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Hydrodynamic properties of the Triton X-100-solubilized chloroplast phosphate translocator

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The molecular weight of the phosphate translocator isolated from spinach envelope membranes was measured in the nonionic detergent Triton X-100. The Stokes' radius of the protein-detergent complex was estimated by gel filtration. The partial specific volume was estimated by equilibrium centrifugation and by differential sedimentation in sucrose gradients containing H_2O and ^2H_2O and the sedimentation coefficient was estimated from the same centrifugation experiments. The phosphate translocator-Triton X-100 complex has an apparent molecular weight of 177500. Its high partial specific volume (0.86 cm^3/g) suggests that bound detergent contributes significantly to the mass. Correcting for the bound detergent (1.9 g/g protein), a molecular weight of 61000 for the protein moiety of the complex was calculated. These results suggest that the isolated phosphate translocator exists as a dimer. The shape of the dimer is described as a prolate ellipsoid of revolution with semiaxes calculated to be 6.59 and 1.59 nm in length.

Introduction

The phosphate translocator is located in the inner membrane of the chloroplast envelope and catalyzes the export of fixed carbon as triose phosphates from the chloroplast into the cytosol in exchange with inorganic phosphate (for review see Ref. 1). This protein has been identified as a major component of the envelope membrane with a molecular weight of 29000 as determined by SDS gel electrophoresis [2]. Recently, the phosphate translocator has been isolated in the nonionic detergent Triton X-100 and reconstituted into liposomes in a functional state [3,4]. In the present study, the hydrodynamic properties of the isolated phosphate translocator-Triton X-100 micelle are described. Based on these measurements, the molecular weight of the protein-Triton complex can be determined. Correcting for bound detergent, it is suggested that the phosphate translocator is a dimeric protein composed of two polypeptide subunits.

Materials and Methods

The radiochemicals were obtained from New England Nuclear; Triton X-100 from Sigma, and Sephacryl S-300 and Sephadex G-25 from Pharmacia, Sweden. Hydroxylapatite (Bio-Gel HTP) and Affi-Gel 501 were purchased from Bio-Rad Laboratories. All other reagents were of the highest purity available.

Envelope membranes from spinach chloroplasts were prepared according to the method of Douce et al. [5]. When indicated, the phosphate translocator was specifically labelled by treatment with 2,4,6-trinitrobenzenesulfonic acid/NaB^3H_4 [3]

Abbreviations: SDS, sodium dodecyl sulfate; Mops, 4-morpholinepropanesulfonic acid.

prior to the isolation of the protein, which was performed as described earlier with a sucrose density centrifugation as the last purification step. At this stage, the phosphate translocator is purified to apparent homogeneity [3]. In order to get rid of the excess of free Triton X-100 micelles, the pooled fractions of the sucrose gradient containing the purified phosphate translocator were first concentrated by ultrafiltration to about 1 ml and then subjected to column chromatography on Sephadex G-25 (1.5 × 20 cm) in order to remove dithiothreitol which had been included in the sucrose density centrifugation step. Subsequently, the phosphate translocator protein was bound to an Affi-Gel 501 column (0.5 × 6 cm) equilibrated with 0.2 M NaCl/0.2% Triton X-100/20 mM Mops (pH 7.4). Free Triton X-100 micelles were removed by washing with 20 ml equilibration buffer. For the elution of the phosphate translocator, the equilibration buffer contained in addition 10 mM dithiothreitol. This preparation exhibited a Triton X-100/protein ratio of less than 10 and was used for the centrifugation studies and the estimation of the Stokes' radius by column chromatography.

