Biochimica et Biophysica Acta, 736 (1983) 130–136
Elsevier

BBA 71908

A STUDY ON THE RENATURATION OF MEMBRANE PROTEINS AFTER SOLUBILIZATION IN SDS OR FOLLOWING POLYACRYLAMIDE GEL ELECTROPHORESIS IN SDS, WITH SPECIAL REFERENCE TO A PHOSPHATASE FROM *ACHOLEPLASMA LAIDLAWII*

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(Received June 27th, 1983)

Key words: Denaturation; Renaturation; Membrane protein; Phosphatase; SDS solubilization; SDS electrophoresis; (A. laidlawii)

It may be easier to renature SDS-denatured hydrophobic proteins than to renature SDS-denatured water-soluble proteins. This paper presents some support for this hypothesis in the form of literature reports and an experiment of our own with an intrinsic membrane protein (a phosphatase from *Acholeplasma laidlawii*), that could be completely renatured, to judge from the restored activity, which was equal to (or higher than) that of the untreated enzyme. If this hypothesis is correct it might be possible to devise general methods to reverse the SDS denaturation of hydrophobic membrane proteins. This would be a breakthrough in the purification of at least some membrane proteins, because the high-resolving polyacrylamide gel electrophoresis in SDS could then be used to prepare membrane proteins in a native state. The method used for the renaturation of the SDS-denatured, entirely inactive, phosphatase comprised removal of SDS with the aid of conventional dialysis against a buffer containing the neutral, very efficient and non ultraviolet light-absorbing detergent G3707. For renaturation of the enzyme following an SDS-electrophoresis in polyacrylamide the gel was immersed in the same buffer for several hours; by staining for phosphatase the enzyme could easily be localized in the gel in the form of a yellow band, coinciding with a protein zone.

Introduction

Unexpectedly few intrinsic membrane proteins have been purified to homogeneity in spite of great efforts from many researchers in different disciplines. The reason for this low output is not that existing separation methods have too low a resolving power, but the difficulty to find agents (detergents) that not only solubilize the membrane but also split complexes (native or artificial) between different membrane proteins without denaturing them, since any attempt to isolate a protein will fail if the protein is not in free form but associated with other proteins. One should also consider that if a detergent has the ability to solubilize proteins in a carrier-free medium, it need not necessarily retain this ability in the presence of a chromatographic or electrophoretic bed; instead, aggregates may be formed [1]. SDS is probably the most powerful detergent in the sense that it in general splits very efficiently protein complexes and prevents the formation of aggregates of proteins. From this point of view SDS is the detergent of choice for purification of membrane proteins. However, a consequence of the strong solubilizing property is its denaturing power. Therefore, if fractionations are to be performed in the presence of SDS (which is highly desirable for the reasons mentioned), one must find methods to renature the membrane proteins. Fortunately, it seems to be easier to renature intrinsic membrane proteins than water-soluble proteins. This view is supported by literature data (see Discussion) and by the experiment described herein, which was undertaken to investigate

whether the activity of a hydrophobic membrane protein, a phosphatase from *Acholeplasma laidlawii*, could be completely recovered after solubilization in SDS. This would provide additional support for the hypothesis that it may be relatively easy to renature membrane proteins after SDS treatment. In addition we were interested in the purification of this particular enzyme by SDS electrophoresis since many other fractionation methods used have failed.

Materials and Methods

QAE-Sephadex A50 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden; *p*-nitrophenylphosphate from Sigma, Saint Louis, U.S.A.; sodium dodecyl sulfate (SDS) from Merck, Darmstadt, F.R.G.; Tris from Labkemi AB, Stockholm, Sweden; Tween 20 and G3707 (neutral detergents) from Atlas Chemie, Everberg, Belgium; the dialysis bag (Visking; diam. 3.5 cm) had an exclusion limit corresponding to proteins of molecular weights around 10000 and was bought from Kebo-Grave, Stockholm, Sweden.

