various densities between 1.0 and 1.22 g/cm^3 for each detergent. Within this range the $d\ln c/dr^2$ slope values switch from negative to positive. Hence, $\rho_{HM} = \rho_{solution}$ is met for $d\ln c_{detergent}/dr^2 = 0$. The intersection was determined graphically. Figs. 1 and 2 illustrate the procedure followed. Fig. 1A displays the schlieren pattern of the SV of 0.3% DDM in PBS revealing the micellar sedimenting boundary. Fig. 1B shows the schlieren pattern of the SE of 0.3% DDM in PBS/Nyc. A graphical evaluation of ρ_{HM} is shown in Fig. 2

Table 1 summarizes the density values obtained for DDM and OG-micelles at concentrations exceeding critical micelle concentration (CMC). The ρ_{HM} values obtained were reproducible and well comparable at the various conditions, indicating the influence of densifier or rotor speed to be negligible. However, in all cases the experimental values determined in Suc or Nyc were about 50% lower than those found for the corresponding detergent micelles in buffer without densifiers [10–12], and a comparison to a micelle density established by pycnometry was not feasible without precisely knowing the effective micelle concentration [13].

For the determination of ρ_{HM} of DDM and OG micelles a higher detergent concentration was used than for the determination of the molecular weight of the MPs. In the case of DDM it was 0.3% instead of 0.03% thus yielding an optical refraction signal appropriate for detection. In the case of OG which has a CMC of about 1.2% [5] 2% was used to be well above CMC.

It may be worth mentioning that the filling height of the cells can become crucial. At high speed in a long solution column the distribution of the detergent concentration may reach values lower than CMC. This effect is avoided when using relatively

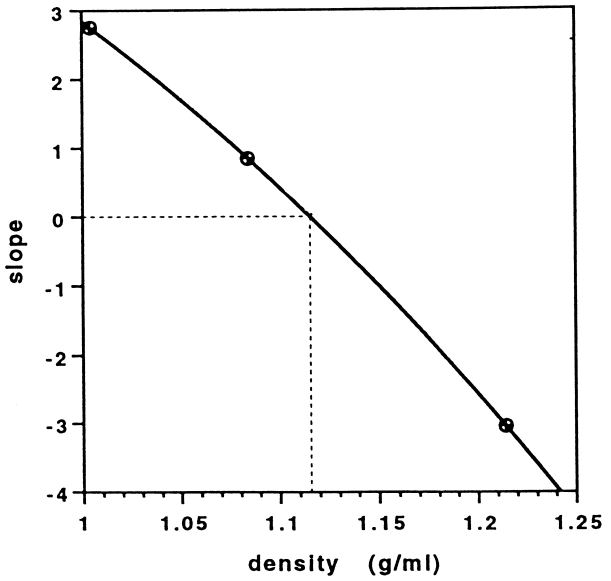

Fig. 2. Graphic determination of the density ρ_{HM} at gravitational transparency ($d\ln c_{detergent}/dr^2 = 0$) of 0.3% DDM HMs by SE. In PBS/Suc in a SS cell at 24 000 rpm, 20°C.

Table 2
Molecular weights and sedimentation velocities $S_{20\,detergent}$ determined experimentally at gravitational transparency (via micelle density)

MP	Detergent (%)	Solution	Optics	rpm	Slope $d\ln c/dr^2$	ρ (g/cm^3) at intersection	$S_{20\,obs}$	Part. spec. vol. (V)	Experimental Mw (kDa)	Reported values (kDa)
PS II	DDM 0.03	Mes	abs	48 000			12.6			
PS II	DDM 0.03	Mes/Nyc	abs	9 000	0.58	1.11		0.757	199	247 [14]
RC-rhsp	DDM 0.03	Tris	abs	56 000			7.1			
RC-rhsp	DDM 0.03	Tris/Nyc	abs	9 000	0.3	1.11		0.757	103	102 [15]
RC-rhsp	DDM 0.03	Tris/Nyc	abs	18 000	1.25	1.11		0.757	107	102
RC-spinach	DDM 0.03	Mes	abs	56 000			9.0			
RC-spinach	DDM 0.03	Mes/Nyc	abs	20 000	1.7	1.11		0.757	118	103 [16]
bR	OG 1.2	PBS	abs	56 000			2.1			
bR	OG 1.2	PBS/Nyc	abs	24 000	0.55	1.08		0.757	23.4	26.5 [17]
OmpF-porin	OG 1.2	PBS	abs	56 000			6.2			
OmpF-porin	OG 1.2	PBS/Nyc	int	10 000	0.42	1.08		0.757	103	110 [2,18]
Bovine rho	OG 1.2	PBS	abs	56 000			4.0			
Bovine rho	OG 1.2	PBS/Suc	abs	22 000	0.9	1.08		0.757	40	42 [19]

