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Purification of membrane proteins in SDS and subsequent renaturation

Stellan Hjertén, Marianne Sparrman and Jia-li Liao *

Institute of Biochemistry, University of Uppsala, Biomedical Center, Uppsala (Sweden)

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A prerequisite for the purification of any protein to homogeneity is that the protein is not non-specifically associated with other proteins especially during the final stage(s) of the fractionation procedure. This requirement is not so often fulfilled when nonionic detergents (for instance Triton X-100) are used for solubilization of membrane proteins. The reason is that these detergents are not efficient enough to prevent the protein of interest from forming aggregates with other proteins upon contact with chromatographic or electrophoretic supporting media, which, due to their polymeric nature, have a tendency to induce aggregation of other polymers, for instance, hydrophobic proteins. The aggregation can be avoided if sodium dodecyl sulfate (SDS) is employed as detergent. We therefore suggest that membrane proteins should be purified by conventional methods in the presence of SDS and that the purified proteins, which are in a denatured state, are allowed to renature. There is good chance to renature internal membrane proteins since they should not be so susceptible to denaturation by detergents as are water-soluble proteins because the natural milieu of the former proteins is lipids which in fact are detergents. In this paper we present a renaturation method based on the removal of SDS by addition of a large excess of G 3707, a nonionic detergent. By this technique we have renatured a 5′-nucleotidase from *Acholeplasma laidlawii* and a neuraminidase from influenza virus. The enzyme activities were higher (up to 6-fold) after the removal of SDS than prior to the addition of SDS.

Introduction

Due to the hydrophobic nature of intrinsic membrane proteins they have a tendency to form aggregates. To prevent aggregation and keep the proteins in solution one often utilizes neutral detergents, which have the advantage that they seldom denature proteins. However, all detergents form micelles, the presence of which can be very disturbing, for instance, in purification studies. It would therefore be advantageous to solubilize membrane proteins without the use of agents which form micelles. From this point of view a mixture of a buffer and an organic solvent would be preferable as a solubilizing medium, especially an aqueous solution of ethylene glycol (which breaks hydrophobic bonds) containing an appropriate concentration of a salt (which breaks electrostatic bonds) [1], since this medium rarely causes denaturation. In fact, it often stabilizes labile enzymes [1]. Unfortunately, many membrane pro-

* Present address: Biology Department, Jinan University, Guangzhou, China.

Correspondence: S. Hjertén, Institute of Biochemistry, University of Uppsala Biomedical Center, P.O. Box 576, S-751 23 Uppsala, Sweden.

teins cannot be solubilized with either neutral detergents or ethylene glycol supplemented with salt (even if the proteins are soluble in free solution in the presence of these agents, they may precipitate upon contact with electrophoretic or chromatographic bed materials [1,2]). In such cases one is often obliged to use the highly solubilizing detergent SDS to keep the membrane proteins in solution. The drawback of SDS is its strong denaturing effect. We have therefore tried to find methods to renature membranes proteins following their exposure to SDS. In a previous paper we showed that this can sometimes be accomplished by removal of SDS by dialysis against G 3707, a neutral detergent [3]. In the same paper we also presented evidence that mere addition of the latter detergent could reactivate a phosphatase from membranes of *Acholeplasma laidlawii* following polyacrylamide gel electrophoresis in SDS. In the present study we show that this renaturation method is applicable also with SDS-denaturated 5′-nucleotidase and neuraminidase. The availability of efficient renaturation methods is of great importance for the purification of membrane proteins. For instance, by electrophoresis in polyacrylamide gels in the presence of SDS one can separate most membrane proteins with high resolution. From such a gel the protein of interest can easily be recovered without dilution and in almost 100% yield [4–8]. There is a good chance that the SDS-denatured protein can be renatured by the method described herein (alternatively one can of course, purify membrane proteins by chromatography in SDS and then apply the renaturation method). In the case of water-soluble proteins there is no particular reason to perform fractionation in SDS. Furthermore, it seems to be difficult to renature water-soluble proteins after exposure to SDS.

