Sebald, W., Graf, T., & Lukins, H. B. (1979) *Eur. J. Biochem. 93*, 587-599.
Sigrist-Nelson, K., & Azzi, A. (1979) *Biochem. J. 177*, 687-692.
Sigrist-Nelson, K., Sigrist, H., & Azzi, A. (1978) *Eur. J. Biochem. 92*, 9-14.
Skipski, V. P., Peterson, R. F., & Barclay, M. (1964) *Biochem. J. 90*, 374-378.
Sone, N., Yoshida, M., Hirata, H., & Kagawa, Y. (1979) *J. Biochem. (Tokyo) 85*, 503-509.
Stekhoven, F. S., Waitkus, R. F., & van Moerkerk, H. T. B. (1972) *Biochemistry 11*, 1144-1150.
Stiggall, D. L., Galante, Y. M., & Hatefi, Y. (1978) *J. Biol. Chem. 253*, 956-964.
Swank, R. T., & Munkres, K. D. (1971) *Anal. Biochem. 39*, 462-477.
Tzagoloff, A., & Akai, A. (1972) *J. Biol. Chem. 247*, 6517-6523.
Weber, K., & Osborn, M. (1976) *Proteins, 3rd Ed. 2*, 179-223.

Molecular Weight and Hydrodynamic Parameters of the Adenosine 5′-Diphosphate–Adenosine 5′-Triphosphate Carrier in Triton X-100†

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ABSTRACT: The Triton-solubilized ADP–ATP carrier from beef heart mitochondria was studied as the carboxyatractylate (CAT) complex by sedimentation in sucrose gradients, gel filtration, and analytical ultracentrifugation. The measurements of Triton binding and sedimentation velocity were not possible unless excess Triton and phospholipid were separated by an additional sucrose density gradient centrifugation. The absorption optical system of the analytical ultracentrifuge was used to analyze a multicomponent system consisting of protein, bound Triton, free Triton micelles, and Triton monomers. The isolated protein binds high amounts of Triton (1.5 g/g of protein) and phospholipid (0.25 g/g of protein) as would be expected for an intrinsic membrane protein. The sedimentation coefficient, measured on sucrose gradients and in the analytical ultracentrifuge, was $s_{20,w} = 3.9 \times 10^{-13}$ s. The diffusion coefficient, calculated from the Stokes' radius (R_S = 65 Å) and determined in the analytical ultracentrifuge, was (3.4–3.5) $\times 10^{-7}$ cm^2 s^{-1} in both cases. The partial specific volume $\bar{v}^*$ of the protein–Triton–phospholipid complex was evaluated from the fractions of the individual components and from sedimentation equilibrium measurements in solutions of different densities ($\bar{v}^* = 0.845$ cm^3/g). The molecular weight was calculated from the Svedberg equation (M^* 184 000) and from sedimentation equilibrium measurements (M^* 172 000). After subtraction of the bound Triton and phospholipid, M_r 67 000 (63 000) was found for the protein moiety, indicating that the Triton-solubilized CAT–protein complex exists as a dimer composed of two peptide subunits. From geometrical considerations it is concluded that the protein is completely enveloped in an ellipsoid micelle of ~150 Triton and ~16 phospholipid molecules.

The ADP–ATP carrier from mitochondria has been isolated in the undenatured form as a carboxyatractylate–protein complex by using the nonionic detergent Triton X-100 (Klingenberg et al., 1974; Riccio et al., 1975b). It contains, according to NaDodSO$_4$[1]–polyacrylamide gel electrophoresis, a single polypeptide of M_r 30 000. This polypeptide is the most abundant one in beef heart mitochondria, accounting for about 9% of total protein. From the amount of carboxyatractylate bound, a molecular weight of ~59 000 was calculated. Therefore, it was proposed that the isolated protein exists as a dimer with two subunits and one binding site.

With the isolated protein, studies in our laboratory were undertaken with the aim of elucidating the mechanism of this carrier, comprising functional and structural investigations (Klingenberg et al., 1975a,b, 1977, 1979; Aquila et al., 1978). For example, it was shown by reconstitution that the isolated carrier molecule is fully competent for transport (Krämer & Klingenberg, 1977).

The solubilization of the protein required high amounts of an efficient detergent and complete disruption of the phospholipid matrix. This was indicative for the strong hydrophobic nature of the protein in accordance with the expected deep embedment of this carrier in the membrane. Another particular feature of the solubilized CAT–protein was its nonadsorption to hydroxylapatite, in contrast to most other mitochondrial proteins; this is utilized as a main purification step. These results were explained by a large detergent coat shielding the protein (Klingenberg et al., 1978).

In the present study the molecular weight and the hydrophobic properties comprising the protein–detergent interaction were investigated by sucrose density gradients, gel filtration, and analytical ultracentrifugation. The CAT complex of the ADP–ATP carrier was used because of its particular stability. This analysis had to take into account the high amount of detergent and phospholipid bound to the protein and required

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[1] Abbreviations used: CAT, carboxyatractylate; Mops, 4-morpholinepropanesulfonic acid; EDTA, ethylenediaminetetraacetate; k, Boltzmann constant; R, gas constant; A_{280}, absorbancy at 280 nm; η, viscosity; ω, angular velocity; T, absolute temperature; ρ_s, solvent density; NaDodSO$_4$, sodium dodecyl sulfate.

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particular methodological efforts to analyze this multicomponent system.

Materials and Methods

Materials

Triton X-100 was obtained from Sigma Chemical Co.; [^{3}H]Triton X-100 was a gift from Dr. W. Lyman (Rohm and Haas Co.). [^{14}C]Sucrose was obtained from the New England Nuclear Co.; Sepharose 6B was from Bio-Rad and AcA-34 was from LKB. The calibration proteins were obtained from Boehringer Mannheim.

Methods

Protein concentrations were measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Sodium dodecyl sulfate was added to prevent precipitation of Triton (Helenius & Simons, 1972). Deviations due to sucrose were corrected by an external calibration with sucrose-containing solutions using bovine serum albumin and the CAT–protein as standards.

Phosphate was determined by the method of Chen (1956).

NaDodSO$_4$–polyacrylamide gel electrophoresis was carried out as described by Neville & Glossmann (1974). Gels (5 × 100 mm) with 11% acrylamide and 0.1% bis(acrylamide) were used.