Determination of the Stokes' radius. The ^{3}H-labelled phosphate translocator protein (200 μg, 13 μCi/mg protein) together with marker proteins were loaded on a Sephacryl S-300 column (1.0 × 95 cm) which had been previously equilibrated with 20 mM Mops/0.2 M NaCl/0.02% NaN_3/1 mM EDTA/0.2% β-mercaptoethanol/5 mM KH_2PO_4/0.2% Triton X-100 (pH 7.4). The marker proteins used were *Escherichia coli* β-galactosidase (R_S = 6.9 nm), phosphorylase a (R_S = 6.4 nm), catalase (R_S = 5.2 nm), aldolase (R_S = 4.6 nm) and cytochrome *c* (R_S = 1.7 nm). The hydrodynamic properties for the calibrating proteins are from Ref. 6. The column was then eluted with the equilibration buffer at a flow-rate of 2 ml/h. Fractions of 0.5 ml were collected and analyzed for radioactivity as well as for marker proteins. β-Galactosidase, phosphorylase *a* and aldolase were assayed enzymatically and catalase and cytochrome *c* were located spectrophotometrically at A_{546} and A_{420}, respectively. Blue dextran and [^{14}C]sorbitol were used for the estimation of the void volume V_0 (30.5 ml) and the total volume V_t (75.0 ml) of the column.

Determination of $\bar{v}$ and $s_{20,w}$ of the phosphate translocator-Triton X-100 complex. The estimation of the partial specific volume $\bar{v}$ was performed by two different approaches. Firstly, it was determined by equilibrium centrifugation. 60 μg of the purified phosphate translocator was introduced into a 4.5 ml 10–45% (w/w) sucrose gradient comprising 0.25 M NaCl/2 mM dithiothreitol/0.02% NaN_3/1 mM EDTA/4 mM KH_2PO_4/0.3% Triton X-100/20 mM Mops (pH 7.4). Centrifugation was performed for 78–90 h at 325 000 × *g* (5°C) in a Beckman SW-56 rotor. After centrifugation, 0.14-ml fractions were collected. The concentration of sucrose in the various fractions was determined refractometrically. The phosphate translocator was located by running an aliquot of each fraction on a SDS-polyacrylamide gel [7]. After staining, the protein was quantified by scanning at 578 nm. Secondly, the determination of $\bar{v}$ in combination with that of the sedimentation coefficient $s_{20,w}$ was carried out by sucrose density centrifugation in a Beckman SW-56 rotor using gradients made up in H_2O or ^2H_2O. Sucrose gradients (4.2 ml) of 8–20% (w/v) sucrose in 0.3% Triton X-100/1 mM EDTA/2 mM dithiothreitol/0.25 M NaCl/0.02% NaN_3/20 mM Mops (pH 7.4) were prepared either with H_2O or 65% ^2H_2O as the solvent. Standard proteins (200 μl) including the phosphate translocator were layered on top of the gradients. The centrifugation time was 24 h at 340 000 × *g* at 5°C. After centrifugation, fractions of about 140 μl were collected. The marker proteins used were alcohol dehydrogenase from horse liver (4.99 S, 0.75 cm^3/g) bovine serum albumin (4.58 S, 0.75 cm^3/g), malate dehydrogenase from pig heart (4.32 S, 0.74 cm^3/g), peroxidase from horse radish (3.42 S, 0.699 cm^3/g) and cytochrome *c* from horse heart (2.1 S, 0.73 cm^3/g). Alcohol dehydrogenase and malate dehydrogenase were assayed by standard enzymatic assays, cytochrome *c* spectrophotometrically at A_{420} and bovine serum albumin, peroxidase and the phosphate translocator by running an aliquot of each fraction on a SDS-polyacrylamide gel. Alternatively, if the phosphate translocator was applied as the [^{3}H]trinitrophenyl derivative, it was located by radioactive counting. From the sedimentation of the phosphate translocator-Triton X-100 complex in relation to that of the marker proteins in sucrose density gradients made up in