Materials and Methods

Chemicals. Sodium dodecyl sulfate (SDS) was obtained from Merck Darmstadt, F.R.G. Tween 20 and heptaoxyethylene lauryl ether (G 3707) were a gift from Dr. D. de Coster, Atlas Chemie, Everberg, Belgium. The dialysis bag (Visking; diameter 3.5 cm) had an exclusion limit corresponding to proteins of molecular weights around 10 000 and was bought from Kebo-Grave, Stockholm, Sweden. [2-^{3}H]AMP (443 GBq/mmol) was from Amersham International, Amersham Bucks, U.K. Liquid Scintillator Supersolve was from Koch-Light Laboratories Ltd., Haverhill Suffolk, U.K. *N*-Acetylneuraminyl-lactose (grade I) was purchased from Sigma, U.S.A. Lactose/galactose test combination, catalogue number 176.303, was from Boehringer, Mannheim, F.R.G.

Influenza virus strains. The following strains of influenza virus were obtained from the National Bacteriological Laboratory (SBL), Stockholm, Sweden, partially purified by differential centrifugation: A/England/42/72 (H3N2), B/Hong Kong/8/73/B and the hybrids A/Hong Kong/68 (H3N2) (X-31) and A/Texas/1/75 (H3N2) (X-49). A/Finland/7/69 was obtained from Orion Pharmaceutical Co., Helsinki, Finland. All virus preparations used were formaldehyde inactivated.

Culturing of Acholeplasma laidlawii and the preparation of plasma membranes.
The procedure was essentially that described by Razin et al. [9].

5′-Nucleotidase assay. 5′-Nucleotidase was assayed by the method of Avruch and Wallach [10], as modified by Newby et al. [11]. The assay was performed at 37 °C in 300 μl of a medium containing 200 μM AMP, 50 mM Tris-HCl (pH 8.0), tracer [2-^{3}H]AMP (20 000–30 000 cpm) and 0.18 mM $MgCl_2$. The reaction was initiated by adding 10 μl of the sample preparation and terminated after 30-min incubation by addition of 60 μl of 0.15 M $ZnSO_4$. Protein, released phosphate and unhydrolyzed AMP were precipitated by addition of 60 μl of 0.15 M barium hydroxide, whereas adenosine remained in the supernatant (centrifugation was performed at $3000 \times g$ for 10 min). An aliquot of 250 μl of the supernatant was added to 3 ml of scintillant (Supersolve) and the radioactivity was determined in a Packard Liquid Scintillation Counter, TRI-CARB 460 C.

Neuraminidase assay. A fluorometric procedure for quantitating the amount of *N*-acetylneuraminic acid enzymatically released by the neuraminidase from *N*-acetylneuraminyl-lactose was used as suggested by José A. Cabezas et al. [12]. The liberated lactose was hydrolyzed with β-galactosidase, and the released galactose oxidized

with β-galactose dehydrogenase and NAD^+. The NADH produced was measured as the increase in fluorescence at 465 nm (excitation 340 nm) in an Aminco SPF-500 spectrofluorometer.

The effect of SDS and G 3707 on the enzymes in the neuraminidase assay. The effect of SDS and G 3707 on the enzymes β-galactosidase and β-galactose dehydrogenase was investigated in the following way: Denaturation and reactivation experiments were performed as described under Experiments and Results with 0.7 nM lactose as sample instead of viral neuraminidase. Controls were run in parallel. The presence of SDS and G 3707 at the concentrations used (0.01 M SDS for denaturation and 0.003 M SDS and 8% G 3707 for reactivation) had no effect on the neuraminidase assay.