[^{35}S]CAT and [^{3}H]CAT were prepared as described by Riccio et al. (1973) and Babel and Klingenberg (unpublished experiments).

The isolation of the CAT–protein was similar to that previously described by Riccio et al. (1975b). The Triton/protein ratio was lowered from 4 to 3 g/g of protein in the solubilization buffer. The Triton concentration in the elution buffers was reduced from 5 to 1 g/L.

For sucrose density gradient centrifugation linear gradients of 5–20% or 5–12% sucrose were prepared. The linearity was examined by addition of [^{14}C]sucrose. The gradient solutions were equilibrated with a buffer containing 1 g/L Triton, 0.1 M NaCl, 10 mM Mops, and 0.5 mM EDTA, pH 7.2. The isolated CAT–protein complex was concentrated by pressure dialysis, layered on top of the gradient, and centrifuged at 5 °C for 20–27 h in an Omega 60 Ti or 70 Ti rotor at 56 000 rpm or for 14–17 h in a Beckman 50 Ti vertical rotor at 48 000 rpm. Aliquots were collected from the bottom of the tubes, and protein, absorption, and radioactivity were determined. The relative mobility of the CAT–protein complex was evaluated from the ratio of the peak fraction number to total fraction numbers. The sedimentation coefficients were then calculated by using the tables of McEwen (1966).

Gel filtration on Sepharose 6B, AcA-34, and Sephadex G-200 columns was used for the determination of the Triton binding and the Stokes' radii (Nozaki et al., 1976). The elution buffers contained 1–2 g/L Triton, 0.1–0.5 M NaCl, 5 mM Mops, and 0.5 mM EDTA, pH 7.2. For the determination of the Stokes' radii of the protein–Triton complex and the Triton micelle, the columns were calibrated with ferritin, phosphorylase *a*, catalase, aldolase, hexokinase, bovine serum albumin, α-chymotrypsinogen, and cytochrome *c*. The void volume (V_0) was determined with blue dextran, and the total volume (V_{tot}) was determined with [^{14}C]sucrose or ^3H_2O.

Analytical ultracentrifugation was performed in a Beckman Model E. In combination with the Schlieren optical system, the An-H Ti rotor equipped with a Kel-F standard double sector or a synthetic boundary capillary type centerpiece was used. Together with the photoelectric scanner the An-F Ti rotor, equipped with three Kel-F standard double sector or six-channel equilibrium centerpieces, could be used. The viscosity of the solutions was determined in a capillary viscometer. The density measurements were carried out in a precision densimeter, Model DMA O2C.

The partial specific volume $\bar{v}^*$ of a multicomponent complex can be calculated from the sum of its individual components *i*, *j*, and *k* (Tanford et al., 1974):

$$\bar{v}^* = \sum x_{i,j,k}\bar{v}_{i,j,k} \tag{1}$$

The $x_{i,j,k}$ and $\bar{v}_{i,j,k}$ are the weight fractions and partial specific volumes of the individual components. The measurements in H_2O/D_2O solutions were evaluated by using the equations derived by Edelstein & Schachmann (1967)

$$\bar{v}^* = \frac{k - \left(\frac{\mathrm{d}\ln A_{280}}{\mathrm{d}r^2}\right)_{D_2O} \Big/ \left(\frac{\mathrm{d}\ln A_{280}}{\mathrm{d}r^2}\right)_{H_2O}}{\left(\frac{\mathrm{d}\ln A_{280}}{\mathrm{d}r^2}\right)_{D_2O} \rho_{D_2O} \Big/ \left(\frac{\mathrm{d}\ln A_{280}}{\mathrm{d}r^2}\right)_{H_2O} - \rho_{H_2O}} \tag{2}$$

where k (= 1.0155) was corrected for the part of the exchangeable protons in the protein–Triton complex.

The molecular weight M^* of the protein–Triton–phospholipid complex is considered the sum of its components (Tanford et al., 1974). The molecular weight of the protein moiety is then calculated from

$$M_r = x_p M^* \tag{3}$$

where

$$x_p = 1(1 + \sum\delta_i) \tag{4}$$

is the protein weight fraction and δ_i's are the protein-bound components in grams per gram of protein.

The M^* was calculated from sedimentation equilibrium by

$$M^* = \frac{2RT(\mathrm{d}\ln A_{280}/\mathrm{d}r^2)}{\omega^2(1 - \bar{v}^*\rho_s)} \tag{5}$$

and from the sedimentation and diffusion coefficients by

$$M^* = \frac{s_{20,w}RT}{D_{20,w}(1 - \bar{v}^*\rho_s)} \tag{6}$$

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

$$D = kT/(6\pi\eta R_S) \tag{7}$$

The molecular weight of the protein moiety can be calculated according to Reynolds & Tanford (1976) from sedimentation equilibrium measurements in solutions of different densities.

$$M_r(1 - \phi'\rho_s) = M_r[(1 - \bar{v}_p\rho_s) + \delta_{Tx}(1 - \bar{v}_{Tx}\rho_s) + \delta_{pl}(1 - \bar{v}_{pl}\rho_s)] = \frac{2RT\,\mathrm{d}\ln A_{280}}{\omega^2\,\mathrm{d}r^2} \tag{8}$$

By extrapolation to the density

$$\rho_s = 1/\bar{v}_{Tx} \tag{9}$$

the detergent term vanishes and M_r can be calculated without the knowledge of the detergent binding.

