Metabolic Probes, American Chemical Society Publication of ACS Symposium, Division of Fluorine Chemistry, August 26, 1975.
Kun, E., Dummel, R. J., and Battaglia, W. L. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 1257.
Kun, E., Kearney, E. B., Lee, N. M., and Weidemann, I. (1970), *Biochem. Biophys. Res. Commun. 38,* 1002–1008.
Kun, E., Kearney, E. B., Weidemann, I., and Lee, N. M. (1969), *Biochemistry 8,* 4443–4449.
Lee, N. M., Weidemann, I., and Kun, E. (1971), *FEBS Lett. 18,* 81–83.
Lehninger, A. L. (1964), The Mitochondrion Molecular Basis of Structure and Function, New York, N.Y., W. A. Benjamin, p 170.
Lehninger, A. L. (1970), *Biochem. J. 119,* 129–138.
Lin, D. C., and Kun, E. (1973a), *Biochem. Biophys. Res. Commun. 50,* 820–825.
Lin, D. C., and Kun, E. (1973b), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3450–3453.
Lin, S., Lin, D. C., Spudich, J. A., and Kun, E. (1973), *FEBS Lett. 37,* 241–243.
Lowenstein, J., Scholte, H. R., and Wit-Peeters, E. M. (1970), *Biochim. Biophys. Acta 223,* 432–436.
Mellors, A., Tappel, A. L., Sawant, P. L., and Desai, I. D. (1967), *Biochim. Biophys. Acta 143,* 299–309.
Pederson, P. L., and Schnaitman, C. (1969), *J. Biol. Chem. 244,* 5065–5073.
Pfaff, E., and Klingenberg, M. (1968), *Eur. J. Biochem. 6,* 66–79.
Pfaff, E., Klingenberg, M., and Vogell, W. (1968), *Eur. J. Biochem. 5,* 222–232.
Reed, W. P., and Lardy, H. A. (1973), *J. Biol. Chem. 247,* 6970–6977.
Rose, I. (1968), *Proc. Natl. Acad. Sci. U.S.A. 61,* 1079–1086.
Rubin, H. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3551–3555.
Sanui, H. (1970), *J. Cell. Physiol. 75,* 361–368.
Schnaitman, C., and Greenawalt, H. N. (1968), *J. Cell Biol. 38,* 158–175.
Scholte, H. R. (1969), *Biochim. Biophys. Acta 178,* 137–144.
Schuster S. M., and Olson M. S. (1973), *J. Biol. Chem. 248,* 8370–8377.
Skilleter, D. N., and Kun, E. (1972), *Arch. Biochem. Biophys. 152,* 92–104.
Stein, W. D. (1967), The Movement of Molecules Across Cell Membranes, New York, N.Y., Academic Press, pp 231–235.
Thiers, R. E., Reynolds, E. S., and Vallee, B. L. (1960), *J. Biol. Chem. 235,* 2131–2133.
Thiers, R. E., and Vallee, B. L. (1957), *J. Biol. Chem. 226,* 911–920.
Weiner, M. W., and Lardy, H. A. (1974), *Arch. Biochem. Biophys. 162,* 568–577.
Wieland, O. H. (1975), *FEBS Lett. 52,* 44–47.

Retention of Enzyme Activity by Detergent-Solubilized Sarcoplasmic Ca^{2+}-ATPase†

Marc le Maire, Jesper V. Møller,‡ and Charles Tanford*

ABSTRACT: The Ca^{2+}-activated ATPase of sarcoplasmic reticulum can exist in true solution in the presence of some nonionic detergents, with retention of enzymatic activity for several days. The soluble active particles retain about 30 mol of phospholipid per mol of polypeptide chain even in the presence of a large excess of detergent, indicating the existence of relatively strong attractive forces between protein and lipid, as previous work from other laboratories has already suggested. Deoxycholate is much more effective than nonionic detergents in removing protein-bound lipid and, when used at solubilizing concentrations, completely delipidates and inactivates the ATPase. Preliminary molecular weight measurements indicate that the Ca^{2+}-ATPase exists as an oligomer in the native membrane: fully active enzyme in Tween 80 has a minimal protein molecular weight of about 400 000, corresponding to a trimer or tetramer of the ATPase polypeptide chain, and even the inactive enzyme in deoxycholate contains a substantial fraction of dimeric protein.

This paper is part of a long-term project to characterize membrane proteins by the traditional methods of protein physical chemistry, using ultracentrifugation as the primary tool. To accomplish this objective it is necessary to have the protein in true solution, i.e., dispersed in particles that contain only a single copy of the protein molecule. This solubilization must be accomplished as far as possible without disruption of the native conformation of the protein, and it is hoped that this can be done with the aid of suitable detergents. It should be noted that our criteria for what is a suitable detergent are more stringent than those that apply to "reconstitution" experiments in which detergent solutions are used for purification procedures but the detergent is ultimately replaced by lipids, and membrane-bounded vesicles each containing many copies of the protein are reformed. In such experiments the protein need not have its native conformation in the detergent solution: it is necessary only that whatever change it has undergone be reversible. For our objectives the protein must remain undenatured in the detergent solution itself, by whatever criteria are available. In the case of the sarcoplasmic Ca^{2+}-ATPase, which is the subject of this paper, we have used the Ca^{2+}-ac-

† From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710. *Received December 23, 1975.* This work was supported by Grant AM-04576 from the National Institutes of Health.
‡ Institute of Medical Biochemistry, University of Aarhus, Denmark.
* Research Career Awardee, National Institutes of Health.

tivated splitting of ATP as such a criterion. Intactness of that part of the molecule that provides a passage for transfer of Ca^{2+} from one side of the sarcoplasmic membrane to the other cannot be tested directly when the ATPase is in true solution.