Experiments and Results

A. Denaturation and renaturation of a 5′-nucleotidase

Distribution of 5′-nucleotidase activity in fractions obtained by extractions of Acholeplasma laidlawii membranes with the neutral detergents Tween 20 and G 3707

A suspension (0.5 ml) of *A. laidlawii* membranes (40 mg protein/ml), prepared as described under Materials and Methods was mixed with 0.5 ml of 0.1 M Tris-HCl (pH 8.0) containing 5% (w/v) Tween 20 (3). After 2 h the mixture was centrifuged at 130000 × *g* (the supernatant is called the Tween 20-extract throughout this paper). The pellet was extracted by stirring for 1 h with 2 ml of water containing 0.5% (w/v) G 3707. The suspension was centrifuged for 30 min at 130000 × *g*. The supernatant (called the G 3707 extract) was collected and the pellet suspended in 1 ml of 0.02 M sodium phosphate (pH 7.4) containing 0.15 M sodium chloride (PBS). The 5′-nucleotidase activity was determined in all of the fractions obtained (see Table I). All of the above operations were performed at room temperature. The Tween extract and the G 3707 extract were used for the renaturation experiments to be described.

TABLE I

DISTRIBUTION OF 5′-NUCLEOTIDASE ACTIVITY IN DIFFERENT MEMBRANE FRACTIONS

Sample	5′-Nucleotidase activity (%)
I *Acholeplasma laidlawii* membranes	100
Tween 20 extraction:	
II supernatant after centrifugation ('Tween 20 extract')	106
III pellet suspended in 0.5% G 3707	60
G 3707 extraction:	
IV supernatant after centrifugation of pellet III ('G 3707 extract')	22
V pellet obtained in IV, suspended in PBS	14

Renaturation of an SDS-denatured 5′-nucleotidase from Acholeplasma laidlawii by (1) addition of an excess of G 3707 (concentration 7%) and by (2) dialysis of this solution against a low concentration of G 3707 (0.5%)

Sample: The G 3707 extract. 800 µl of the G 3707 extract (containing 0.5% (w/v) G 3707 and about 2 mg protein/ml) was, after addition of 40 µl of 1 M Tris-HCl (pH 8.0), incubated with 100 µl of 0.1 M SDS (final SDS concentration: 0.011 M) at room temperature. The enzyme activity was measured immediately before the addition of SDS and 15 and 30 min after (Fig. 1). The renaturation experiments were then started by addition of a 25% (w/v) solution of G 3707 in 0.05 M Tris-HCl (pH 8.0) to a final G 3707 concentration of 7%. The solution was divided into two aliquots: one was kept at room temperature and agitated occasionally and the other was dialyzed for two days at room temperature against 300 ml of 0.5% (w/v) G 3707 in 0.05 M Tris-HCl (pH 8.0) followed by

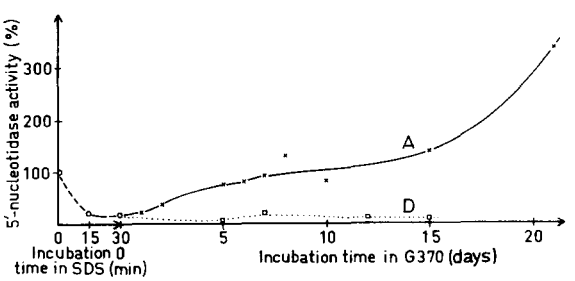

Fig. 1. Denaturation by SDS of a 5′-nucleotidase in the G 3707 extract and attempts at renaturation by (1) addition of G 3707 to a concentration of 7% (curve A) and (2) by dialysis of this solution against 0.5% G 3707 (curve D).

two days at 4° C against a fresh 300-ml portion of the same solution. Aliquots were removed periodically for measurement of 5′-nucleotidase activity. As shown in Fig. 1, the activity was recovered following incubation with 7% G 3707 but not following dialysis against 0.5% G 3707. The figure also shows that the activity recovered was more than three times that before the treatment with SDS. Parallel control experiments (without G 3707 extract) did not show any enzyme activity.