H_2O or ^2H_2O, the partial specific volume and the sedimentation coefficient of the phosphate translocator-Triton X-100 complex were calculated using the equations given by Clarke [8]. The molecular weight of the phosphate translocator-Triton X-100 complex was calculated according to the following equation:

$$M_r = \frac{6\pi N \eta_{20,w}}{1 - \bar{v} \cdot \rho_{20,w}} \cdot R_S \cdot s_{20,w}$$

where R_S is the Stokes' radius, N is Avogadro's number ($6.02252 \cdot 10^{23}$), $\eta_{20,w}$ the viscosity of water at 20°C (0.01 0002 $g \cdot cm^{-1} \cdot s^{-1}$) and $\rho_{20,w}$ the density of water at 20°C (0.998203 g/cm^3). The frictional ratio for the anhydrous micelle was calculated by the equation:

$$f/f_0 = R_S(4\pi N/3M_r\bar{v})^{1/3}$$

The relation between the diffusion coefficient and Stokes' radius is given by:

$$D = k \cdot T/(6\pi\eta \cdot R_S)$$

where k is the Boltzmann's constant and T is 293 K.

Determination of the Triton X-100 binding. The amount of Triton X-100 bound to the phosphate translocator was determined by measuring the ^3H-labelled detergent which eluted together with the phosphate translocator from an Affi-Gel 501 column (0.5 × 5 cm). This column had been pre-equilibrated with 0.3 M NaCl/5 mM KH_2PO_4/1 mM EDTA/0.02% NaN_3/0.05% Triton X-100/20 mM Mops (pH 7.4) and was loaded with about 0.3 mg of the purified phosphate translocator. The column was equilibrated further with the same buffer containing 0.05% [^{3}H]Triton X-100 with a specific activity of 77 μCi/g at a flow-rate of 8 ml/h for 20 h. The phosphate translocator was then eluted with 20 mM dithiothreitol in the equilibration buffer. The protein contents of the eluted fractions were measured as described in Ref. 9.

Results and Discussion

For the estimation of the size and shape of a membrane protein, it is important to ensure that the native structure is retained during solubilization and purification. This can be achieved by use of a mild nonionic detergent which replaces the lipid bilayer and closely simulates the native environment of the membrane protein [10]. It can then be characterized by evaluation of the hydrodynamic properties of the isolated protein-detergent complex. Rate sedimentations in sucrose gradient containing H_2O and ^2H_2O provide a reliable method to determine the sedimentation coefficient and the partial specific volume as well [8]. To relate both values to the molecular weight of the protein-detergent complex, the Stokes' radius must be known. This can be estimated by gel exclusion chromatography. For the calculation of the molecular weight of the protein moiety of the protein-detergent complex, the amount of detergent bound to the protein under defined conditions must also be known.

For the determination of the Stokes' radius, the phosphate translocator-Triton X-100 complex was applied to a Sephacryl S-300 chromatography. Calibration of the column in the presence of Triton X-100 was achieved by co-chromatography of proteins of known Stokes' radii that do not bind the detergent in the elution buffer. A plot of $1 - K_{av}$ versus the Stokes' radii of the calibration proteins is shown in Fig. 1. From this, a Stokes' radius of 6.32 nm for the protein-detergent complex was measured. The diffusion coefficient (D, calculated as described in Materials and Methods) was $3.39 \cdot 10^{-7} cm^2/s$.

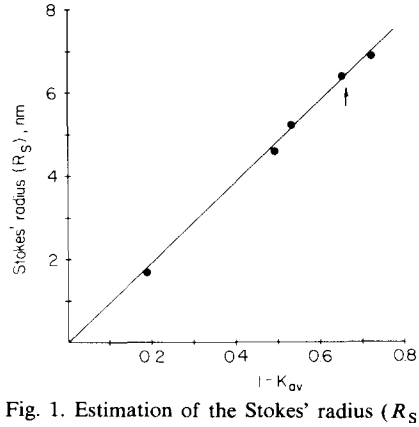

Fig. 1. Estimation of the Stokes' radius (R_S) by gel filtration on Sephacryl S-300. The arrow indicates the position of the phosphate translocator-Triton complex. For details see Materials and Methods.