The culturing of *Acholeplasma laidlawii* and the preparation of the plasma membranes were done essentially as described by Razin et al. [2]. A crude preparation of *p*-nitrophenylphosphatase was obtained as follows. About 2 ml of a suspension of the membranes (approx. 30 mg protein/ml) was mixed with 2 ml of 0.1 M Tris-HCl, pH 8.0, containing 5% Tween 20. After 2 h at room temperature the suspension was centrifuged for 30 min at $130000 \times g$. Most of the membrane proteins (but less than 5% of the phosphatase activity) were found in the supernatant. The pellet was extracted with stirring for 1 h at room temperature with 4 ml of water, containing 0.5% G3707, a neutral detergent the structure of which is given in Ref. 3. After centrifugation most of the phosphatase activity was found in the supernatant, which was used as sample in the studies presented in this paper.

The phosphatase activity was assayed as described by Ne'eman et al. [4]. As a measure of the activity we have used the absorption at 420 nm after incubation with *p*-nitrophenylphosphate for 30 min at 37°C. To determine the distribution of phosphatase activity among the protein bands following polyacrylamide gel electrophoresis we used a very accurate, previously published method [5].

Experiments and Results

Removal of SDS (in the absence of proteins) by dialysis

The removal of SDS should proceed sufficiently slowly that the protein molecules have time to return to their native conformation [6]. For that reason we have used a dialysis procedure. In preexperiments performed at room temperature we first determined the rate at which SDS passes the dialysis membrane both in the absence and presence of G3707. The procedures were as follows. 10 ml of 0.01 M Tris-HAc, pH 8.0, containing 0.01 M SDS, was transferred to a dialysis bag. The dialysis was carried out against 500 ml of the same buffer without SDS. One-ml aliquots were taken from the dialysis bag 0, 1, 3, 5, and 20 h after the start of the dialysis and analyzed for the content of SDS by addition of 37 mg of KCl. The degree of the opalescence formed is a measure of the concentration of the dodecyl sulfate, since potassium dodecyl sulfate is very poorly soluble in water [7]. The opalescence was estimated by 'absorption' measurements in a spectrophotometer at 450 nm. The relation between the concentration of SDS in the dialysis bag and the 'absorption' was obtained from a standard curve. In a parallel experiment the buffer outside the dialysis bag contained also 2 g of QAE-Sephadex for a more efficient removal of the dodecyl sulfate.

From the results shown in Fig. 1a, it is evident that most of the SDS dialyses out within 25 h, particularly when an anion exchanger was included in the outer solution. In the latter case the dodecyl sulfate concentration in the dialysis bag reached the limit for detection by the method used which is about $2 \cdot 10^{-4}$ M dodecyl sulfate (the sensitivity can, however, be increased by increasing the amount of KCl added).

Fig. 1b shows another preexperiment where G3707 was present not only in the sample in the dialysis bag but also in the solution outside the bag. The experimental conditions were similar to those described in the next section. The sample, which did not contain protein, consisted of 10 ml of 0.05 M Tris-HCl, pH 8.0, containing 0.5% (w/v)