Sample: The Tween 20 extract. This experiment was performed similarly and in parallel with the experiment mentioned above with the main difference that the sample consisted of 800 µl of Tween extract (containing about 5 mg protein and 2.5% (w/v) Tween 20) instead of 800 µl of the G 3707 extract. The result (Fig. 2) agrees with that shown in Fig. 1 in the sense that the activity cannot be recovered by dialysis against buffer containing 0.5% G 3707 but is restored by incubation with a high concentration of G 3707 (in the latter case the activity was almost six times higher than before the addition of SDS).

B. Denaturation and renaturation of a neuraminidase

Denaturation by SDS of a neuraminidase from membranes of influenza virus A / England / 42 / 72

225 µl of influenza virus A/England/42/72, diluted 1 : 100 with 0.05 M Tris-HCl (pH 8.0) to a final concentration of 9.4 µg protein/ml was incubated with 25 µl of 0.1 M SDS in water for a period up to 90 min. The results of occasional neuraminidase activity determinations during this time are presented in Fig. 3. As shown, after about 30 min the activity had dropped to a constant value equal to about 20% of the activity of untreated, native enzyme.

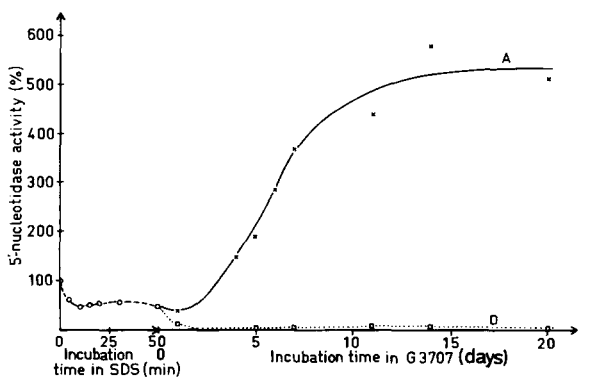

Fig. 2. Denaturation by SDS of a 5′-nucleotidase in the Tween 20 extract and attempts at renaturation by addition of G 3707 to a concentration of 7% (curve A) and by dialysis of this solution against 0.5% G 3707 (curve D).

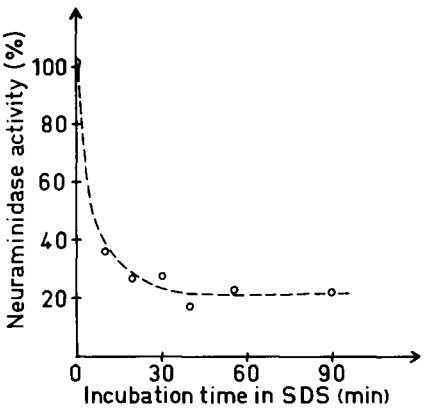

Fig. 3. Neuraminidase activity in influenza virus A/England/42/72 at different times after addition of SDS.

Renaturation of an SDS-denatured neuraminidase from influenza virus by addition of a large excess of G 3707

75 µl of 0.1 M SDS in water was added to 675 µl of influenza virus A/England/42/72, diluted 1 : 100 with 0.05 M Tris-HCl (pH 8.0). After 30 min (which was the incubation time required for a constant, low activity according to Fig. 3) an aliquot of 75 µl was withdrawn for the enzyme assay. After addition of 375 µl of a 25% (w/v) solution of G 3707 to the remainder of the enzyme solution, the activity was measured after 5, 10, 20, 30, 90 and 180 min. Fig. 4 shows that the enzyme activity was restored within 5 min and leveled off at about 110% of the activity of the untreated, native enzyme.