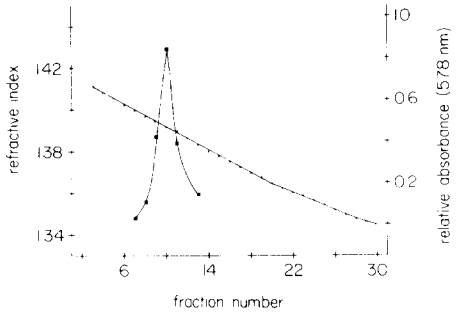

Fig. 2. Determination of the partial specific volume by sucrose gradient centrifugation. 60 μg of the purified phosphate translocator-Triton complex was centrifuged for 85 h at 325000 × *g* (5°C) in a 10–45% (w/w) sucrose gradient containing 0.3% Triton X-100. For further details see Materials and Methods.

The partial specific volume ($\bar{v}$) of the protein-Triton X-100 complex was determined by two methods. Firstly, the complex was subjected to equilibrium density centrifugation in sucrose gradients. After 78–90 h, the complex was located at a sucrose concentration of 36.7% (w/w) (Fig. 2). This value corresponds to a density of 1.160 g/cm^3, giving a partial specific volume for the protein-Triton X-100 complex of 0.862 cm^3/g ($n = 5$). Secondly, the determination of both $\bar{v}$ and the sedimentation coefficient $s_{20,w}$ was performed by sedimentation of the phosphate translocator-Triton X-100 complex together with proteins of known hydrodynamic properties into sucrose gradients made up in H_2O and ^2H_2O. This method was first described by Edelstein and Schachmann [11] and Meunier et al. [12].

The results of those sucrose gradient centrifugations are shown in Fig. 3. In the H_2O-containing gradient, the phosphate translocator sediments between peroxidase and cytochrome *c*, and in the ^2H_2O-containing gradient, near cytochrome *c*. The change in the relative position of the complex in comparison to that of the calibration proteins on the H_2O and ^2H_2O gradient indicate that the partial specific volume of the complex is higher than those of the protein standards. Analysis of the data presented in Fig. 3 and calculated as described in Ref. 8 reveal a sedimentation coefficent of 3.5 S and a partial specific volume of 0.858 cm^3/g for the phosphate translocator-Triton X-100 complex ($n = 7$), in close agreement with the first

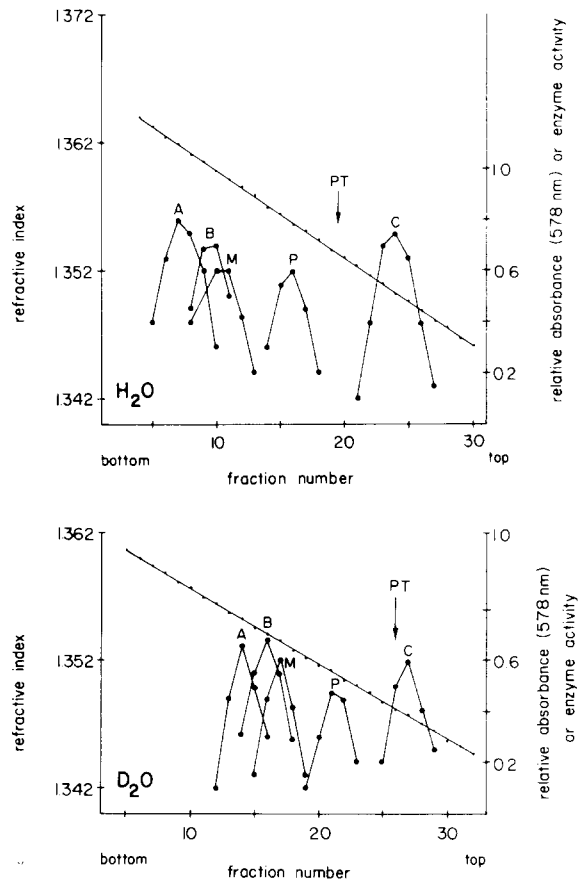

Fig. 3. Sucrose density gradient centrifugation of the phosphate translocator-Triton complex in the presence of calibration proteins in H_2O (top) and ^2H_2O (bottom). The calibration proteins employed were alcohol dehydrogenase (A), bovine serum albumin (B), malate dehydrogenase (M), peroxidase (P) and cytochrome *c* (C). The arrows indicate the position of the phosphate translocator-Triton complex (PT). For experimental details see Materials and Methods.

method. In the determination of $\bar{v}$, it is assumed that changes due to the deuteration of the components are insignificant and that the binding of detergent is independent of the sucrose concentration. The conceivable change in detergent binding to the protein due to substituting ^2H_2O for H_2O, however, is explicitly considered by the analysis method used for the calculation of $\bar{v}$ [8].