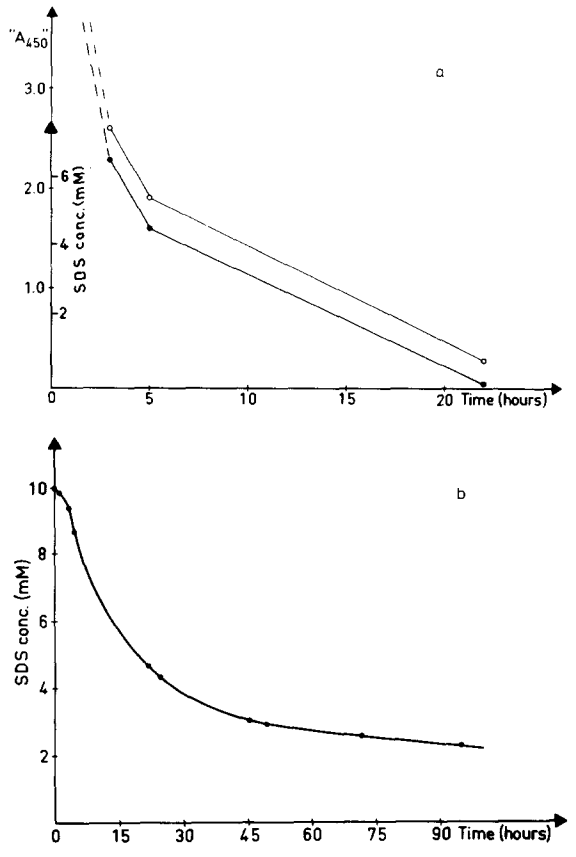

Fig. 1. (a) Removal of dodecyl sulfate (in the absence of proteins) by dialysis against a buffer with (●———●) and without (○———○) an anion exchanger. 'A_{450}' is a measure of the concentration of dodecyl sulfate in the dialysis bag. The SDS concentration was measured by the KCl method (see text). (b) Removal of dodecyl sulfate (in the absence of proteins) by dialysis against a G3707-containing buffer. The SDS concentration in the dialysis bag was measured by the basic fuchsin method [8].

G3707 and 0.01 M SDS. The G3707-containing dialysis buffer was replaced by a fresh one after 25 and 50 h. For the determination of SDS in the dialysis bag the above very simple KCl method did not work satisfactorily, probably because SDS now formed mixed micelles with G3707. Therefore we turned to the more sensitive, but also more laborious basic fuchsin method [8]. An aliquot of the retentate was diluted 100-fold with water in order for the concentration to fall below the critical micelle concentrations for the SDS-micelles, the G3707-micelles and the mixed micelles and to get most of SDS in free form. Under these conditions the SDS concentration determined by the fuchsin method very closely represented the total SDS concentration in the dialysis bag (when the retentate was dilute with 0.5% G3707 (w/v) instead of water the method gave too low SDS values probably due to the fact that SDS then formed mixed micelles with G3707).

For the SDS determinations we used 1 ml of the 100-fold diluted aliquots, which were treated according to the procedues described in Ref. 8 (the aliquots were withdrawn occasionally during a period of 90 h). The water phase was not aspirated off until the opalescence in the chloroform phase disappeared which took 30–60 min after the tubes had been brought from the 60°C water bath to another water bath of room temperature. The result is shown in Fig. 1b.

Renaturation of an SDS-denatured membrane protein, a phosphatase from Acholeplasma laidlawii, following removal of SDS by dialysis

About 1.8 ml of the G3707 extract was mixed with 0.2 ml of 0.05 M Tris-HCl pH 8.0, containing 0.5% (w/v) G3707; 100 μl was used for measurement of the phosphatase activity, which corresponded to $A_{420} = 3.8$. Another 1.8-ml portion of the G3707 extract was then mixed with 0.2 ml of 0.1 M SDS. This mixture, which had no phosphatase activity, was dialyzed for 16 h at room

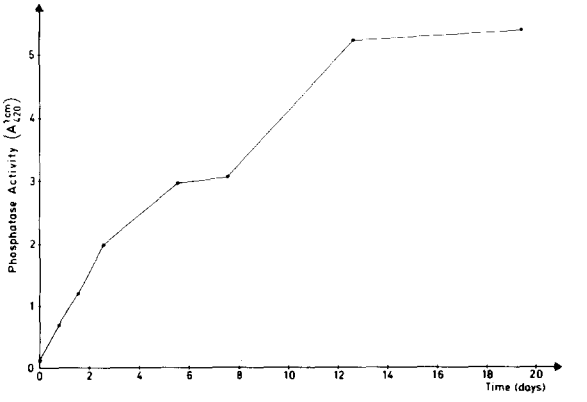

Fig. 2. Renaturation of the phosphate activity by removal of SDS with the aid of dialysis against a buffer containing the neutral detergent G3707. As shown, after dialysis for about twelve days there was no further increase in activity (the restored activity was higher than that of the non-SDS treated enzyme).