abs = absorption; int = interference
PS II (Photosystem II complex); RC-rhsp (reaction center of *R. sphaeroides* R26); RC-spinach (spinach photosystem II reaction center); bR (bacteriorhodopsin); OmpF-porin; Bovine rho (rhodopsin from bovine retina)

moderate rotor speeds and filling heights of 1.5 to 2.5 mm.

3.2. Auxiliary SV measurements

Prior to SE, each MP was tested for monodispersity by SV in the detergent/buffer mixture and at a density close to that of the buffer, i.e., in absence of Nyc or Suc (Fig. 3). The resulting sedimentation coefficient S_{obs} are summarized in Table 2. It should be borne in mind that these S-values are not solely protein specific but depend on the sedimentation behavior of the protein-detergent complex (protein detergent micelles), in contrast to e.g. globular proteins soluble in the absence of detergents. Moreover, such sedimentation coefficients of MP's obtained by SV may be pressure dependent, as it was the case with OmpF-porin in octyl-POE (Rosenbusch and Lustig, unpublished).

3.3. Determination of the molecular weight of MPs

The molecular mass of a MP can in principle be determined by a single SE run, if carried out in Suc or Nyc solution at the density equal to ρ_{HM}, i.e., at gravitational transparency of the hydrated detergent micelle. Actually, a series of SE runs were carried out at different solution densities bracketing ρ_{HM}. The value $d\ln c_{protein}/dr^2$ at ρ_{HM} was then determined from a plot of these SE results by interpolation, and the molecular weight of the MP was calculated from the latter.

In the course of this study, the molecular weights of six different MP's (photosystem II complex, reaction center of *Rhodobacter sphaeroides* R26, spinach photosystem II reaction center (core complex), bacteriorhodopsin, OmpF-porin and rhodopsin from

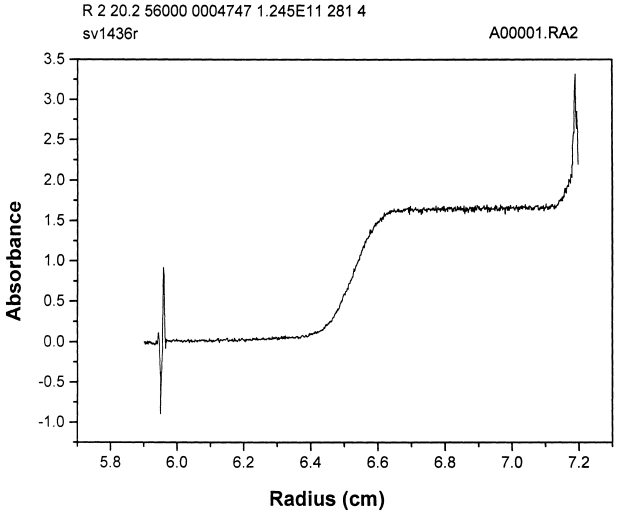

Fig. 3. SV of reaction center from *R. sphaeroides* R26 (RHPS-rc) in Tris-HCl/0.03 DDM used as purity test: Absorption scan recorded at 281 nm after 58 min at 56 000 rpm, 20°C. $S_{20} = 7.1$.