The events that occur upon addition of increasing amounts of detergents to biological membranes have recently been summarized by Helenius and Simons (1975). When the molar ratio of detergent to lipid is small, the detergent is incorporated into the membrane without disrupting it. Further addition of detergent may disrupt the membrane and lead progressively to some or all of the following: soluble membrane fragments containing many protein molecules, protein–lipid–detergent complexes containing only a single protein, and lipid-free protein–detergent complexes. Lipid removed from the protein complexes becomes incorporated into mixed lipid–detergent micelles.

These successive stages of solubilization may occur for some membrane proteins without evident structural change; e.g., microsomal cytochrome b_5 appears to retain its active site and other major structural features in lipid-free complexes with a variety of benign detergents (Robinson and Tanford, 1975; Visser et al., 1975). Some other membrane proteins have structures that are much more sensitive to the immediate environment of the molecule, and sarcoplasmic Ca^{2+}-ATPase is one of these. For example, the solubilization of the ATPase by sodium deoxycholate is accompanied by loss of enzymatic activity (Martonosi, 1968) and, if lipid is removed, the inactivation is irreversible (Hardwicke and Green, 1974). On the other hand if sodium deoxycholate is removed while lipid is still present, vesicular preparations can be obtained that exhibit ATPase activity and are able to transport Ca^{2+} with accumulation inside the vesicle (Martonosi, 1968; MacLennan, 1970; MacLennan et al., 1971; Meissner et al., 1973; Warren et al., 1974a). The most striking demonstration of the sensitivity of the ATPase to its environment comes from the work of Warren et al. (1974a,b), in which it was shown that reconstituted vesicles in which the endogenous lipid has been replaced by dioleoyl phospholipids retain both ATPase activity and the ability to accumulate Ca^{2+}, but that inactivation (which is reversible) occurs when dimyristoyl phospholipids are used. In the same experiments, little effect of phospholipid head groups on activity was observed.

It is not certain whether the solubilized protein–lipid–sodium deoxycholate complexes that were formed in the course of the reconstitution experiments cited above themselves possessed ATPase activity, since the enzyme was always diluted for activity measurements into an assay medium that did not contain sodium deoxycholate. The sodium deoxycholate concentration is thereby reduced to below the level required to maintain solubility and all the available evidence indicates that vesicular or particulate lipid–protein complexes are then reformed. The same question applies to previous work in which the nonionic detergent Triton X-100 was used for solubilization (McFarland and Inesi, 1970; Ikemoto et al., 1971). The danger of irreversible inactivation appears to be much less with this detergent than with sodium deoxycholate, but activity was again assayed by dilution into a detergent-free medium so that retention of activity in the presence of solubilizing concentrations of detergent was not established.

The experiments reported in this paper are exploratory in nature, intended to seek conditions under which active ATPase can be obtained in the form of soluble particles of molecular dimensions. Previously purified Ca^{2+}-ATPase in vesicular form was solubilized by several different detergents. Gel exclusion chromatography was used to separate solute particles on the basis of their dimensions, and concurrent lipid analysis was used to determine the extent of delipidation. Enzyme activity was measured on appropriate chromatographic fractions (as well as during solubilization) without dilution of the detergent. Preliminary molecular weight data for both inactive and active forms of the ATPase are also reported.

Experimental Section

Materials. Sodium deoxycholate was purchased from Fisher Scientific Co., purified by charcoal treatment and recrystallized from 80% acetone. Triton X-100 (Rohm and Haas, Co.), Tween 80 (Sigma Corp.), and Lubrol WX (Sigma Corp.) were commercial samples. Dodecyl octaoxyethylene glycol monoether ($C_{12}E_8$)[1] was a nominally pure compound prepared by Nikko Chemicals Co., Tokyo, Japan, and it was recrystallized from hexane before use. The corresponding detergent with six oxyethylene groups ($C_{12}E_6$) was obtained in radioactive form through the courtesy of Dr. W. J. Griebstein, Procter & Gamble Co., Cincinnati, Ohio. It was used as a tracer in one experiment to indicate the distribution of $C_{12}E_8$ among column fractions. It was not used as a primary detergent because, while its behavior is generally like that of $C_{12}E_8$, its micelles tend to self-associate at room temperature to form large aggregates (Corkill and Walker, 1972), whereas $C_{12}E_8$ micelles do not do so. Micelles consisting predominantly of $C_{12}E_6$ thus tend to spread over a wide volume when gel exclusion chromatography is carried out, which would have interfered with our chromatographic procedure.

Preparation of ATPase Vesicles. Sarcoplasmic reticulum vesicles were prepared by zonal centrifugation from a homogenate of rabbit skeletal muscle as described by Meissner et al. (1973). The preparation was extracted twice with a low concentration of sodium deoxycholate to remove proteins other than the ATPase from the sarcoplasmic membranes (method 2 of Meissner et al., 1973). The extracted membrane preparation (Ca^{2+}-ATPase vesicles) was essentially free of other proteins ($\simeq$95% Ca^{2+}-ATPase) as judged by gel electrophoresis (Weber and Osborn, 1969). The lipid content of the preparation, determined as organic phosphate (Bartlett, 1959), was about 78 mol per 10^5 g of protein.