temperature against 500 ml of 0.05 M Tris-HCl, pH 8.0, containing 0.5 % (w/v) G3707. The dialysis was then continued in the cold (+4°C) at the same time as the G3707-containing dialysis buffer was replaced by a fresh one (the temperature at the start of the dialysis cannot be as low as +4°C as SDS then will precipitate (9)). Aliquots (100 μl) were removed periodically from the dialysis bag during a period of about 20 days, and were assayed for *p*-nitrophenylphosphatase activity. The result is shown in Fig. 2, where the activity is seen to become maximal after about 12 days.

Renaturation and localization of the phosphatase following a polyacrylamide gel electrophoresis in SDS

The electrophoresis chamber consisted of a quartz tube of the dimensions 4 mm (i.d.) × 100 mm. The run was performed in a polyacrylamide gel of the composition $T = 4\%$; $C = 5\%$ (for definition of these parameters, see Ref. 10). As buffer we used 0.05 M Tris-HCl, pH 8.0, containing 0.01 M SDS, and as sample 75 μl of the crude G3707 extract. The duration of the run was 90 min at a voltage of 80 volts (4 mA). The gel was taken out of the glass tube and immersed in 0.05 M Tris-HCl, pH 8.0, containing 0.5% (w/v) G3707, for removal of SDS and an attempt at renaturation. After 16 h with stirring the gel rod was tested for phosphatase activity by a method which correlated the activity pattern with the ultraviolet light pattern. This method [5] has the advantage of showing whether an activity peak coincides with an ultraviolet light peak. This information is important in order to get an idea of the homogeneity of the enzyme. The pattern obtained (Fig. 3) shows that (1) the enzyme could be renatured after electrophoresis in SDS and that (2) the activity peak corresponded to one of the protein peaks. When the experiment was repeated but with the difference that the gel rod was immersed in 0.05 M Tris-HCl, pH 8.8 (without G3707) no activity was observed.

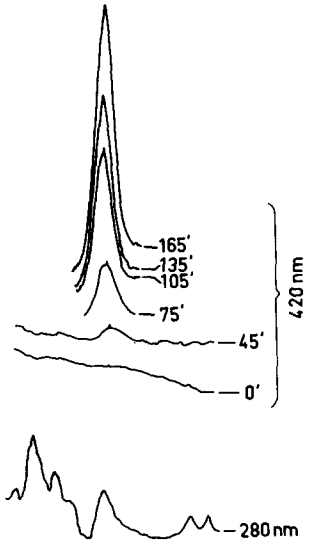

Fig. 3. Renaturation and localization of the phosphatase following a polyacrylamide gel electrophoresis in SDS. After an SDS electrophoresis, the polyacrylamide gel was immersed in a buffer containing the neutral detergent G3707, for removal of SDS and renaturation of the enzyme. The enzyme was then localized by a method described previously [5]. As shown, the enzyme activity could be restored and the activity peak corresponded to one of the protein peaks.

Discussion

Intrinsic membrane proteins (in contrast to water-soluble proteins), are in their native state partly surrounded by lipids. Since lipids resemble detergents in the sense that they consist of a hydrophilic part and a hydrophobic part, one might expect that the conformation of membrane proteins would be less affected by detergents than are water-soluble proteins. Or in other words: hydrophobic (membrane) proteins should be less susceptible to denaturation by detergents (or organic solvents) than are ordinary water-soluble proteins (and the risk of denaturation should be smaller the larger the hydrophobic (and the smaller the hydrophilic) segment(s) of the protein). I will now refer to some experiments (taken from literature) which support this hypothesis.