For the determination of the amount of Triton x-100, which is associated with the phosphate translocator, the protein-Triton X-100 complex was bound to an affinity chromatography column and

equilibrated with [^{3}H]Triton as described in Materials and Methods. A mean value of 1.90 ± 0.05 g Triton X-100 bound per g of protein ($n = 9$) was calculated, corresponding to 180 mol of Triton bound per mol of protein. These data suggest that the phosphate translocator binds a high amount of detergent, as is expected for an integral transmembrane protein with an extensive membrane contact area. Analysis of the sedimentation data together with that of the gel filtration measurement of the Stokes' radius and the determination of the detergent binding are summarized in Table I. From these data, a molecular weight of 177 500 for the phosphate translocator-Triton X-100 complex can be estimated. After correcting for the bound detergent (1.90 g/g of protein), a molecular weight of about 61 000 is calculated for the protein moiety of the complex. Since the isolated phosphate translocator exhibits a molecular weight of 29 000 when subjected to SDS-polyacrylamide gel electrophoresis, it appears that the phosphate translocator exists as a dimer made up of two identical polypeptide chains. Such dimeric structures have been found also for other membrane proteins which traverse biological membranes (for review see Ref. 13).

The data shown in Table I allow considerations on the possible shape of the phosphate translocator in the Triton X-100 micelle. From the calculated frictional ratio (f/f_0) of 1.60, it is evident that the phosphate translocator-Triton X-100 complex deviates significantly from a spherical shape. Three factors contribute to the frictional ratio f/f_0. These are the protein shape (i.e., the deviation from spherical which determines largely the shape of the micelle), the binding of detergent and binding of lipid (negligible in this case, results not shown) and the binding of solvent. These contributions can be separated into a volume function $f(V)$ and a shape function $g(\rho)$ which are independent of each other. Therefore, the frictional ratio f/f_0 can be expressed as a linear combination of both according to the following equation:

$$f/f_0 = f(V)^{1/3} \cdot g(\rho)$$

where ρ is the axial ratio of an ellipsoid of revolution. The volume function $f(V)$ can be expressed as follows:

$$f(V) = \frac{V_{\text{micelle}}}{V_{\text{protein}}} = \left(1 + \delta_D \frac{\bar{v}_{\text{protein}}}{\bar{v}_D} + \delta_s \frac{\bar{v}_{\text{protein}}}{\bar{v}_s}\right)$$

where δ_D and δ_s are the amounts of protein bound detergent and solvent (g/g), respectively, and $\bar{v}_D$ and $\bar{v}_s$ are the corresponding partial specific volumes. The value for δ_D was determined to be 1.9 g/g of protein, $\bar{v}_D$, the partial specific volume of the detergent Triton X-100 is 0.908 cm^3/g [14], the values for $\bar{v}_{\text{protein}}$ and δ_s can be taken as 0.738 cm^3/g [15] and 0.3 g/g, respectively [16], and $\bar{v}_s$, the partial specific volume of the solvent was 0.984 cm^3/g [17]. From these data, the numerical value for the volume function ($f(V)$) can be calculated as 2.77.