ATPase Assay. Ca^{2+}-activated ATPase activity was measured as the difference between the rates of production of inorganic phosphate in the presence of 10^{-4} M Ca^{2+} and 10^{-4} M EGTA, respectively. Other constituents of the assay medium were 0.1 M KCl, 0.01 M Taps (pH 8.0), or Tes (pH 7.0–7.5) buffer, and sufficient detergent to maintain the concentration at the desired level. The Ca^{2+}-ATPase preparation was added to the incubation media to give a final concentration of 0.005–0.015 mg of protein/ml. The reaction was started by addition of a 100 mM stock solution of Mg–ATP to a final concentration of 5 mM and, when nonionic detergents were present, was terminated by addition of the perchloric–molybdovanadate reagent described by Lecocq and Inesi (1966). The absorption at 340 nm of the color developed after addition of the reagent was measured after butanol extraction as described by Louis et al. (1974) to avoid interference by the detergent. In sodium deoxycholate solutions, the reaction was terminated by addition of 1 ml of 1 M perchloric acid and inorganic phosphate was determined by the method Fiske and

[1] Abbreviations used are: $C_{12}E_8$, dodecyl octaoxyethylene glycol monoether; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Taps, 3-[tris(hydroxymethyl)methyl]aminopropanesulfonic acid; Tes, *n*-tris(hydroxymethyl)methyl-2-aminomethanesulfonic acid; cmc, critical micelle concentration.

Subbarow (1925). Corrections were made for pre-formed phosphate in the Mg-ATP stock solution.

Gel Chromatography. 1.5 × 90 cm columns of Sepharose 4B were used. Blue dextran and β-mercaptoethanol were used as void and total volume markers. Between 2 and 5 mg of protein in a volume of 1 ml or somewhat less was placed on the column which had been preequilibrated with the same solution as was used for elution. The flow rate was about 5 ml/h and fractions of about 1.2 g were collected.

Protein Concentration. Protein concentrations were measured on the basis of the method of Lowry et al. (1951). Ca^{2+}-ATPase protein concentrations were calculated from standard curves of bovine serum albumin, allowing for the 1.2-fold higher color yield of the ATPase protein (Hardwicke and Green, 1974). Standard curves were always run in the same medium, including detergent, in which the ATPase concentration was measured.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. The procedure of Weber and Osborn (1969) was used, except in one experiments where the β-mercaptoethanol normally present was omitted. The bulk of the protein always appeared as a single band of apparent molecular weight near 100 000. The satellite band reported by Louis and Shooter (1972) was not observed with ATPase prepared by the procedure used here.

Sedimentation Equilibrium. A Beckman Model E analytical ultracentrifuge, equipped with photoelectric scanner, was used. The slopes of equilibrium plots yield a quantity that is formally equal to $M(1 - \phi'\rho)$, where M is the molecular weight of the protein component of the sedimenting particle (excluding bound water, detergent, or lipid), ϕ' is the effective specific volume per g of protein, and ρ is the solvent density. As previously described (Tanford et al., 1974), the contributions of bound detergent and bound lipid (δ_D and δ_L g/g of protein, respectively) can be allowed for by the relation

$$M(1 - \phi'\rho) = M[(1 - \bar{v}_P\rho) + \delta_D(1 - \bar{v}_D\rho) + \delta_L(1 - \bar{v}_L\rho)] \quad (1)$$

where $\bar{v}_P$ is the partial specific volume of protein, and $\bar{v}_D$ and $\bar{v}_L$ are the partial specific volumes of bound detergent and lipid, respectively. A value of $\bar{v}_P = 0.740$ cm^3/g was calculated on the basis of the amino acid composition of the ATPase by the method of Cohn and Edsall (1943). Values of $\bar{v}_D$ for sodium deoxycholate (0.778 cm^3/g) and Tween 80 (0.896 cm^3/g) and the value of $\bar{v}_L$ (0.975 cm^3/g) were taken from Tanford et al. (1974), the latter being based on the lipid composition of MacLennan et al. (1971).

Results

Purified ATPase vesicles had initial Ca^{2+}-dependent ATPase activity of about 3 μmol of P_i per mg of protein per min when measurements were made at 23 °C at the optimal pH of 7.4 (MacLennan, 1970). Taking into account the activation energy of the reaction (Inesi et al., 1973), this is comparable to the activity of 5.5 μmol of P_i per mg per min observed at 32 °C by Meissner et al. (1973) or the figure of 10–14 μmol of P_i per mg per min obtained at 37 °C by Warren et al. (1974a).[2] A very slow loss of activity with time was generally observed, similar to that reported by MacLennan (1970). The rate of inactivation was slower at lower temperature and vesicles or detergent-solubilized protein were, therefore, generally stored in the cold.

Experiments Using Sodium Deoxycholate. The conditions used to obtain active or readily reactivatable ATPase in previously published work involve high concentrations of protein and lipid, and dilution is necessary for most of the measurements we wish to make. In particular, the first step in our procedure is to use gel chromatography to assess size homogeneity and to separate molecular complexes into sized fractions if needed, and this process automatically involves dilution. The initial ATPase-lipid-sodium deoxycholate complex is in a state of dynamic equilibrium with mixed lipid-sodium deoxycholate micelles, as Warren et al. (1974b) have already pointed out, since their lipid exchange experiments depend on such equilibration. The required dilution would then have to be carried out at constant thermodynamic activities of both sodium deoxycholate and phospholipid if the composition of the enzyme particle has to be kept within narrow limits to maintain its initial solubilized state, and this condition cannot be translated into operational terms (i.e., expressed in terms of concentrations rather than activities) on the basis of presently available data. It should also be noted that oligomer ⇄ monomer equilibria may be important in this system, in which case dilution can alter the initial state even if sodium deoxycholate and lipid activities are maintained constant.