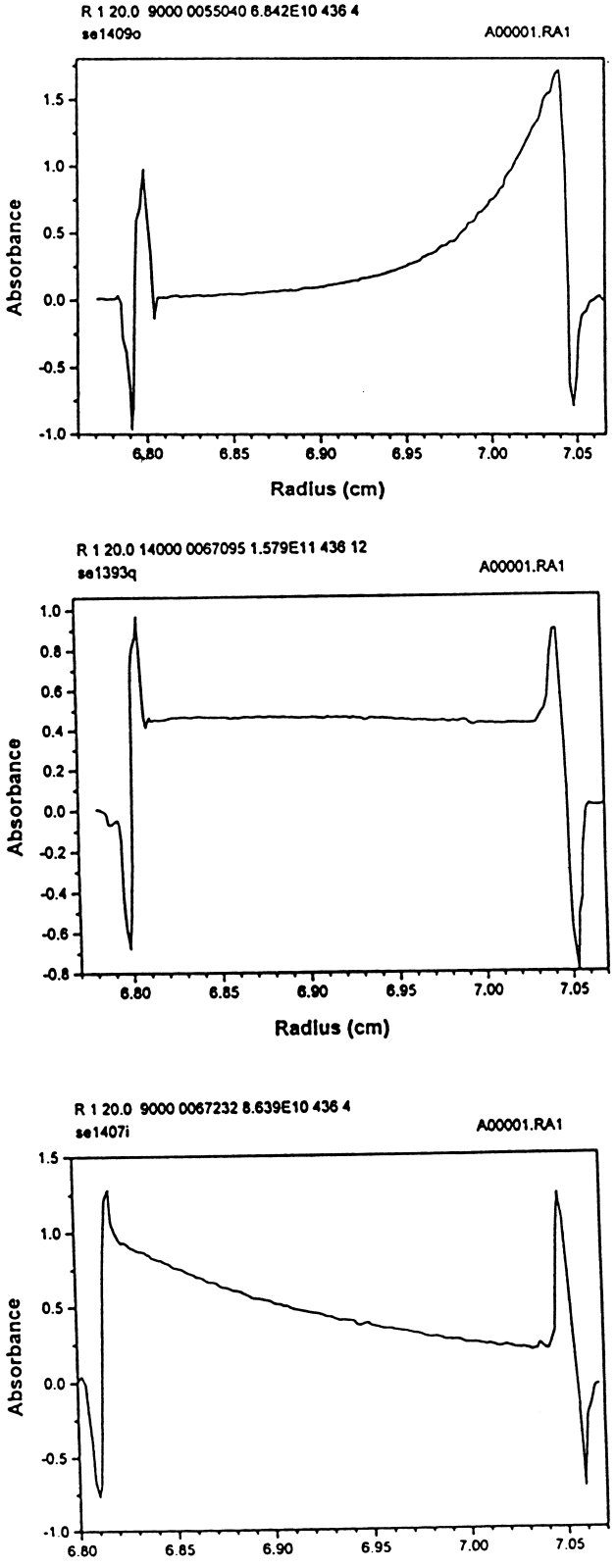

Fig. 4. Influence of solution density on SE. SE of photosystem II complex (PS II) in MES/0.03% DDM in 4 mm DS cells, 20°C, scanned at 436 nm. The Nyc concentration was varied. (top) 9000 rpm, solution density $\rho = 1.004$ g/cm^3. Slope = 1.55, in sedimentation direction; (middle) 14 000 rpm, solution density $\rho = 1.17$ g/cm^3. Slope = close to zero, at gravitational transparency; (bottom) 9000 rpm, solution density $\rho = 1.21$ g/cm^3. Slope = −0.543, in flotation direction.
←

bovine retina) were determined by SE at gravitational transparency (Table 2), which was deduced from SE runs at different densities achieved by addition of densifiers (Table 1).

For each MP at least three slope values were determined at different solution densities, usually ranging within 1–1.25 g/cm^3. Fig. 4 illustrates the SE behavior of PS II in Nyc below (Fig. 4A), approximately at (Fig. 4B), and above (Fig. 4C) gravitational transparency. The final evaluation of the slope $d\ln c_{protein}/dr^2$ at ρ_{HM} for porin OmpF in PBS/1 2% octyl-glucoside/Nyc solution is documented in Fig. 5.

It may be worth mentioning that in Fig. 5, the ρ-value at $d\ln c_{protein}/dr^2 = 0$ corresponds to the density of the MP-detergent-hydrated complex.