Results

Sucrose Density Gradient Centrifugation. The CAT–protein as usually isolated by the procedure of Riccio et al. (1975b) was further treated by sucrose density gradient centrifugation for the following reasons. Though nearly pure after gel chromatography on agarose, the protein can be still further

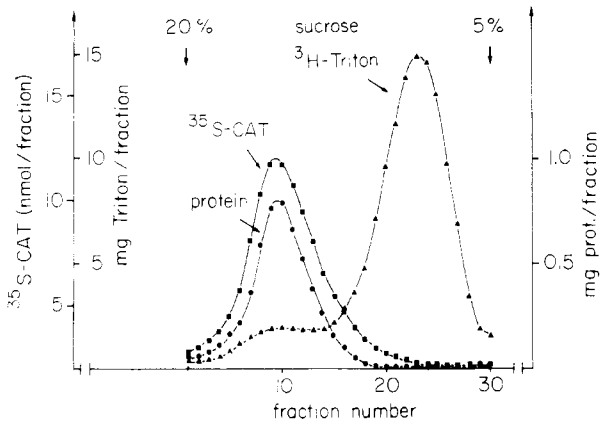

FIGURE 1: Sucrose density gradient centrifugation of the CAT–protein complex. 0.5 mL of a solution containing 5.8 mg of protein (15.6 nmol of CAT per mg of protein) and 82.4 mg of Triton and thereafter 0.5 mL of H_2O was layered on 11 mL of a linear sucrose gradient, which was equilibrated with a buffer containing 1 g/L Triton. The solution was centrifuged for 27 h and at 5 °C in an Omega 70 Ti rotor at 56000 rpm. 0.4-mL fractions were collected and 0.02-mL aliquots were taken for protein and radioactivity determinations. By pooling of the fractions 6–14, the protein yield was 4.9 mg (84%) whereas the amount of Triton was diminished from 14.3 to 3.2 g/g of protein. The CAT/protein ratio remained nearly unchanged after centrifugation (16.7 nmol/mg). The sedimentation coefficient was $s_{20,w}$ = 2.4×10^{-13} s.

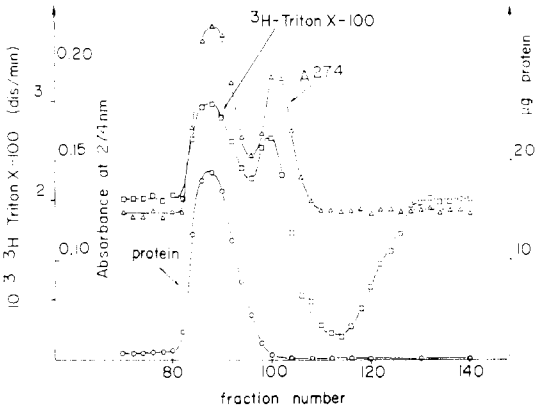

FIGURE 2: Triton binding by gel filtration. A column (100 × 1 cm) of AcA-34 was equilibrated with a buffer containing 1 g/L [^{3}H]Triton with a specific activity of 19000 dpm/mg. 0.5 mL of sucrose gradient pretreated CAT–protein (2 mg of protein and 4.6 mg of Triton) was applied to the column and eluted at 5 °C with an elution velocity of 3 mL/h. 0.05-mL aliquots were taken from the 0.5-mL fractions for protein and absorption measurements, and 0.1 mL was taken for the radioactivity assay. The Triton/protein weight ratio (1.45 ± 0.07 from the radioactivity measurements and 1.56 ± 0.10 from the UV absorption measurements) was constant from fractions 84–90 in the protein peak.

purified from small contaminants. Also, the sucrose gradient is more efficient in removing excess Triton and phospholipid than the preceding chromatography on hydroxylapatite and filtration on agarose. This removal is a prerequisite for the accurate determination of Triton binding by gel filtration. In addition, the protein–Triton complex is not sedimentable in the analytical ultracentrifuge as monitored by the Schlieren optical system unless the amount of free Triton and phospholipid is drastically diminished.

The high concentrations of hydrophobic protein, Triton, and phospholipid during centrifugation may produce nonideal behavior. The high viscosity of the solution observed after centrifugation indicates strong intermolecular interactions. The state of the protein is not changed by the sucrose gradient centrifugation. Before the gradient purification step, identical sedimentation coefficients were measured in the analytical ultracentrifuge, using the UV absorption with dilute protein solutions. A gradient centrifugation of a CAT–protein preparation is shown in Figure 1. [^{35}S]CAT was used as a marker for the CAT–protein complex, and the Triton distribution is given by the ^{3}H-labeled compound. A clear separation was obtained because the CAT–protein complex migrates faster than the Triton micelle due to its higher density and molecular weight. The amount of Triton in the protein-containing fraction is reduced from 14.3 to 3.2 g/g of protein. In general, by this treatment the amount of Triton and phospholipid is diminished about 5- to 10-fold. The following experiments were all performed with CAT–protein preparations, treated in this manner for removing excess Triton and phospholipid.

Triton and Phospholipid Binding. The binding of Triton to the protein was first measured in sucrose gradients. A minimal value of 1.5 g of Triton per g of protein was found, but the results from several experiments were not consistent. The Triton binding was more exactly determined by gel filtration of the preparation after sucrose density gradient centrifugation (Figure 2). Without the centrifugation step, the large amounts of free Triton micelles could not be clearly separated during the gel chromatography. In addition, the high phospholipid content seems to make the Triton micelles more polydispersed so that they overlap with the protein–Triton complex. Also, a large excess of micelles may disturb the ideal distribution between the two phases and therefore reduce the separation efficiency of the column.

For the determination of the Triton binding, the elution buffer was equilibrated with ^{3}H-labeled Triton. Additionally, the UV absorption of Triton was monitored and corrected for the protein absorption which was known from spectra in UV-translucent amine oxide detergents. Nearly identical Triton binding was measured by radioactivity (1.45 g/g of protein) and UV absorption (1.56 g/g of protein). The very high binding corresponds to 150 molecules of Triton per one dimeric CAT–protein complex.

In other experiments CAT–protein was solubilized and purified in [^{3}H]Triton in order to eliminate possible nonequilibration of ^{3}H-labeled and unlabeled Triton in the elution buffer. The results were in good agreement with those of Figure 2. Furthermore, the CAT binding was monitored by [^{35}S]CAT which can be used in conjunction with [^{3}H]Triton.

The amount of phospholipid was determined to be 0.28 ± 0.04 μmol of phosphate per mg of protein in seven preparations. With an average molecular weight of 900 per phospholipid molecule, this gives a weight ratio of 0.25. The phospholipid content could not be further reduced by a second sucrose density gradient centrifugation.