Our attempts to arrive empirically at suitable dilution conditions were not successful. In the absence of added lipid, particles of molecular size always lost activity very rapidly. Addition of egg yolk phosphatidylcholine slowed the rate of inactivation (indicating that delipidation is one factor in loss of activity) but not sufficiently for our purposes. When the sodium deoxycholate concentration was reduced in effects to avoid inactivation, the ATPase emerged in the void volume on Sepharose 4B, indicating that vesicles or other very large particles had been formed. Attempts to meet our objectives by use of sodium deoxycholate as solubilizing agent were therefore abandoned.

In the course of this work, a few experiments were carried out to obtain a preliminary characterization of sodium deoxycholate-inactivated ATPase. Figure 1 shows a typical gel chromatogram on a Sepharose 4B column equilibrated and eluted with 5 mM sodium deoxycholate. ATPase vesicles were layered on the column at a level of 6 mg of protein/ml and a sodium deoxycholate/protein weight ratio of 1.25. It is seen that phospholipid has become completely dissociated from the protein, emerging as particles of small size, presumably in the form of mixed micelles with sodium deoxycholate. Most of the protein elutes as a single peak with a prominent shoulder, which can be resolved into two peaks with distribution coefficients of about 0.6 and 0.5, respectively. Material from nonoverlapping regions of the two peaks, shown by the shaded regions on the figure, was collected and re-chromatographed, as shown in Figure 2. No redistribution of material occurred, showing that the two peaks represent separate components that are not interconvertible under the conditions and within the time (~48 h) of these experiments. Sodium dodecyl sulfate gel electrophoresis of the two peaks under standard conditions gave identical results: the ATPase polypeptide chain of apparent molecular weight near 100 000 was the only component present in significant amount.

To further characterize the protein under these conditions, preliminary sodium deoxycholate binding measurements were made using sodium deoxy[^{14}C]cholate. Binding was determined by equilibrium dialysis or by column chromatography

[2] Considerably higher activities have been reported by MacLennan (1970), perhaps because he used a lower Ca^{2+} concentration in his assay medium. The Ca^{2+} dependence of activity measurements is discussed by MacLennan et al. (1972).

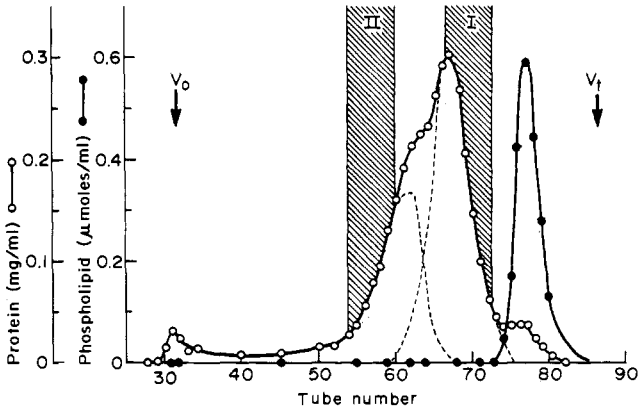

FIGURE 1: Typical gel chromatogram of inactive enzyme in sodium deoxycholate on Sepharose 4B. The sample placed on the column had a protein concentration of 5.8 mg/ml and a sodium deoxycholate/protein weight ratio of 1.25. The column was preequilibrated and eluted with 5 mM sodium deoxycholate, 0.1 M KCl, 0.01 M Taps (pH. 8.0), 0.001 M EDTA, 0.001 M NaN_3. The arrows indicate elution positions of markers for the void volume (V_0) and total volume (V_t). Shaded areas indicate fractions pooled for re-chromatography.

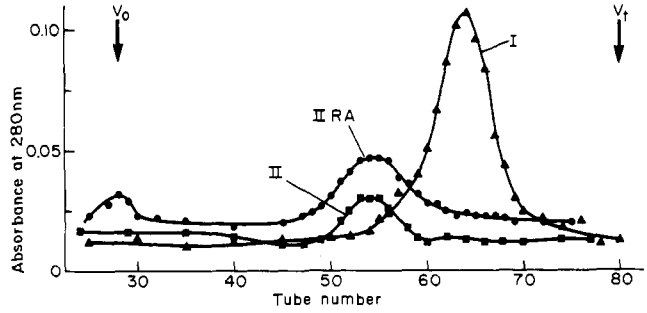

FIGURE 2: Re-chromatography of fractions I and II from Figure 1. The pooled samples were concentrated by ultrafiltration to a volume of 1 ml before being applied to the column. Curve II RA represents an aliquot of fraction II that was exposed to dithiothreitol and alkylated by iodoacetamide before chromatography. Absorbance at 280 nm was used to indicate protein concentration, and corrections for background absorbance were not made.

under conditions similar to those of Figure 1. The results indicated binding of 0.30 ± 0.04 g of sodium deoxycholate/g of protein at an equilibrating sodium deoxycholate level of 5 mM. Aliquots of each pooled peak were subjected to equilibrium ultracentrifugation and correction for bound detergent was made by using eq 1, given in the Experimental Section. The sedimentation equilibrium plots were slightly curved, indicating that neither peak contained entirely homogeneous protein. Average molecular weights of 140 000 and 270 000 were obtained for the protein moieties of the two peaks. A subsequent determination, using protein collected from narrower chromatographic fractions, gave molecular weight values of 120 000 and 230 000. Studies in progress in our laboratory indicate that the true molecular weight of the ATPase polypeptide chain is close to 120 000, i.e., slightly higher than the apparent molecular weight determined by sodium dodecyl sulfate gel electrophoresis. It is thus evident that the protein-detergent complexes of peaks I and II of Figure 1 contain one and two polypeptide chains, respectively.