The frictional ratio f/f_0 can now be corrected for the volume contribution resulting in a value for the shape function $g(\rho)$ which can be considered to be the volume-corrected frictional ratio $(f/f_0)^*$ for the phosphate translocator-Triton X-100 micelle [18]:

$$(f/f_0)^* = g(\rho) = f/f_0 / f(V)^{1/3}$$

The value for $(f/f_0)^*$ is calculated to be 1.14. This describes the shape of the protein-detergent micelle, which is very close to the shape of the protein [10]. From this corrected frictional ratio, the axial ratio ρ can be calculated as 3.38 for a prolate ellipsoidal shape ($r_1 > r_2$) and as 3.56 for

TABLE I

HYDRODYNAMIC PROPERTIES OF THE PURIFIED PHOSPHATE TRANSLOCATOR-TRITON X-100 COMPLEX

Physical parameter	
Sedimentation coefficient, $s_{20,w}$ (S)	$3.50 \cdot 10^{-13}$ s
Partial specific volume ($\bar{v}$)	0.860 cm^3/g
Stokes' radius (R_S)	6.32 nm
Triton X-100 binding	1.90 g/g protein
Frictional ratio (f/f_0)	1.60
Frictional ratio $(f/f_0)^*$ (volume corrected)	1.14
Axial ratio (prolate shape)	3.38
Diffusion coefficient (D)	$3.39 \cdot 10^{-7}$ cm^2/s
Molecular weight	177 500
Protein moiety	61 000
Minimal molecular weight (SDS-PAGE)	29 000

an oblate ellipsoidal shape ($r_1 < r_2$) [19].

These possibilities cannot be distinguished a priori. But since the surface area of an oblate ellipsoid exposed to the hydrophobic core of the membrane is much larger than that of a prolate one, the assumption of a prolate shape seems to be more reasonable.

The volume of the protein is given by $V = M(\bar{v} + \delta_s/\rho_{H_2O})/N$) and is calculated to be $10.52 \cdot 10^{-20}$ cm^3. If a prolate ellipsoidal shape is assumed ($V = (4/3)\pi \cdot r_1 \cdot r_2^2$), the semimajor axis ($r_1$) and the semiminor axis (r_2) of the ellipsoid are calculated to be 6.59 and 1.95 nm, respectively. Fig. 4 shows a model for the dimeric phosphate translocator according to these data. It is suggested that the phosphate translocator is a transmembrane protein penetrating through the membrane and connecting the stromal and the cytosolic compartment. Although a large portion of the dimer is assumed to be embedded in the hydrophobic core of the membrane, the amount protruding from both sides of the membrane seems to be sufficient to render the translocator accessible to its substrates.

Membrane proteins, especially those like the phosphate translocator which span a membrane, are thought to have large hydrophobic surface areas reflecting the direct interaction with the membrane lipid layer. Assuming a prolate ellipsoid of revolution, the surface area A of the phosphate translocator protein can be calculated according to the following equation [20]:

$$A = 2\pi r_2^2 \cdot r_1 \left(r_1^2 - r_2^2\right)^{-1/2} \ln \frac{r_1 + \left(r_1^2 - r_2^2\right)^{1/2}}{r_1 - \left(r_1^2 - r_2^2\right)^{1/2}}$$

This calculation yields a value of 95 nm^2. Assuming that one molecule of Triton X-100 covers an area of about 0.5 nm^2 at an air/water interface as shown in Ref. 21, and taking into account the amount of bound Triton X-100 in the protein detergent complex (180 mol Triton X-100/mol protein), then more than 90% of the surface of the protein could indeed be covered by the detergent in the micelle.

It appears from these model calculations that the phosphate translocator, as an intrinsic membrane protein, has an extensive hydrophobic surface area. As shown in Fig. 4, and in accordance with its function, the phosphate translocator is shown to traverse the membrane thereby mediating the communication between two compartments separated by the lipid bilayer.

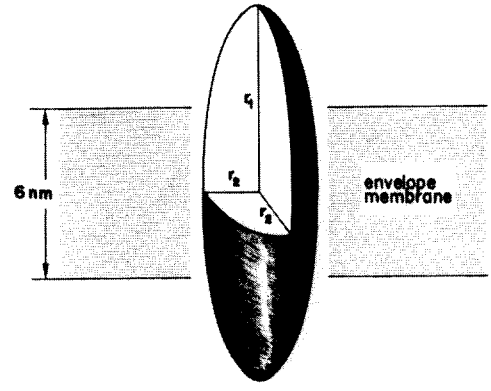

Fig. 4. Model of the dimeric phosphate translocator located in the inner envelope membrane. $r_1 = 6.59$ nm, $r_2 = 1.95$ nm. For details see text.