Diffusion and Sedimentation Coefficients. For measurement of the Stokes' radius of the protein–Triton complex, gel filtration columns (Sepharose 6B and AcA-34) were calibrated with soluble proteins of known Stokes' radii. The elution volumes of these proteins were not influenced by the presence of Triton in the elution buffer. The intactness of the calibration proteins was also confirmed by sedimentation velocity studies under identical conditions in the analytical ultracentrifuge. A plot of $1 - K_{av}$ vs. the Stokes' radii of the calibration proteins is shown in Figure 3. Stokes' radii of 65 Å for the protein–Triton complex and 53 Å for the pure Triton micelles were found. The diffusion coefficient was calculated from the Stokes' radius, combining the Einstein–Sutherland equation and the Stokes' law:

$$D_{20,w} = 3.43 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$$

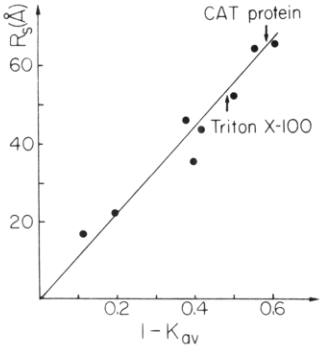

FIGURE 3: Stokes' radius by gel filtration. A column (100 × 1 cm) was packed with agarose gel (Sepharose 6B) and equilibrated with a buffer containing 1 g/L Triton and 0.2 M NaCl. The exclusion volume (V_0 = 22.25 mL) was determined with blue dextran. The total volume (V_{tot} = 71.50 mL) was measured with [^{14}C]sucrose or ^3H_2O. For calibration the following proteins were used: cytochrome *c*, 17 Å; α-chymotrypsinogen, 22 Å; bovine serum albumin, 35 Å; hexokinase, 43 Å; aldolase, 46 Å; catalase, 52 Å; phosphorylase *a*, 64 Å; ferritin, 65 Å.

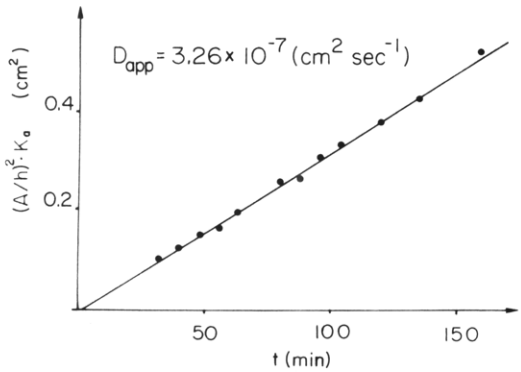

FIGURE 4: Diffusion coefficient by analytical ultracentrifugation. A synthetic boundary (capillary type) double-sector cell was filled with 0.15 mL of a solution containing 2.45 g/L protein–Triton complex and then allowed to diffuse into the dialysate buffer at 4000 rpm and 20 °C. The diffusion velocity was monitored by taking Schlieren photos at 8-min intervals. A plot of the height/area ratio vs. the time was used to evaluate the diffusion coefficient. From the slope D_{app} = 3.26 × 10^{-7} cm^2 s^{-1} was calculated. After correction for the viscosity of the solution, a $D_{20,w}$ = 3.42 × 10^{-7} cm^2 s^{-1} was found.

It was also determined in the analytical ultracentrifuge using a synthetic boundary capillary type double sector cell. The broadening of the boundary was monitored by the Schlieren optical system. The apparent diffusion coefficient was evaluated from a plot of the area/height ratio vs. time (Figure 4) as

$$D_{app} = 3.26 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$$

The value corrected to standard conditions is

$$D_{20,w} = 3.42 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$$

This is in good agreement with the diffusion coefficient calculated from the Stokes' radius.

The sedimentation coefficient of the protein–Triton complex was determined by analyzing the sedimentation behavior on sucrose density gradients and in the analytical ultracentrifuge. It was calculated from the mobility of the protein–Triton complex on linear 5–20% (w/v) sucrose density gradients. The sedimentation coefficient calculated from the purification gradient step was (2.2–3.0) × 10^{-13} s. On further sucrose gradient centrifugation of the now purified protein–Triton complex, the sedimentation coefficient increased to 3.9 × 10^{-13} s. The anomaly is also reflected in the observation that before the gradient purification step the protein–Triton complex did not sediment in the analytical ultracentrifuge, as monitored by the Schlieren optical system.

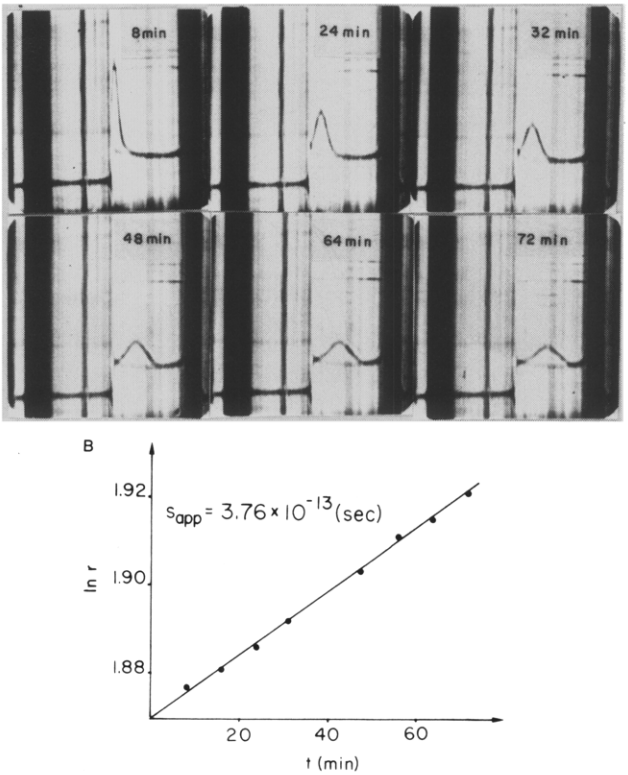

FIGURE 5: Sedimentation coefficient using the Schlieren system. (A) A solution of 1.4 g/L protein and 2.3 g/L Triton was obtained after equilibrium dialysis of a density gradient purified CAT–protein preparation. The sedimentation of the protein–Triton complex at 20 °C and 52 000 rpm was monitored by taking Schlieren photos at 8-min intervals. (B) The sedimentation coefficient was evaluated from a plot of the logarithm of the distance from the center of rotation vs. time. From the slope of the straight line s_{app} = 3.76 × 10^{-13} s was calculated. After correction to standard conditions, $s_{20,w}$ = 3.85 × 10^{-13} s was found.