Results similar to those of Figure 1 were obtained in about a dozen experiments of this type. The positions of the two peaks were always the same, but the relative amounts of dimer and monomer were somewhat variable, and in several experiments the dimer was the dominant species. There appeared to be no correlation between monomer/dimer distribution and the temperature at which the column was run or the sodium deoxycholate/protein weight ratio on the solution loaded onto the column. The latter was varied from 0.6:1 to 3.3:1. There was an increase in the amount of protein eluting in the void volume at low sodium deoxycholate levels, but no significant effect on the included peak. It may be noted that a qualitatively similar result for sodium deoxycholate inactivated protein has been reported by Hardwicke and Green (1974). Sedimentation velocity measurements under conditions similar to ours showed the presence of two independently sedimenting peaks, with sedimentation rates that are not incompatible with the interpretation that they might represent monomeric and dimeric species.

The failure to observe interconversion between monomer and dimer is puzzling and at present unexplained. Peak II material was subjected to sodium dodecyl sulfate gel electrophoresis with reducing agent *omitted*. A faint slow-moving band appeared, that was not seen when the standard medium including β-mercaptoethanol was employed. However, the bulk of the protein migrated as before, which shows that interchain disulfide bonds are not responsible for the formation of the dimeric peak II complex. Another experiment leading to the same conclusion is shown in Figure 2. The reducing agent dithiothreitol was added to peak II material and free SH groups were alkylated. No effect on the elution position was observed. Another possible explanation for lack of interconversion is that there exist two distinct polypeptide chains that are indistinguishable by sodium deodecyl sulfate gel electrophoresis but differ in their chemical properties. This possibility would seem to be remote. Phosphorylation, the binding of ATP and Ca^{2+}, and the rapid cleavage into two fragments by trypsin (Meissner, 1973; Inesi and Scales, 1974) all occur quantitatively, suggesting a single population of polypeptides.

Nonionic Detergents. The behavior of the ATPase in the presence of nonionic detergent is less sensitive to the exact experimental conditions than it is in the presence of sodium deoxycholate, probably because the rate of interaction between these detergents and the protein–lipid system is slow. Three quite distinct effects were observed, but their relative importance was different for each of the four nonionic detergents that were tested. The three observable effects were (1) an initial increase in specific activity, as previously observed for Triton X-100 and other nonionic detergents in other laboratories (Martonosi et al., 1968; McFarland and Inesi, 1970; Ikemoto et al., 1971); (2) disruption of the membrane to form soluble complexes; and (3) loss of enzymic activity. Disruption of the membrane did not occur at all within a reasonable time for two of the detergents: suspensions of vesicles remained turbid in Lubrol WX or Tween 80 even after 30 min incubation at detergent/protein ratios as high as 100:1 by weight. Lack of solubilization for these two detergents was confirmed by centrifugation at 100 000*g* for 60 min: 75–80% of the protein was pelleted, almost as much as the 80–85% pelleted by similar treatment of the original vesicle preparation without added detergent. On the other hand vesicle suspensions containing 0.37 mg/ml protein were completely clarified in 2 min when $C_{12}E_8$ or Triton X-100 was added at a detergent/protein weight ratio of 1.5 or greater, and subsequent gel chromatography showed that complexes of molecular dimensions had been formed. It may be noted that resistance to disruption of sarcoplasmic reticulum vesicles by detergent-like amphiphiles has been previously observed by Deamer (1973), who used lysophosphatidylcholine for purification of the ATPase from lobster abdominal muscle, and found that the lysolipid entered

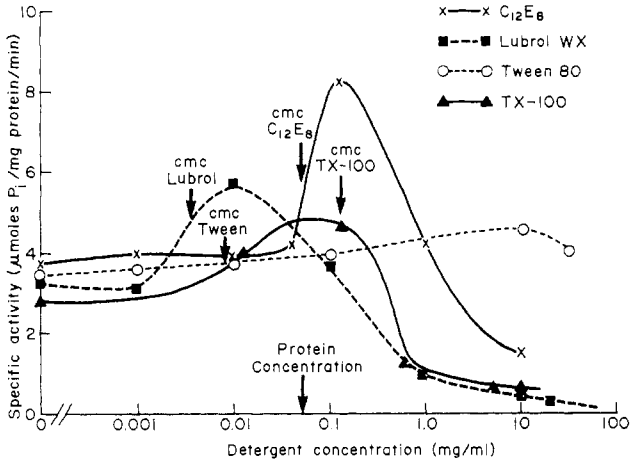

FIGURE 3: Effect of detergent concentration in the incubation medium on Ca^{2+}-activated ATPase activity. ATPase vesicle suspensions (protein content 10 mg/ml) were diluted in the detergent-containing medium to a final protein concentration of 0.015 mg/ml. The medium contained 0.1 M KCl, 0.01 M Taps (pH 8.0), and 10^{-4} M $CaCl_2$, and the incubation time was 2 min at 32 °C.

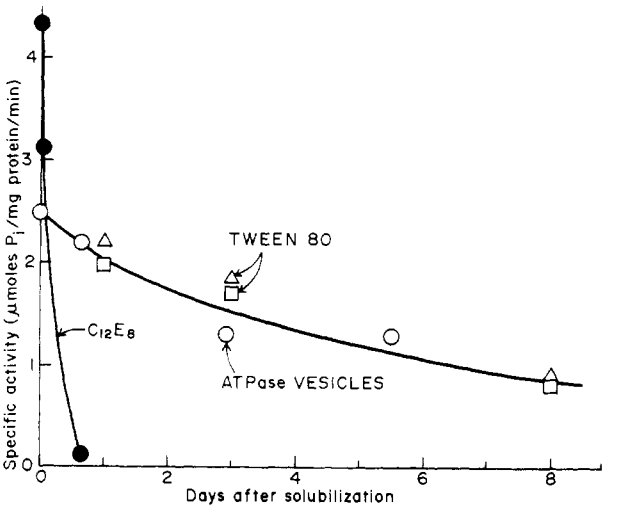

FIGURE 4: Time dependence of Ca^{2+}-stimulated ATPase activity, measured at 23 °C in 0.1 M KCl, 0.01 M Tes (pH 7.0), 10^{-4} M $CaCl_2$. All preparations were stored in the cold between measurements. Open circles represent purified vesicles without added detergent; filled circles represent vesicles solubilized by $C_{12}E_8$ at a detergent concentration of 10 mg/ml. Triangles and squares represent two chromatographic fractions from an experiment similar to that shown in Figure 5, where $C_{12}E_8$ had been replaced by Tween 80. In the latter experiments the first measurements were made 1 day after solubilization, this being the time elapsed during chromatography (done at 4 °C).