Denaturation by SDS of neuraminidases from different influenza virus strains

The enzyme activities of the virus strains X-31, X-49, and A/Finland/7/69, diluted 1 : 50 with 0.05 M Tris-HCl (pH 8.0), were determined and set equal to 100%. The activity measurements were then repeated after different times of incubation

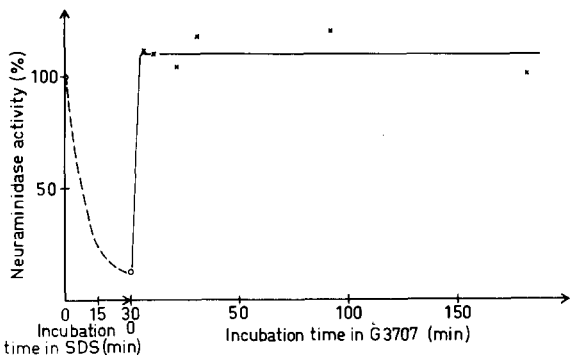

Fig. 4. Denaturation by SDS of neuraminidase in influenza virus A/England/42/72 and renaturation by addition of a large excess of G 3707.

with SDS. The result presented in Fig. 5 shows that neuraminidases from different strains respond very differently to exposure to SDS, one enzyme being activated (A/Finland/7/69), one completely inactivated (X-49), and one partially inactivated (X-31). The protein concentrations in the strains (after dilution) were 8.6, 24, and 6.4 μg/ml, respectively).

Renaturation by incubation with G 3707 of SDS-denatured neuraminidases from different influenza virus strains

The enzyme activities of the virus strains X-31, X-49, A/Finland/7/69 and B/Hong Kong/8/73/B, diluted in 0.05 M Tris-HCl (pH 8.0), were determined and set equal to 100%. After incubation in 0.01 M SDS for 30 min the activities were redetermined and then again after subsequent incubation for 15 min and 26 h in 10% G 3707. The data in Table II indicate that the neuraminidases from all of the strains were only partially in-

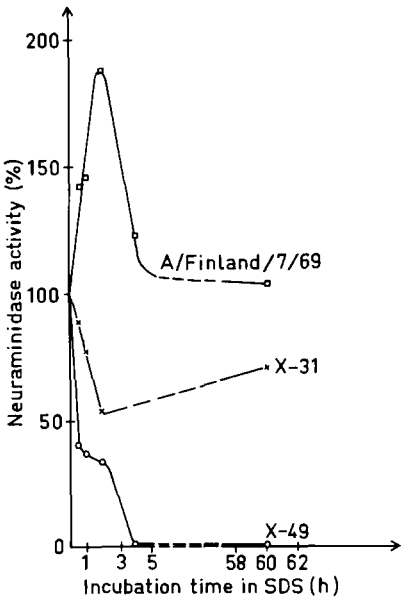

Fig. 5. Neuraminidase activity in different strains of influenza virus at different times after addition of SDS.

activated upon incubation for 30 min with SDS and that all of the neuraminidase species, except those from virus B/Hong Kong/8/73/B, can be reactivated to 100% after incubation for only 15 min with high concentrations of G 3707.

C. Purification studies

Purification of neuraminidase from influenza virus by high-performance molecular-sieve chromatography in the presence of SDS and subsequent renaturation

Agarose beads (12%) were prepared as previously described [13] and crosslinked with divinyl-

TABLE II

THE ACTIVITY OF NEURAMINIDASES FROM DIFFERENT INFLUENZA STRAINS FOLLOWING EXPOSURE TO SDS AND SUBSEQUENT TREATMENT WITH G 3707.

All the virus strains were diluted in 0.05 M Tris-HCl (pH 8.0).