Sedimentation velocity experiments in the analytical ultracentrifuge were performed over a wide concentration range using the Schlieren optical system (1.5–10 g of protein per L) or the photoelectric scanner (0.02–0.2 g of protein per L). Besides the evaluation of the sedimentation coefficient, these experiments should give information about the purity of the protein preparation.

The Schlieren figures of a sedimentation velocity experiment are shown in Figure 5A. A single band of the protein–Triton complex was observed. This means that in this protein preparation the concentration of free Triton micelles was reduced to nearly zero. In other sedimentation velocity experiments (not shown), a second peak was found, which migrates much slower ($s_{20,w}$ = 1.3 × 10^{-13} s) than the protein–Triton complex ($s_{20,w}$ = 3.93 × 10^{-13} s) and which can be attributed to free Triton micelles. The data from the sedimentation velocity experiments of Figure 5A give a straight line by plotting the logarithm of the distance of the center of rotation vs. the time (Figure 5B). The linearity indicates that no appreciable effects of pressure and dilution have to be considered. The apparent sedimentation coefficient from this experiment was s_{app} = 3.76 × 10^{-13} s. After correction to standard conditions the value was $s_{20,w}$ = 3.85 × 10^{-13} s.

Because of the high binding of Triton, it was possible to use the UV absorption of the protein-bound Triton as a marker

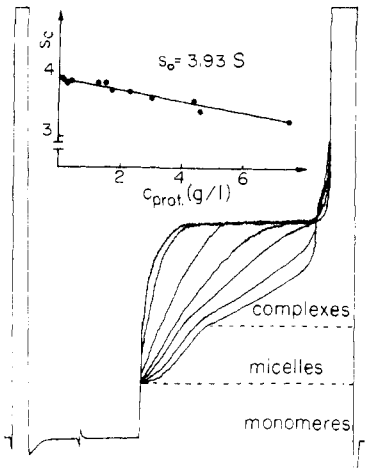

FIGURE 6: Sedimentation measurements using UV absorption. A sucrose gradient pretreated CAT–protein preparation was diluted sixfold with a Tritonless buffer to give 0.11 g/L protein and 0.38 g/L Triton. The sedimentation velocity of the two moving boundaries at 52 000 rpm and 23 °C was monitored by its absorption at 280 nm. Beginning at 52 000 rpm (t = 0), scans were recorded at t = 11, 36, 59, 86, 109, and 156 min. The apparent sedimentation coefficients calculated from this experiment were s_{app}(complex) = 4.02×10^{-13} s and s_{app}(Triton micelles) = 1.18×10^{-13} s. Correction to standard conditions give $s_{20,w} = 3.88 \times 10^{-13}$ and 1.23×10^{-13} s. The dependence of the sedimentation coefficient on the protein concentration is shown in the insert. s values at concentrations below 0.2 g/L protein were evaluated from the UV absorption; above 1.4 g/L protein they were evaluated from the Schlieren optical system.

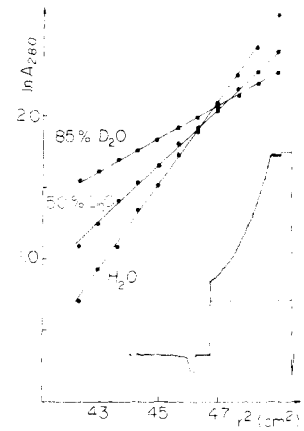

FIGURE 7: Sedimentation equilibrium in H_2O/D_2O solutions. In this experiment the CAT–protein was diluted as described in Figure 6 with a solution containing 0, 50, or 85% D_2O. The sedimentation equilibrium patterns at 7200 rpm and 5 °C were recorded with the photoelectric scanner after 63 h. The exponential equilibrium distribution of the protein–Triton complex in the H_2O buffer is shown in the lower right. The dashed line indicates the base line, determined by accelerating the rotor to 52 000 rpm after recording the sedimentation equilibrium patterns. The logarithm of the absorption of the protein–Triton complex is plotted against the square of the distance from the axis of rotation. The partial specific volume and the molecular weight of the protein–Triton complex were calculated as described under Methods.

for the whole protein–Triton complex. The CAT–protein complex was diluted in these experiments to about 0.02–0.2 g of protein per L. A typical sedimentation velocity experiment as monitored by the photoelectric scanner is shown in Figure 6. Three species can be distinguished by their different sedimentation velocities (as indicated by the dotted lines). The upper boundary represents the protein–Triton complex which sediments most rapidly ($s_{app} = 4.02 \times 10^{-13}$ s). In the middle part one observes the more slowly sedimenting free Triton micelles ($s_{app} = 1.18 \times 10^{-13}$ s). The monomeric Triton molecules in the lower part do not migrate.

In this experiment the concentration of the monomeric, of the micellar, and of the protein-bound Triton can be determined by using the absorption of Triton. For instance, the critical micelle concentration of Triton was calculated to be 0.13 g of Triton per L. With an average molecular weight of 628 for Triton, this corresponds to 20 mM monomeric Triton. This value is smaller than that reported for Triton in pure water [0.16 g/L (Simons et al., 1973)], probably because of the presence of 0.2 M NaCl in the solution.

The ratio of free Triton micelles to protein-bound Triton can be evaluated after correction for the protein absorption. This ratio may be critical for the stabilization of the Triton–protein complex by the free Triton micelles. In the present experiment the protein-bound Triton exceeds the free micellar Triton by 1.4. The concentration of the Triton micelle could be diminished to nearly zero in other experiments without any observable aggregation products (see also Figure 5A). The discrimination between bound and free Triton also permitted the measurement of the Triton binding. After subtracting the protein absorption (20%), 1.46 g of Triton per g of protein was obtained. The mean value of several experiments was 1.45 g of Triton per g of protein.

The sedimentation coefficient of the protein–Triton complex was only slightly dependent on the protein concentration over a concentration range of 0.02–10 g of protein per L. The extrapolation from 11 different protein concentrations to infinite dilution gives $s_0 = 3.93 \times 10^{-13}$ s (Figure 6, insert).