Nyc which absorbs strongly in the UV interferes

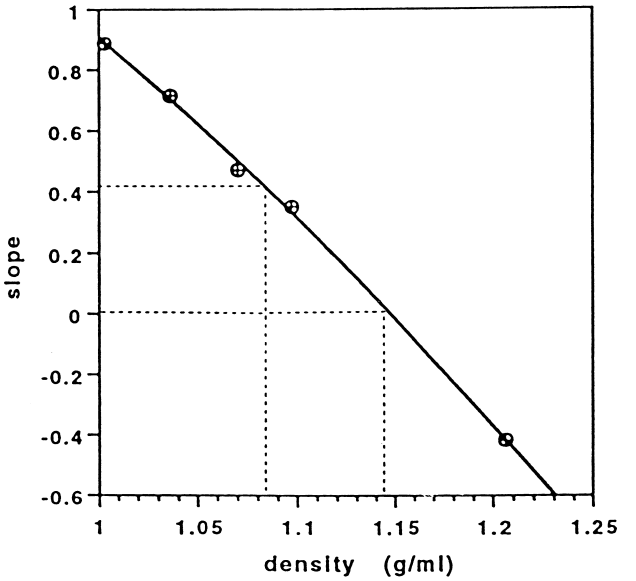

Fig. 5. Graphical determination of the molecular weight of MPs by SE. OmpF-porin in PBS/1.2% octyl-glucoside/Nyc solution. SE in 4 mm DS cells, 10 000 rpm, 20°C. Intersection: $d\ln c_{protein}/dr^2$ at $\rho = 1.082$ g/cm^3, slope = 0.42. Intersection: $\rho = 1.142$ g/cm^3 for $d\ln c_{protein}/dr^2 = 0$.

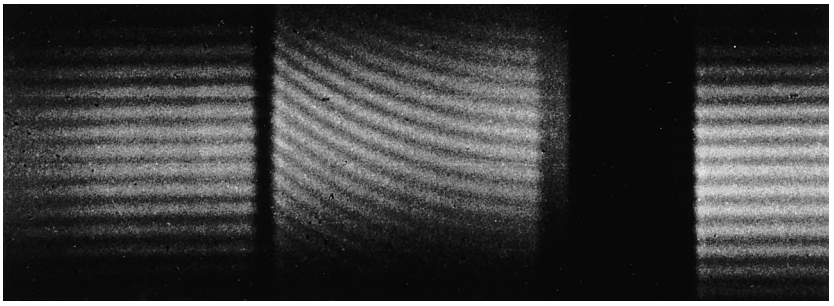

Fig. 6. SE of OmpF-porin at 4.5 mg/ml in PBS/1.2% octyl-glucoside/Nyc, $\rho = 1.21$ g/cm^3 using interference optics, recorded at equilibrium in a 4 mm DS cell at 16 000 rpm, 22°C in floating direction.

with protein absorption in the UV. In absorption optics the use of Nyc is thus restricted to proteins absorbing in the visible region (Table 2). Alternatively, interference optics can be used (Fig. 6). Nyc is less viscous than Suc, which may be advantageous.

4. Discussion

The present study introduces a new approach for the estimation of the molecular mass of MP's by AU, which is complementary to reported procedures having been used for measuring the molecular mass of specific MP's [1,3,4]. All methods are based on the principle of gravitational transparency, i.e., on 'blanking out' of the contribution of bound detergent. In contrast to the earlier methods, the use of densifiers such as Nyc or Suc for increasing the solution density seems to be much more versatile than the use of heavy water or of specific detergents such as octyl-POE. Moreover, with densifiers such as Nyc or Suc, densities well above that of D_2O and even $D_2^{18}O$ can be achieved. In consequence, this new approach appears to be more widely applicable and further opens the possibility to apply it to MP systems, that involve dense solubilizing detergents such as OG or DDM.

The use of densifiers for achieving gravitational transparency is complicated by the fact that at ρ_{HM} the system would be transparent for fully hydrated proteins, but not for partially hydrated proteins such as MP's. The value for the partial specific volume of fully hydrated proteins would therefore be valid for calculating the molecular mass at ρ_{HM} as long as the water bound to the protein exchanges with the heavy water in solution, thus adjusting the density of the bound water accordingly. In contrast to heavy water molecules, densifiers do not exchange with the bound water. Hence, the system will not become transparent for the water bound as a hydration shell to the MP. The partial specific volume of the MP has thus to be corrected for partial hydration. Since there is no easy way for measuring the partial hydration of a specific MP, we accounted for it in an empirical fashion. Assuming a 50% hydration of the MP, we used the arithmetic mean between the reported values for the partial specific volume for fully hydrated and for non-hydrated proteins for calculation of the molecular mass. This empirical approach of delineating the state of hydration of a MP was demonstrated to give reliable values for the molecular masses of a variety of MP's, and within a reasonable range of error with respect to the published values.

Acknowledgements

The authors thank Prof. Ezra Daniel (Tel Aviv University) for critical reading of the manuscript and valuable suggestions, Leo Faletti and Hans Vogt for modifying the DS cells, and Franz Biry and Dr. Gernot Hänisch for computing assistance. We are indebted to Profs. Jurg Rosenbusch and Jürgen Engel for their continuous encouragement. Last, but not least we would express our thanks to Peter Acherman, Beckman Instruments Inc. and his office, who never failed to help running the instruments. This work was supported by the M.E. Müller Foundation of Switzerland, and the Canton of Basel-stadt.

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