The most fully characterized transversal membrane transport system is the mitochondrial ATP/ADP antiporter [13,14,22,23]. Like the chloroplast phosphate translocator, the mitochondrial ATP/ADP translocator catalyzes a strict counter-exchange and has a dimeric structure [14]. It is supposed to exist as a fixed carrier in the membrane exhibiting a C_2 rotational axis perpendicular to the membrane plane [13]. This transmembrane arrangement allows the formation of a straight hydrophilic channel along the symmetry axis between the two subunits providing the translocation path for the substrate. The gating of this pore is triggered by substrate binding in such a way that the binding site is exposed to the one or the other side of the membrane in turn. It is tempting to suppose that the function of the dimeric chloroplast phosphate translocator may also be described by the gated pore model.

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References

1 Flügge, U.I. and Heldt, H.W. (1984) Trends Biochem. Sci. 9, 530–533
2 Flügge, U.I. and Heldt, H.W. (1979) in Function and Molecular Aspects of Biomembrane Transport (Quagliariello, E., Palmieri, F., Papa, S. and Klingenberg, E.M., eds.), pp. 373–382, Elsevier-North Holland, Biomedical Press, Amsterdam
3 Flügge, U.I. and Heldt, H.W. (1981) Biochim. Biophys. Acta 638, 296–304
4 Flügge, U.I., Gerber, J. and Heldt, H.W. (1983) Biochim. Biophys. Acta 725, 229–237
5 Douce, R., Holtz, B.R. and Benson, A.A. (1973) J. Biol. Chem. 248, 7215–7222
6 Sober, H.A. (ed.) (1970) Handbook of Biochemistry, The Chemical Rubber Co., Cleveland
7 Laemmli, U.K. (1970) Nature (Lond.) 227, 680–685
8 Clarke, S. (1975) J. Biol. Chem. 250, 5459–5469
9 Neuhoff, V., Philipp, K., Zimmer, H.G. and Meseke, S. (1979) Hoppe-Seyler's Z. Physiol. Chem. 360, 1657–1670
10 Tanford, C. (1977) in Structure of Biological membranes (Abrahamsson, S. and Pascher, J., eds.), pp. 497–508, Plenum Press, New York
11 Edelstein, S.J. and Schachmann, H.K. (1967) J. Biol. Chem. 242, 306–311
12 Meunier, J.C., Olsen, R.W. and Changeux, J.P. (1972) FEBS Lett. 24, 63–68
13 Klingenberg, M. (1981) Nature 290, 449–454
14 Hackenberg, H. and Klingenberg, M. (1980) Biochemistry 19, 548–555
15 Finney, J.L. (1979) in Water: A Comprehensive Treatise (Frank, F., ed.), Vol. 6, chapter 2, pp. 47–123, Plenum Press, New York
16 Teeter, M.M. (1984) Proc. Natl. Acad. Sci. USA 81, 6014–6018
17 Handbook of Chemistry and Physics (1979) CRC Press Inc., Section D
18 Perrin, F. (1934) J. Phys. Radium 5, 497–518
19 Scheraga, H.A. (1981) Protein Structure, Academic Press, New York
20 Smigel, M. and Fleischer, S. (1977) J. Biol. Chem. 252, 3689–6396
21 Neer, E.J. (1974) J. Biol. Chem. 249, 6527–6531
22 Klingenberg, M. (1976) in Enzymes of Biological Membranes: Membrane Transport, Vol. 3 (Martonosi, A.N., ed.), pp. 383–438, Plenum Press, New York
23 Klingenberg, M., Aquila, H. and Riccio, P. (1979) Methods Enzymol. 56, 229–233