Sedimentation Diffusion Equilibrium Measurements. An unambiguous determination of the molecular weight of the protein–Triton complex should be possible by sedimentation equilibrium measurements. The Schlieren optical system proved to be unsuitable in sedimentation equilibrium experiments with the protein–Triton complex. The molecular weights evaluated from measurements at different rotor speeds were not consistent. The high concentrations of Triton and hydrophobic protein (≥3 g/L) required by this method probably caused nonideal behavior.

These problems were avoided by measuring the UV absorption of the protein–Triton complex. With the photoelectric scanner, very dilute protein preparations could be used ($c_{protein}$ ≥ 0.02 g/L). The discimination between bound and free Triton was also employed in the equilibrium measurements. In control runs without protein, it was shown that free Triton micelles and monomers do not contribute to the exponential equilibrium distribution of the protein–Triton complex, except by shifting the base line. The displaced base line was determined, as shown in Figure 6, by a subsequent sedimentation velocity run of the solution after reaching equilibrium. The equilibrium distribution with the corrected base line (dashed line), from which the molecular weight could be calculated, is shown in the insert of Figure 7. Double-sector centerpieces were used in these experiments in order to determine the base line more accurately. Measurements at different rotor speeds yielded nearly identical results. Plots of the logarithm of the optical density at 280 nm vs. the square of the distance from the center of rotation are given in Figure 7 for three different densities of the solution. Straight lines give evidence for the monodisperse protein preparation.

Partial Specific Volume. The partial specific volume of the CAT–protein complex is composed of protein, Triton, and phospholipid

$$v^* = x_p\bar{v}_p + x_{Tx}\bar{v}_{Tx} + x_{pl}\bar{v}_{pl} \qquad (10)$$

where x_p, x_{Tx}, and x_{pl} = weight fraction of the individual components (protein, Triton, phospholipid) and $\bar{v}_p$, $\bar{v}_{Tx}$, and

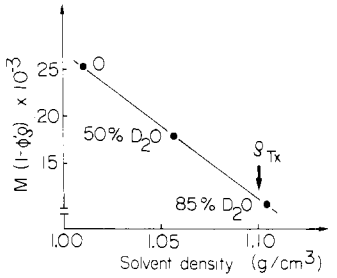

FIGURE 8: Plot of $M_r(1 - \phi'\rho_s)$ vs. the solvent density. The results from the sedimentation equilibrium measurements $2RT(d \ln A_{280}/dr^2)/\omega^2 = M_r(1 - \phi'\rho_s)$ are plotted against the solvent densities. The density of Triton $\rho_{Tx} = 1/\bar{v}_{Tx}$ is indicated by the arrow. The molecular weight of the protein moiety was calculated from this point.

$\bar{v}_{pl}$ = partial specific volume of the components. The partial specific volume of Triton determined by density measurements was 0.908 cm³/g, in agreement with Tanford et al. (1974). The value for the phospholipid (0.975 cm³/g) was calculated from the composition (45% phosphatidylcholine, 34% phosphatidylethanolamine, 7% phosphatidylinositol, and 14% diphosphatidylglycerol) by using Traube's rule (Traube, 1899). The partial specific volume of the protein ($\bar{v}_{p(index)}$ = 0.73 cm³/g) was calculated from the amino acid composition (Cohn & Edsall, 1943). On the basis of these data, the partial specific volume of the total protein–Triton–phospholipid complex amounts to v^* = 0.849 ± 0.015 cm³/g.

v^* was determined more directly by experiments using solutions of different densities by replacing H_2O with D_2O. The slopes of sedimentation equilibrium measurements in H_2O and D_2O solutions (Figure 7) were evaluated according to Edelstein & Schachmann (1967). A mean value of v^* = 0.846 ± 0.015 cm³/g was found.

v^* was also calculated from sedimentation velocity experiments in H_2O/D_2O solutions: the result with linear 5–20% (w/v) sucrose density gradients gave v^* = 0.845 cm³/g. From analytical ultracentrifugation, using the absorption optic, v^* = 0.836 cm³/g was found. For evaluation of the molecular weight, a mean value from the various methods and experiments was computed: v^* = 0.845 cm³/g.

Molecular Weight. The molecular weight of the protein–Triton–phospholipid complex M^* was calculated from sedimentation equilibrium and hydrodynamic measurements.

From sedimentation equilibrium experiments at different rotor speeds, a mean value of the complex molecular weight, M^* 172 000 ± 13 000, was determined. From $M_r = x_p M^*$ an M_r of 64 000 ± 4000 follows.

The molecular weight of the protein moiety can also be evaluated by plotting the sedimentation equilibrium measurements vs. the solvent densities (Figure 8) (Reynolds & Tanford, 1976). By extrapolation of the density of the solvent to

$$\rho_s = 1/\bar{v}_{Tx} \text{ g/cm}^3 \quad (9)$$

M_r can be calculated without the need for determining the detergent binding. The result was an M_r of 63 000.

The molecular weight of the protein–Triton complex was also calculated from sedimentation and diffusion velocity measurements by using the Svedberg equation, M^* 182 000 and M_r 67 000, with a relative error of about 10%.

The frictional ratio f/f_0 was calculated from three combinations of the molecular weight, the sedimentation coefficient, and the diffusion coefficient. A mean value of f/f_0 = 1.56 ± 0.05 was found.

A summary of the measured parameters is shown in Table I.

Table I: Characteristics of the Purified CAT-Protein-Triton X-100 Complex

parameters	values
Gel Filtration	
Stokes' radius	65 Å
detergent binding	1.47 g/g of protein
	150 mol/mol of protein
phospholipid binding	0.25 g/g of protein
	16 mol/mol of protein
Analytical Ultracentrifugation	
diffusion coefficient	3.43×10^{-7} cm²/s
sedimentation coefficient	3.93×10^{-13} s
partial specific volume	0.845 cm³/g
molecular weight (s/D)	184 000
molecular weight (sed equil)	172 000
protein moiety (s/D)	67 000
protein moiety (sed equil)	64 000
frictional ratio	1.56

Discussion

Triton in Hydrodynamic Measurements. Detergents used in the isolation of intrinsic membrane proteins must fulfill two main requirements, solubilization power and conservation of the native state of the solubilized protein. In this respect the efficiency of various detergents was compared by using the CAT–protein complex of beef heart mitochondria (Riccio et al., 1975a). As a result of these studies, Triton X-100 was found to be the most suitable detergent in solubilizing the carrier and leaving the protein–inhibitor complex intact. Because of these advantages, Triton has been extensively used for the solubilization and purification of membrane proteins [for reviews see Helenius & Simons (1975) and Tanford & Reynolds (1976)]. Triton is difficult to handle, however, due to its low critical micelle concentration and its high aggregation number. Therefore, one has to be careful using Triton in the molecular characterization of membrane proteins which is clearly demonstrated by the present studies.