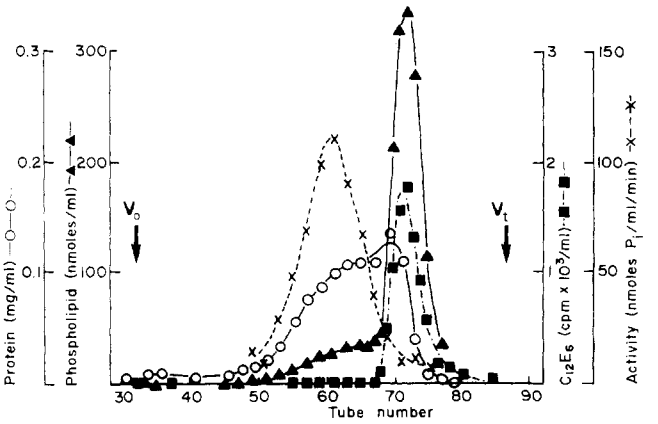

FIGURE 5: Chromatography of ATPase in Tween 80 on Sepharose 4B at 4 °C. The solution loaded onto the column contained ATPase vesicles (final protein concentration 3 mg/ml) dissolved in $C_{12}E_8$ (final concentration 12 mg/ml). The column was preequilibrated and eluted with a solution containing 0.1 M KCl, 0.01 M Tes (pH 7.0), 10^{-4} M $CaCl_2$, and Tween 80 at a concentration of 0.1 mg/ml. Radioactive $C_{12}E_6$ was present in the $C_{12}E_8$ as a tracer: the distribution of these two detergents on the column should be identical.

the vesicles, but did not disrupt them even at a 2:1 weight ratio of lysolipid to protein.

Figure 3 shows the effect of the nonionic detergents on ATPase activity after 2 min of incubation at 32 °C. These experiments were done at pH 8.0, where the initial activity is about one-third below that attainable at the optimal pH (MacLennan, 1970). The results show that Lubrol WX inactivates the enzyme even though it does not solubilize it. Another interesting feature of Figure 3 is the lack of any general correlation between the cmc of a detergent and the concentration at which rapid inactivation sets in.

As previously noted, the resistance to solubilization of the ATPase may have a kinetic rather than a thermodynamic origin, and $C_{12}E_8$ and Triton X-100 may differ from the other two detergents only in the degree of resistance. The concentrations of $C_{12}E_8$ or Triton X-100 that are required for solubilization lead to complete inactivation after storage for a period of hours (Figure 4) but, at least in the case of $C_{12}E_8$, the solubilizing concentration is much higher than the detergent concentration required to maintain the enzyme in soluble form. Short exposure to a solubilizing level of $C_{12}E_8$, followed by dilution to a lower detergent concentration, leads to a product that retains a virtually constant high level of activity for more than a day, and, in contrast to what was found when similar procedures were attempted with sodium deoxycholate, reaggregation to vesicular or other large particles does not occur.

The best results were obtained when ATPase was solubilized by $C_{12}E_8$ and the $C_{12}E_8$ was then displaced by Tween 80. As Figure 4 shows, preparations of this kind had enzymatic activity essentially identical with that of the original vesicles, and the slow loss of activity with time that was observed was also comparable with the similar inactivation of the detergent-free starting material. The procedure used for the detergent substitution (and analysis of the product) is illustrated by Figure 5. ATPase vesicles were dissolved in $C_{12}E_8$ at a detergent concentration of 12 mg/ml and the solution was immediately placed on a Sepharose 4B column, preequilibrated and subsequently eluted with Tween 80 at a concentration of 0.10 mg/ml. The protein eluted as a broad peak, similar to that observed when sodium deoxycholate was used (Figure 1), but extending to smaller elution volumes, indicative of a larger average particle size. Most of the phospholipid appears as a sharp peak, eluting later than the protein, but incompletely separated from it. This peak presumably represents mixed $C_{12}E_8$–lipid micelles and the poorer separation from the protein peak, in comparison with the results in sodium deoxycholate, can be ascribed to the fact that $C_{12}E_8$ micelles are considerably larger than sodium deoxycholate micelles, so that the mixed micelles are also larger. The $C_{12}E_8$ used in this experiment contained [^{14}C]$C_{12}E_6$ as tracer, and the resulting radioactivity is seen to be entirely associated with the lipid–detergent mixed micelle peak, indicating that no $C_{12}E_8$ remains associated with the protein. In contrast to this, some phospholipid does remain associated with the protein peak, at a level of 0.2–0.3 μmol per mg of protein. The appearance of lipid in association with the protein peak is the most striking analytical

difference between these results and those of Figure 1 and may be the reason why inactivation did not occur. Chromatography of active ATPase in solutions in which $C_{12}E_8$ had been diluted but not replaced by Tween 80 showed lipid retention to a similar extent.