Virus strain	X-31	X-49	A/Finland/7/69	B/Hong Kong/8/73/B
Dilution	1:50	1:50	1:50	1:5
Approx. protein content (μg/ml)	8.6	24	6.4	104
Initial enzyme activity (%)	100	100	100	100
Activity after a 30-min incubation with 0.01 M SDS	70	50	90	20
Activity after a subsequent 15-min incubation with 10% G 3707	110	100	110	40
Activity after an additional 26-h incubation with 10% G 3707	110	140	120	40

sulfone [14]. The beads were sized by elutriation in water and those with diameters between 5 and 10 μm were collected and used to pack a 0.6-cm column to a height of 24 cm. The bed was equilibrated with 0.05 M Tris-HCl (pH 8.0) containing 0.01 M SDS. About 200 μl of a solution of influenza virus A/England/42/72 in this buffer was applied (protein concentration. 0.94 mg/ml) The chromatogram was developed with the same buffer. Detection was performed at 280 nm. Fractions of 0.15 ml were collected. The flow rate was low (0.05 ml/min) in order to minimize dilution of the sample and thus allow reliable measurements of the neuraminidase activity. Fractions of 150 μl were collected. Each fraction was divided into two 75-μl portions, one portion being used directly for activity measurements and the other after addition of G 3707. Enzyme activity was observed only in peak N in the chromatogram upon renaturation with G 3707 (Fig. 6).

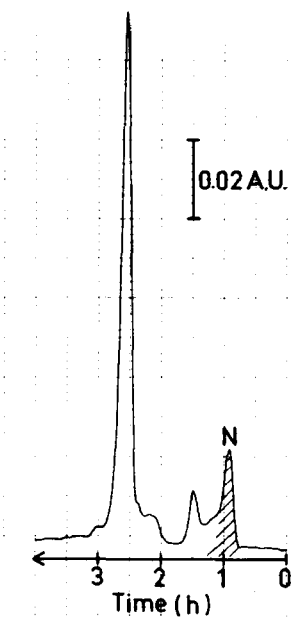

Fig. 6. Purification of a neuraminidase by high-performance molecular-sieve chromatography in the presence of SDS and subsequent renaturation. Bed material: 5–10 μm crosslinked 12% agarose beads. G 3707, a neutral detergent, was added to each of the chromatographic fractions. By this rapid reactivation technique the enzyme was localized to fractions corresponding to peak N. This figure illustrates the possibility to perform a purification of a hydrophobic membrane protein in the presence of the highly solubilizing (but unfortunately denaturing) detergent SDS and then to renature the protein with the aid of G 3707 or another non-denaturating detergent.

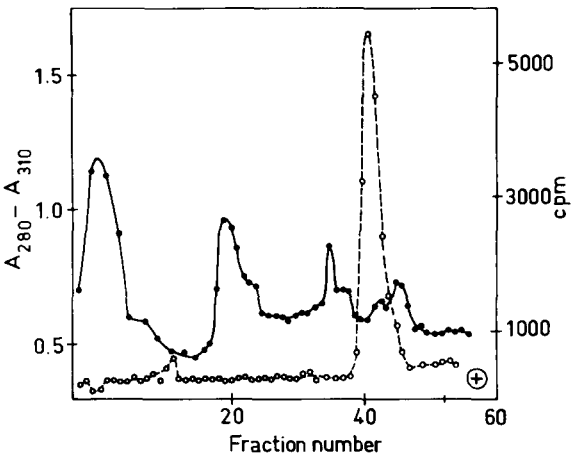

Fig. 7. Agarose suspension electrophoresis of the Tween 20 extract of *A. laidlawii* membranes in the presence of G 3707. Supporting medium: 0.17% agarose. This figure illustrates that the detergent G 3707 can be used not only for removal of SDS but sometimes also as a solubilizing agent for membrane proteins, although it is not as efficient as SDS.