The mixed Triton–phospholipid micelles formed during the solubilization of the phospholipid matrix of the membrane were isolated together with the protein–Triton complex. The very short purification procedure based on polar interactions (hydroxylapatite chromatography) and particle size (gel filtration) did not separate the excess Triton and phospholipid. As a result, the protein solution exhibited anomalous behavior in gel filtration and in sedimentation experiments. These problems were overcome by introducing sucrose density gradient centrifugation as an additional purification step. The separation of excess Triton and phospholipid was then possible due to their smaller density and molecular weight. Similar problems may not be found with other membrane proteins which have to be purified by more complicated procedures (Von Jagow et al., 1978). In these cases, one purification step may be involved which inadvertently separates excess detergent and phospholipid.

The UV absorption of Triton was regarded as a major obstacle in the characterization of membrane proteins, because it interferes with the measurement of the protein absorption, particularly in the analytical ultracentrifuge (Tanford et al., 1974; Tanford & Reynolds, 1976). Previous ultracentrifugation studies in Triton were therefore limited to membrane proteins with chromophoric prosthetic groups, such as cytochromes (Von Jagow, 1977, 1978) and rhodopsin (Osborne et al., 1974; Reynolds & Stoeckenius, 1977). The present experiments demonstrate that the very UV absorption of the protein-bound Triton can be used as a marker for the whole protein–Triton complex. Moreover, additional data can be extracted from the measurements of the UV absorption of the

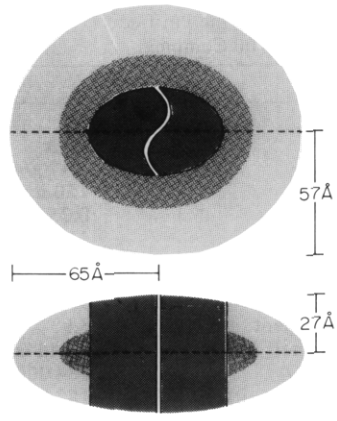

FIGURE 9: Geometrical model of the CAT–protein–Triton complex. For details see the text.

Triton during a sedimentation velocity run. This is, for example, a simple method for measuring the critical micelle concentration, as the monomeric Triton molecules do not sediment.

The low concentration of protein used in this method substantiates the s_0 value extrapolated from Schlieren optical measurements. Thus, concentration-dependent dissociation of the protein was excluded. The application of the UV absorption to sedimentation equilibrium experiments proved to be a great advantage. The determination of the partial specific volume and the molecular weight of the protein–Triton complex could thus be confirmed by a method independent of hydrodynamic measurements.

CAT–Protein Complex. So far, only a few intrinsic isolated membrane proteins have also been studied carefully in nondenaturing detergent solutions [for a review see Tanford & Reynolds (1976)]. The CAT–protein complex is the first anion-transporting protein which is characterized in this respect. The present study demonstrates that it exists as a dimer of two peptide subunits, each of M_r 30000. The CAT/protein ratio in the purified preparation indicated originally the existence of one CAT binding site per two subunits, and consequently the existence of a dimer was suggested (Riccio, 1975b). Evidence for a stabilizing effect of the dimeric state of the CAT–protein complex is provided by experiments in which the dissociation of the subunits is provoked by chaotropic reagents (Hackenberg and Klingenberg, unpublished experiments). This supports the idea that in the native state the protein is also dimeric. Moreover, the isolated protein is derived from the CAT–protein complex preformed in the mitochondrial membrane. The dimeric structure is also demonstrated by cross-linking studies of the isolated protein (Hackenberg and Klingenberg, unpublished experiments).

The only other purified CAT–protein complex of *Neurospora crassa* mitochondria seems to be a dimer also, as deduced from the CAT/protein ratio (Hackenberg et al., 1978). Recently, some further membrane proteins have been isolated from mitochondria as dimeric proteins with Triton, such as cytochrome oxidase (Briggs et al., 1975), cytochrome bc_1 complex, and cytochrome *b* (Von Jagow et al., 1977, 1978). Another transport-active protein, the Na^+,K^+-ATPase, has also been solubilized as a dimer in poly(oxyethylene) detergents (Hastings & Reynolds, 1979).

It seems that the dimeric structure of intrinsic membrane proteins is favored for energetic reasons. However, generalizing this concept, as postulated by Steck (1972) from cross-linking studies on erythrocyte membrane proteins, can be misleading, as shown by several examples: rhodopsin (Osborne et al., 1974) and galactosyltransferase and sulfotransferase (Fleischer & Smigel, 1978) of Golgi membranes were solubilized in Triton as active monomers.

CAT–Protein–Triton Complex. The hydrodynamic behavior of the protein deviates appreciably from the properties of water-soluble proteins of comparable size due to the high amount of bound Triton. The remarkably large Stokes' radius of the protein–Triton complex can be explained by the strong hydration and asymmetry of the poly(oxyethylene) detergent. This and the reduced density of the complex due to the bound Triton and phospholipid are responsible for the relatively small sedimentation coefficient.

The high amount of bound Triton and the nonremovable phospholipid indicates that a large part of the surface of the protein is hydrophobic and therefore must be enveloped by the bound phospholipid and Triton. A large hydrophobic surface can be expected for a membrane protein which must be postulated to span the membrane on the basis of its very function as a "gated pore" (Klingenberg et al., 1975b). This Triton binding is similar to that of cytochrome *b* of beef heart mitochondria (n = 150) (Von Jagow et al., 1978) and bacteriorhodopsin (n = 170) (Reynolds & Stoeckenius, 1977). The binding is larger compared to that of bovine rhodopsin (n = 90) (Osborne et al., 1974) or to that of the galactosyltransferase (n = 80) (Fleischer & Smigel, 1978) of Golgi membranes. These data indicate that the size of the protein-bound detergent micelle is influenced by the hydrophobic surface of the individual membrane protein.