1. A C_{55}-isoprenoid alcohol phosphokinase from the membrane of *Staphylococcus aureus* (one of the most hydrophobic proteins known), is active also in the presence of SDS and is not denatured by organic solvents [11]. To the best of my knowledge no experiments have been reported in which an ordinary water-soluble protein was active in SDS.

2. Neuraminidase of influenza B virus (LEE strain) was not inactivated upon disruption of the virus particles by SDS and subsequent electrophoresis in SDS [12]. The activity was also retained after an ether treatment of the virus [13,14].

3. For the hydrophobic protein cytochrome b_5 Robinson and Tanford [15] state: "It is probable than even the binding of SDS to the hydrophobic domain of cytochrome b_5 is a process of this kind, occuring without denaturation of the protein, instead of the cooperative binding with denaturation commonly observed for water-soluble proteins."

4. Helenius and Simons [16] have in a review article on solubilization of membranes drawn attention to the finding that phospholipase A1 of *Escherichia coli* membranes [17] and another phosphatase than that described herein; namely, an alkaline phosphatase from liver cell plasma membranes [18], are resistant to the denaturing action of SDS.

5. Molecular weights of membrane proteins estimated by SDS electrophoresis are often erroneous when water-soluble proteins are used as standards [19,20,36]. This can be explained by assuming that water-soluble proteins are more completely denatured than are membrane proteins in an SDS milieu.

6. Albumin (which resembles membrane proteins in the sense that it has hydrophobic surface patches capable of binding amphiphilic compounds, for instance fatty acids) was not detectably denatured after solubilization in aqueous alcohol solutions containing trichloroacetic acid [21].

7. Rhodopsin was not denatured after solubilization in ether or hexane [22].

Electrophoresis in polyacrylamide gels in the presence of SDS might be the most high-resolving existing method for the analysis of membrane proteins. It could therefore also be a very powerful method for the preparation of pure membrane proteins in a native state if the proteins could be renatured following a run. The experiments described herein and the literature data discussed should be seen against this background and in view of the fact that we have developed a series of methods for the recovery of proteins from gel slices following SDS electrophoresis [23–26].

As a model protein in the renaturation experiment described herein I chose an enzyme, since its activity can be taken as a measure of the degree of denaturation (and renaturation). The enzyme selected was a *p*-nitrophenylphosphatase from *Acholeplasma laidlawii*, because this enzyme seems to be very hydrophobic to judge from its poor solubility in the detergent Tween 20 (which solubilizes almost all other membrane proteins from this organism). In addition, this enzyme has a low molecular weight (around 25000) to judge from an SDS electrophoresis, and therefore probably does not consist of subunits, which should facilitate the attempts at renaturation following an SDS-treatment.

In this paper I have described the use of simple conventional dialysis for removal of SDS. Other methods can certainly be used as well (for instance, that of Clarke, described in Ref. 27), provided that SDS can be replaced by a non-denaturing detergent that can keep the protein in solution, for instance, Triton X-100, G3707, octyl glycoside, or deoxycholate. Triton X-100 has the unfortunate drawback of a strong ultraviolet light absorption, which often is disturbing in preparative fractionation studies and in analytical runs of the type shown in Fig. 3. The non-denaturing detergent need not have so high a solubilizing power as SDS, since after fractionation in SDS the protein is pure or contains probably only few contaminants, which means that the risk that the protein of interest may form complexes with other proteins no longer exists or is at least greatly reduced. If the protein is homogeneous following the purification in SDS it may even be kept in solution without transferring it into a non-denaturing detergent solution. The reason for this is probably that the non-polar patches of a membrane protein associate hydrophobically to form well defined complexes (in the absence of other proteins), for instace tetramers or pentamers. There are several examples of membrane proteins that are soluble in the absence of detergents [16,28–30].