Purification of a 5'-nucleotidase from A. laidlawii by agarose suspension electrophoresis in the presence of G 3707

The electrophoresis method is described in Ref. 15 and its usefulness for the fractionation of membrane proteins is illustrated in Refs. 16 and 17. The glass column tube (and the 'shunt') had an inner diameter of 1.3 cm and a length of 65 cm. The run was performed at 1000 volts (50 mA) in a 0.1 M Tris-HCl buffer (pH 8.0), containing 0.17% agarose. The sample consisted of 0.6 ml of the Tween 20 extract (protein concentration about 5 mg/ml). After electrophoresis for 23 h 1-cm fractions were withdrawn from the top of the glass column and analyzed for enzyme activity. The absorbance of each fraction was also measured at both 280 (A_{280}) and 310 (A_{310}) nm. By using the difference $A_{280} - A_{310}$ instead of A_{280} in a plot against the fraction number (Fig. 7) irregularities in the background due to the presence of agarose could be suppressed [18]. The figure shows that the fractions 40–43 contained most of the enzyme activity.

Discussion

Purification of hydrophobic membrane proteins: an approach

The experiments presented in Figs. 1, 2 and 4 and Table II show that the renaturation method based on the addition of an excess of a neutral detergent works satisfactorily with 5′-nucleotidase and neuraminidase. It is therefore also likely to be successful with many other membrane proteins. It is noteworthy that the neuraminidase activity following renaturation often was 10–20% higher after the renaturation than the activity before denaturation (Fig. 4 and Table II). In the case of the 5′-nucleotidase in the G 3707 extract and in the Tween 20 extract the final activity was about 3 and 6 times higher, respectively (Figs. 1 and 2). One can therefore conclude that a rational approach to purifying hydrophobic membrane proteins might be to perform the fractionation (for instance by chromatography or polyacrylamide gel electrophoresis) in SDS (the highly dissociating and solubilizing ability of which makes the fractionation not much more difficult than for water-soluble proteins in the absence of detergents), and then to renature the denatured protein by addition of a large excess of a non-denaturing, neutral detergent, preferably one without UV-absorption, such as G 3707. An example of this approach is the chromatography experiment presented in Fig. 6. Another example is found in Ref. 3, where we reported that a phosphatase from *Acholeplasma laidlawii* could be reactivated following polyacrylamide gel electrophoresis in SDS by immersing the gel in a solution of G 3707. Also, the 5′-nucleotidase in the Tween 20 extract from the same organism could be renatured in an analogous way after an SDS electrophoresis (not shown herein).

In this connection we would like to refer to a renaturation study by Lance C. Seefeldt and Daniel J. Arp (Department of Biochemistry, University of California). They found that a membrane-bound hydrogenase from *A. vinelandii* could be reactivated following electrophoretic analysis in an SDS-containing polyacrylamide by removal of SDS by soaking the gel in buffer without SDS.

Theoretical aspects of the renaturation of SDS-denatured membrane proteins

We have previously put forward the hypothesis that hydrophobic membrane proteins are less susceptible to denaturation by SDS than are ordinary water-soluble proteins [3]. This hypothesis is based on the following facts and considerations.

Membrane proteins are more or less surrounded by lipids in the native cell membrane. These lipids consist of a hydrophilic head and a hydrophobic tail, which is the characteristic structure of any detergent. Lipids can therefore be regarded as detergents. In other words: detergents are a natural milieu for intrinsic, hydrophobic membrane proteins. From these considerations one would expect that the more hydrophobic the proteins are, the less their structures should be affected by SDS, i.e. the less they should be denatured. This hypothesis was supported by a literature search, which revealed that the most hydrophobic membrane proteins were biologically active also in the presence of SDS (see Ref. 3 and Fig. 5, virus A/Finland/7/69). There are no obvious reasons to assume that this hypothesis can be extended also to those water-soluble proteins which have a pronounced hydrophobic structure. However, we found that β-galactosidase, the surface of which apparently contains many hydrophobic amino acid residues, since it elutes after serum albumin upon hydrophobic-interaction chromatography, showed an activity increase of about 50% upon transfer to a 0.05 M SDS solution (Hjertén, S. and Ganea, E., unpublished data). We do not known of any other water-soluble enzyme which is biologically active in the presence of SDS. There are, however, several papers on the reactivation of water-soluble enzymes following SDS treatment [19–25].