The protein peak in Figure 5 may be divided into two approximately Gaussian components, as was done in Figure 1. The trailing peak is relatively sharp and elutes at a position that suggests it may represent monomeric ATPase; the leading peak, as already noted, is broad and elutes somewhat earlier than the dimer peak in Figure 1. Activity is associated with both peaks, but the specific activity of the leading peak is clearly about tenfold higher than the specific activity of the presumed monomer peak. It may be noted that the experiment shown in Figure 5 was repeated several times under somewhat different conditions, with essentially similar results. However, the position of the leading peak and the fraction of protein in it depended somewhat on the $C_{12}E_8$ concentration used for the initial solubilization. Persistence of larger particles was favored by the use of relatively low solubilizing concentrations.

Material from the peak tube of the leading peak of Figure 5 was subjected to equilibrium ultracentrifugation to obtain a measure of the degree of association of the protein. This cannot be done as simply as for the sodium deoxycholate containing complexes because there is no convenient method for determining the amount of bound Tween 80; i.e., the parameter δ_D of eq 1 is not known. This problem was circumvented by measuring $M(1 - \phi'\rho)$ at different densities, using D_2O–H_2O mixtures. The procedure is an adaptation of the method of Edelstein and Schachman (1967) for the determination of partial specific volume, but in this case the $\bar{v}$ values of eq 1 are all known, so that both M and δ_D can be obtained from the data. The measured $M(1 - \phi'\rho)$ values were 121 000, 106 500, and 94 500, respectively, at ρ = 1.004, 1.031, and 1.058 g/cm^3. Using eq 1, with the $\bar{v}$ values given in the Experimental Section and with δ_L = 0.23 g/g (from Figure 5), we obtained $\delta_D = 0.25 \pm 0.20$ g/g and $M \simeq 400\,000$. Because δ_D is small, the uncertainty in the determination of the molecular weight is much less than the uncertainty in δ_D, probably no more than 15%. The result obtained thus indicates that the state of aggregation of the protein corresponds to a content of three or possibly four polypeptide chains per complex.

Discussion

We have shown that enzymatically active Ca^{2+}-ATPase can be dispersed in true solution, in the form of particles of molecular size, by use of nonionic detergents. This state of the enzyme is likely to be more convenient than reconstituted vesicular ATPase for many kinds of physical and chemical studies that are necessary if the transport function of the enzyme is to be interpreted on a molecular basis. Transport itself of course can be studied experimentally only in the vesicular state.

In terms of characterization of the active state of the ATPase, our data indicate that protein–lipid interaction must be an important factor. The major difference between active protein–detergent complexes and the inactive ones obtained when sodium deoxycholate is used is that the active complexes retain 0.2–0.3 μmol of phospholipid per mg of ATPase, whereas inactive complexes in sodium deoxycholate were completely delipidated. Since the ATPase polypeptide chain has a molecular weight of about 120 000, our binding figure corresponds to about 30 mol of phospholipid per chain and, thus, corroborates previous studies that have demonstrated a similar phospholipid requirement for retention of ATPase activity (Meissner et al., 1973; Warren et al., 1974a,b, 1975). Our conditions provide stronger evidence than previous work that this amount of lipid is not merely a factor in the retention of activity, but actually uniquely *bound* to the protein. A large excess of detergent was present in experiments such as the one illustrated by Figure 5 and retention of lipid in the active complex could have occurred only if the retained lipid molecules (a) were not able to participate in the rapid equilibration between the detergent and the major lipid pool of the system, or (b) had sufficiently high free energy of binding to remain protein-bound despite rapidly equilibrating conditions. Phospholipid exchange experiments such as those described by Warren et al. (1974a,b, 1975) should permit a distinction between these possibilities.

Our preliminary molecular weight measurements indicate that the native state of the enzyme may be an oligomer of the ATPase polypeptide chain, requiring perhaps three or four chains per molecule for full activity. This cannot be regarded as an unequivocal conclusion between a somewhat higher degree of association than that measured for the protein from the column fractions of Figure 5 was attainable when a lower concentration of $C_{12}E_8$ was used for the initial solubilization. Since Tween 80 by itself was not able to disrupt the initial vesicular state of the ATPase, one cannot exclude the possibility that the experimentally observed oligomers were in fact residual membrane fragments containing more than one fully active enzyme molecule. However, the specific activity in Figure 5 and similar experiments always decreased dramatically in the transition between the oligomeric peak and the later eluting peak of smaller sized complexes, and did so without a parallel diminution in the amount of lipid associated with the protein, which strongly suggests that some form of denaturation occurs when the degree of association falls below a critical minimum.

The observation that inactive and delipidated ATPase in sodium deoxycholate exists as a mixture of dimeric and monomeric species may be considered as supporting the possible original existence of the native protein in an associated form. It at least shows that the polypeptide chains are *capable* of self-association, with what appear to be strong and specific interchain bonds; i.e., we are not dealing here with the type of nonspecific association often observed for membrane proteins in aqueous solutions in the absence of detergent, leading to formation of heterogeneous clusters, which are normally disrupted when detergents such as sodium deoxycholate are present.

Freeze fracture electron microscopy of purified sarcoplasmic reticulum membranes also supports the conclusion that the native ATPase is an oligomeric molecule. Packer et al. (1974) and Malan et al. (1975) have shown that membranes in which the Ca^{2+}-ATPase accounts for 70% or more of the membrane protein contain after freeze fracture about 5700 intramembranous particles per μm^2 of fracture plane area. The analytical content of ATPase polypeptides is about three times greater (Malan et al., 1975). If one is willing to assume a one-to-one relationship between intramembranous particles and ATPase complexes, the simplest interpretation is that there are about three polypeptide chains per ATPase complex.