A comparison between Fig. 1a and Fig. 1b shows that the removal of SDS from the dialysis bag is a slower process when the dialysate contains G3707. The reason is that SDS forms mixed micelles with G3707 and that these micelles are too large to pass the dialysis membrane (G3707 must be present on both sides of the dialysis membrane in the renaturation experiments to keep the protein in solution).

Fig. 2 shows that complete renaturation of the phosphatase activity by simple dialysis proceeds very slowly, which may be a prerequisite for efficient refolding [6]. Maximum activity is not ob-

tained until after about twelve days. It is also interesting to note that the restored activity (A_{420} = 5.2) after this period of time is even higher than that of the starting solution (A_{420} = 3.8). The reason for this has not been investigated. It indicates, however, that the renaturation method is efficient for the enzyme studied. This and other techniques can probably be used with advantage also for other membrane proteins. As mentioned above it may possibly be an advantage to remove SDS slowly when the aim of an experiment is complete renaturation. However, a high phosphatase activity was restored following polyacrylamide gel electrophoresis in SDS when the gel was immersed in a G3707 solution for only one hour (the activity was detected by the method outlined in Fig. 3).

The primary reason for the inclusion of the neutral detergent G3707 in the dialysis or extraction buffers is that we wanted to prevent the enzyme from precipitating in the dialysis bag (or in the polyacrylamide gel) when the SDS concentration decreased. For instance, if the proten forms a precipitate in the gel we cannot utilize the recently introduced method to recover it from a gel slice (see Ref. 26, Fig. 1, where such a recovery of the phosphatase is shown). One should observe that Fig. 1b refers to the total concentration of SDS in the dialysis bag, i.e. to the concentration of SDS in the form of free SDS molecules, SDS micelles and mixed SDS-G3707 micelles. From this figure one cannot deduce the amount of SDS bound to protein although it is obvious that the concentration of this bound SDS is much lower than the total SDS concentration. This amount involves both SDS directly bound to the protein and indirectly via G3707 (for instance in the form of mixed micelles). The indirectly bound SDS is not denaturing while the directly bound is (at least when present in relatively large amounts) and therefore of some interest from the view point of renaturation. The amount of the directly bound SDS is, however, extremely difficult to determine and therefore also the rate at which it is released from the protein. It is not unlikely that this rate is higher in the presence of G3707 than in its absence (at least in the beginning of the dialysis) since one can expect bound SDS to be efficiently displaced by the harmless G3707 [16], in the same way as SDS is displaced by Triton X-100 from the minor glycoprotein of erythrocytes [31]. Due to this displacement effect the amount of SDS directly bound to protein may be very low after the renaturation.

Fig. 3 does not indicate any heterogeneity of the enzyme, since the activity peak follows a protein peak. However, more homogeneity tests are required to establish the degree of purity of the enzyme.

I pointed out in the Introduction that it is of fundamental importance to find methods for renaturation of membrane proteins following SDS treatment. However, there is also an interest in renaturation of SDS-denatured water -soluble proteins, which is reflected in several papers on this topic [6,27,32–34]. However, it might be difficult to apply these methods (introduced for water-soluble proteins) to renaturation of hydrophobic membrane proteins, because the experimental conditions often are such that one can expect membrane proteins to form aggregates or precipitates upon the removal of SDS. Examples of such conditions are: the use of supporting media (ion exchangers), which we have recently shown may cause aggregation [1,35]; steps involving precipitation of proteins with organic solvents; the absence of any other detergent to replace the SDS (with one exception [27]), because no detergent is necessary to keep a water-soluble protein in solution.

Acknowledgements

I want to express my sincere thanks to Dr. Yao Kunquan for fruitful collaboration on the development of the method for the extraction of the phosphatase and to Mrs. Karin Elenbring for skilful technical assistance. The determination of SDS by the basic fuchsin method was performed by Mr. Staffan Uhlén. The detergent G3707 was a gift from Dr. D. De Coster, Atlas Chemie. The work has been financially supported by the Swedish Natural Science Research Council and the Von Kantzow Foundation.

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