When removing SDS in connection with renaturation experiments one should at the same time try to create a milieu for the membrane proteins which is as similar as possible to that in the native cell membrane. This could be accomplished by adding to the protein-SDS solution an efficient non-denaturating detergent at a high concentration. The hydrophobic part of this detergent should have a structure similar to that of the hydrophobic tail of the predominant lipid in the cell membrane or the possible lipid specifically

associated with the membrane protein of interest (theoretically and ideally one should employ a mixture of the lipids that occur in the native membrane or a mixture of detergents whose hydrophobic moieties have the same structure as the hydrophobic tails of the membrane lipids). Since the hydrophobic segment of most membrane lipids consists of a long aliphatic chain, a detergent containing such a structure should be suitable to displace SDS in the renaturation of many membrane proteins (cf. activation of a C_{55}-isoprenoid alcohol phosphokinase by phospholipids, fatty acids and detergents [26–28]).

The structure of the hydrophilic segment of the detergent molecule is probably of less importance, since the hydrophilic part of a membrane protein is 'happy' in most hydrophilic milieus.

There should be no disadvantage to adding the detergent in a large excess, since the lipids in a cell membrane are in very high concentration.

Many of the above requirements of a detergent are fulfilled if a 20% solution of G 3707 is added to the SDS-solubilized protein to a final concentration of 5–10% (the properties of this neutral detergent, heptaoxyethylene lauryl ether, is discussed in Ref. 29). G 3707 will then form mixed micelles with the free SDS molecules, the concentration of which thus decreases. The SDS micelles associated with the proteins will accordingly be dissociated rapidly, since the G 3707 concentration is high. In this way the membrane proteins are freed from SDS at the same time as they become surrounded by the non-denaturing, lipid-like hydrophobic tails of the G 3707 molecules.

Some comments on the renaturation experiments

When Tween 20 and G 3707 extracts were treated with SDS the 5′-nucleotidase activity did not approach zero after long incubation times, but leveled off at about 25% (Fig. 1). This residual activity may be explained by the natural assumption that Tween 20 and G 3707 have formed less denaturing mixed micelles with SDS.

In a previous paper [3] we showed that an SDS-denatured phosphatase from *Acholeplasma laidlawii* could be reactivated either by dialysis against a 0.5% G 3707 solution or by addition of this detergent. For the 5′-nucleotidase from the same organism used in this study only the latter renaturation method was useful, as shown in Figs. 1 and 2.

These figures also show that the 5′-nucleotidase activity was higher after renaturation than before the denaturation by SDS. The same observation was made with neuraminidase (Fig. 4 and Table II). The increase in activity can be due to a favorable change in the conformation of the enzyme and/or increased accessibility of the substrate to the enzyme.

In this paper we have used G 3707 for extraction of a 5′-nucleotidase from *A. laidlawii* membranes and for removal of SDS. Due to its highly solubilizing effect [28] this detergent can in some cases be employed with advantage as an additive to buffers used in electrophoresis and chromatography for the separation of membrane proteins, although it is not as efficient as SDS. An example is shown in Fig. 7. A comparison between the UV and activity pattern reveals that a high degree of purification of the enzyme was obtained. The experiment was performed in a supporting medium of very low concentration (0.17% agarose) which decreases the risk of aggregation and precipitation of the membrane proteins [1,2].

The main purpose of this paper was to investigate the possibilities to renature intrinsic membrane proteins following exposure to SDS. The availability of such methods should represent a breakthrough in the purification of these proteins, which often can be well resolved by many fractionation methods in the presence of SDS (for water-soluble proteins there is no particular need for fractionation in SDS). However, these renaturation techniques can also be used in studies of the mechanism of folding of newly synthesized polypeptides and their assembly into biological membranes. This has been discussed by London and Khorana in their successful reactivation of SDS-denatured bacteriorhodopsin [30] (see also Ref. 31).

Acknowledgements

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