The predominance of Triton is also expressed by the similarity of the frictional ratios of the complex (f/f_0 = 1.59). The amount of bound Triton (n = 150) is very similar to that of a pure Triton micelle (n = 143) (Kushner & Hubbard, 1954). One can therefore envisage that the protein-bound Triton exists in a micellar state in which the hydrophobic core is enlarged and distorted by the incorporation of the apolar protein. On this basis a possible geometrical model for the protein–Triton complex is delineated in Figure 9. The increase of the R_S from 53 Å in the pure Triton micelle to the R_S = 65 Å in the complex is conceived to result from the insertion of the protein along the small axis. The complex is visualized as an oblate ellipsoid, as derived from simple geometrical considerations, based on the increased Stokes' radius, the total volume, and the unchanged thickness.

With these parameters, the rotational symmetry becomes slightly distorted. In this model the two faces of the protein are flat with the ellipsoid surface. The protein is assumed to have an elliptical shape which causes the distortion of the micelle. It is clear that such a symmetrical ellipsoid does not take into account the parallel transmembrane asymmetry of the two subunits. Nevertheless, a reasonable and notable result is the total immersion of the protein in the micelle, which may reflect its nearly complete membrane embedment.

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References

Aquila, H., Eiermann, W., Babel, W., & Klingenberg, M. (1978) *Eur. J. Biochem. 85*, 549–560.

Briggs, M., Ramp, P. F., Robinson, N. C., & Capaldi, R. A. (1975) *Biochemistry 14*, 5123–5128.

Chen, P. S. (1956) *Anal. Chem. 28*, 1756.

Cohn, E. J., & Edsall, J. T. (1943) *Proteins, Amino Acids and Peptides*, pp 157–161, 370–375, Reinhold, New York.

Edelstein, S. J., & Schachmann, H. K. (1967) *J. Biol. Chem. 242*, 306–311.

Fleischer, B., & Smigel, M. (1978) *J. Biol. Chem. 253*, 1632–1638.

Hackenberg, H., Riccio, P., & Klingenberg, M. (1978) *Eur. J. Biochem. 88*, 373–378.

Hastings, D. F., & Reynolds, J. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 817–821.

Helenius, A., & Simons, K. (1972) *J. Biol. Chem. 247*, 3656–3661.

Helenius, A., & Simons, K. (1975) *Biochim. Biophys. Acta 415*, 29–79.

Klingenberg, M., Riccio, P., Aquila, H., Schmiedt, B., Grebe, K., & Topitsch, P. (1974) in *Membrane Proteins in Transport and Phosphorylation* (Azzone, G. F., Klingenberg, M., Quagliariello, E., & Siliprandi, N., Eds.) pp 229–243, North-Holland Publishing Co., Amsterdam.

Klingenberg, M., Aquila, H., Riccio, P., Buchanan, B. B., Eiermann, W., & Hackenberg, H. (1975a) in *Electron Transfer Chains and Oxidative Phosphorylation* (Quagliariello, E., Papa, S., Palmieri, F., Slater, E. C., & Siliprandi, N., Eds.) pp 341–438, North-Holland Publishing Co., Amsterdam.

Klingenberg, M., Riccio, P., Aquila, H., Buchanan, B. B., & Grebe, K. (1975b) in *Mechanism of Carrier Transport and the ADP, ATP Carrier* (Hatefi, Y., & Djavadi-Ohaniance, L., Eds.) pp 293–311, Academic Press, New York.

Klingenberg, M., Aquila, H., Krämer, R., Babel, W., & Feckl, J. (1977) in *Biochemistry of Membrane Transport* (Semenza, G., & Carafoli, E., Eds.) pp 567–579, Springer-Verlag, Berlin, Heidelberg, and New York.

Klingenberg, M., Riccio, P., & Aquila, H. (1978) *Biochim. Biophys. Acta 503*, 192–210.

Klingenberg, M., Hackenberg, H., Eisenreich, G., & Mayer, I. (1979) in *Function and Molecular Aspects of Biomembrane Transport* (Palmieri, F., Papa, S., Klingenberg, M., & Quagliariello, E., Eds.) pp 209–303, North-Holland Publishing Co., Amsterdam.

Krämer, R., & Klingenberg, M. (1977) *FEBS Lett. 82*, 363–367.

Kushner, L. M., & Hubbard, W. D. (1954) *J. Phys. Chem. 58*, 1163–1167.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, A. J. (1951) *J. Biol. Chem. 193*, 265–275.

McEwen, C. R. (1966) *Anal. Biochem. 20*, 114–149.

Neville, D. M., Jr., & Glossmann, H. (1974) *Methods Enzymol. 32*, 92–102.

Nozaki, Y., Schechter, N. M., Reynolds, J. A., & Tanford, C. (1976) *Biochemistry 15*, 3884–3890.

Osborne, H. B., Sardet, C., & Helenius, A. (1974) *Eur. J. Biochem. 44*, 383–390.

Reynolds, J. A., & Tanford, C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4467–4470.

Reynolds, J. A., & Stoeckenius, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2803–2804.

Riccio, P., Scherer, B., & Klingenberg, M. (1973) *FEBS Lett. 31*, 11–14.

Riccio, P., Aquila, H., & Klingenberg, M. (1975a) *FEBS Lett. 56*, 129–132.

Riccio, P., Aquila, H., & Klingenberg, M. (1975b) *FEBS Lett. 56*, 133–139.

Simons, K., Helenius, A., & Garoff, H. (1973) *J. Mol. Biol. 80*, 119–133.

Steck, T. L. (1972) *J. Mol. Biol. 66*, 295–305.

Tanford, C., & Reynolds, J. A. (1976) *Biochim. Biophys. Acta 457*, 171–212.

Tanford, C., Nozaki, Y., Reynolds, J. A., & Makino, S. (1974) *Biochemistry 13*, 2369–2376.

Traube, J. (1899) *Samml. Chem. Chem. Tech. Vortr. 4*, 225.

Von Jagow, G., Schägger, H., Riccio, P., Klingenberg, M., & Kolb, H. J. (1977) *Biochim. Biophys. Acta 462*, 549–558.

Von Jagow, G., Schägger, H., Engel, W. D., Machleidt, W., & Machleidt, I. (1978) *FEBS Lett. 91*, 121–125.