Acknowledgment

We are grateful to Thor B. Nielsen for assistance in the preparation of sarcoplasmic reticulum, and to both him and Jacqueline A. Reynolds for numerous constructive suggestions.

References

Bartlett, G. R. (1959), *J. Biol. Chem. 234,* 466.
Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides, New York, N.Y., Reinhold, p 372.
Corkill, J. M., and Walker, T. (1972), *J. Colloid Interface Sci. 39,* 621.
Deamer, D. W. (1973), *J. Biol. Chem. 248,* 5477.
Edelstein, S. J., and Schachman, H. K. (1967), *J. Biol. Chem. 242,* 306.
Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66,* 375.
Hardwicke, P. M. D., and Green, N. M. (1974), *Eur. J. Biochem. 42,* 183.
Helenius, A., and Simons, K. (1975), *Biochim. Biophys. Acta 415,* 29.
Ikemoto, N., Bhatnagar, G. M., and Gergely, J. (1971), *Biochem. Biophys. Res. Commun. 44,* 1510.
Inesi, G., Millman, M., and Eletr, D. (1973), *J. Mol. Biol. 81,* 483.
Inesi, G., and Scales, D. (1974), *Biochemistry 13,* 3298.
Lecocq, J., and Inesi, G. (1966), *Anal. Biochem. 15,* 160.
Louis, C., Buonaffina, R., and Binks, B. (1974), *Arch. Biochem. Biophys. 161,* 83.
Louis, C., and Shooter, E. M. (1972), *Arch. Biochem. Biophys. 153,* 641.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
MacLennan, D. H. (1970), *J. Biol. Chem. 245,* 4508.
MacLennan, D. H., Seeman, P., Iles, G. H., and Yip, C. C. (1971), *J. Biol. Chem. 246,* 2702.
MacLennan, D. H., Yip, C. C., Iles, G. H., and Seeman, P. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 469.
Malan, N. T., Sabbadini, R., Scales, D., and Inesi, G. (1975), *FEBS Lett 60,* 122.
Martonosi, A. (1968), *J. Biol. Chem. 243,* 71.
Martonosi, A., Donley, J., and Halpin, R. A. (1968), *J. Biol. Chem. 243,* 61.
McFarland, B. H., and Inesi, G. (1970), *Biochem. Biophys. Res. Commun. 41,* 239.
Meissner, G. (1973), *Biochim. Biophys. Acta 298,* 906.
Meissner, G., Conner, G. E., and Fleischer, S. (1973), *Biochim. Biophys. Acta 298,* 246.
Packer, L., Mehard, C. W., Meissner, G., Zahler, W. L., and Fleischer, S. (1974), *Biochim. Biophys. Acta 363,* 159.
Robinson, N. C., and Tanford, C. (1975), *Biochemistry 14,* 369.
Tanford, C., Nozaki, Y., Reynolds, J. A., and Makino, S. (1974), *Biochemistry 13,* 2369.
Visser, L., Robinson, N. C., and Tanford, C. (1975), *Biochemistry 14,* 1194.
Warren, G. B., Houslay, M. D., Metcalfe, J. C., and Birdsall, N. J. M. (1975), *Nature (London) 255,* 684.
Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G., and Metcalfe, J. C. (1974a), *Proc. Natl. Acad. Sci. U.S.A. 71,* 622.
Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G., and Metcalfe, J. C. (1974b), *Biochemistry 13,* 5501.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.

Iodination of a Tyrosyl Residue in Staphylococcal α-Toxin†

Paul Cassidy and Sidney Harshman*

ABSTRACT: Iodination of staphylococcal α-toxin by the lactoperoxidase method resulted in the maximal incorporation of about 2.5 atoms of iodine per molecule of α-toxin. The iodination primarily involved a single tyrosine residue as shown by analysis of both cyanogen bromide and tryptic peptides. Iodination at a level of 1.2 iodine atoms per α-toxin molecule led to a dramatic decrease in the hemolytic and lethal activities, although no decrease in the binding of iodinated toxin to rabbit erythrocytes was observed (Cassidy and Harshman (1976), *Biochemistry,* the following paper in this issue). Monoiodinated α-toxin was found to have 15% of the specific hemolytic activity of native α-toxin. Incubation of rabbit erythrocytes with iodinated α-toxin led to a significant protection from the hemolytic activity of native α-toxin added later. The results show that modification of a single unique tyrosyl residue in α-toxin permits the resolution of α-toxin's biological activities from its cell binding activity.

Staphylococcal α-toxin, a protein produced in large amounts in the growth medium of *Staphylococcus aureus,* is a hemolytic toxin which is lethal for most mammalian species (Arbuthnott, 1970). Chemical modification techniques which have been used so successfully in the study of structure-function relationships in enzymes (Glazer, 1970) and neurotoxins (Tu, 1973) have not until now been applied to staphylococcal α-toxin. In fact, the only reported chemical modification of α-toxin involved "toxoiding" or total reaction of the protein with formaldehyde (Bernheimer et al., 1968).

Iodination by the lactoperoxidase method (Morrison et al., 1971) is a protein modification reaction which can be carried out under mild conditions and is a reaction of high specificity. Lactoperoxidase-catalyzed iodination of protein substrates can result in mono or diiodo derivatives of tyrosine and histidine,

† From the Department of Microbiology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232. *Received November 7, 1975.* Preliminary reports of some of the work reported here were given at the 3rd International Symposium on Staphylococci and Staphylococcal Infections, September 8–14, 1975, Warsaw, Poland. This work was supported in part by NIH Grant RO1 AI-11564 and by NSF Grant GB-38902. Paul Cassidy is a Vanderbilt Biomedical Sciences Fellow.