SDS-resistant aggregation of membrane proteins: application to the purification of the vesicular monoamine transporter

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The vesicular monoamine transporter, which catalyses a H^+/monoamine antiport in monoaminergic vesicle membrane, is a very hydrophobic intrinsic membrane protein. After solubilization, this protein was found to have a high tendency to aggregate, as shown by SDS/PAGE, especially when samples were boiled in the classical Laemmli buffer before electrophoresis. This behaviour was analysed in some detail. The aggregation was promoted by high temperatures, organic solvents and acidic pH, suggesting that it resulted from the unfolding of structure remaining in SDS. The aggregates were very stable and could be dissociated only by suspension in anhydrous trifluoroacetic acid. This SDS-resistant aggregation behaviour was shared by very few intrinsic proteins of the chromaffin granule membrane. Consequently, a purification procedure was based on this property. A detergent extract of chromaffin granule membranes enriched in monoamine transporter was heated and the aggregates were isolated by size-exclusion HPLC in SDS. The aggregates, containing the transporter, were dissociated in the presence of trifluoroacetic acid and analysed on the same HPLC column. This strategy might be of general interest for the purification of membrane proteins that exhibit SDS-resistant aggregation.

INTRODUCTION

Protein sequencing provides key data for the identification of unknown proteins. The purity of the material to be sequenced is more important than its activity and proteins that can be identified by covalent labelling or immunodetection may be purified in a denatured state. Two-dimensional PAGE is commonly used for this purpose. However, this technique is less satisfactory with membrane proteins, which often appear as very broad spots. In this case, a multistep purification procedure needs to be defined. Such a difficulty was encountered with the vesicular monoamine transporter from bovine chromaffin granule. The vesicular monoamine transporter is a membrane protein that mediates the transport of monoamines into their secretory organelles. It catalyses a H^+/monoamine antiport, driven by the H^+ electrochemical gradient generated by a vacuolar ATP-dependent H^+-pump [1]. Two cDNAs expressing a H^+/monoamine antiport activity, now designated $VMAT_1$ and $VMAT_2$, have been cloned recently [2,3]. The transporter from the bovine chromaffin granule, on which most biochemical studies have focused [4–6], has been photolabelled using 7-azido-8-[^{125}I]iodoketanserin [^{125}I](AZIK) [7]. Analysis of the photolabelled material by two-dimensional PAGE revealed that it is a minor component of the chromaffin granule membrane, highly glycosylated and migrating as a large diffuse spot. A procedure for purification of the photolabelled transporter was devised [6]. During the course of the purification, we observed spontaneous aggregation of the photolabelled transporter, demonstrated by accumulation of the labelled material at the top of the gel after SDS/PAGE. Because this phenomenon hampered the purification, it was analysed systematically. We show that it is possible to take advantage of this property to purify the vesicular monoamine transporter, because it is shared by very few proteins from the chromaffin granule membrane. Such a strategy might be of general interest for the purification of integral membrane proteins that share the same behaviour.

EXPERIMENTAL

Chemicals

7-Amino-8-[^{125}I]iodoketanserin (2000 Ci/mmol) was from Amersham International (Amersham, Bucks., U.K.). Anhydrous trifluoroacetic acid (TFA) (sequencing grade) was from Pierce (Rockford, IL, U.S.A.). Sulfobetaine 3–12 was from Fluka (Buchs, Switzerland).

Synthesis of [^{125}I]AZIK

[^{125}I]AZIK was synthesized from 7-amino-8-[^{125}I]iodoketanserin as previously described [6,8].

Preparation of chromaffin granule membranes

Bovine chromaffin granule membranes were prepared by osmotic lysis of granules purified by centrifugation through a 1.6 M sucrose layer [9,10]. EDTA (1 mM), leupeptin (5 μg/ml), aprotinin (5 μg/ml), pepstatin (10 μg/ml) and PMSF (1 mM) were included in the lysis buffer. The membrane pellet was resuspended at a protein concentration of 3–5 mg/ml in 10 mM Tris/HCl buffer, pH 7.5, containing the same inhibitors, frozen in liquid nitrogen, and stored at −80 °C.

Photolabelling with [^{125}I]AZIK

Membranes were photolabelled as previously described [6,7]. After SDS/PAGE and autoradiography, the major photolabelled

Abbreviations used: [^{125}I]AZIK, 7-azido-8-[^{125}I]iodoketanserin {7-azido-8-iodo-3-[2(4-fluorobenzoyl-1-piperidinyl)ethyl]-2,4(1*H*,3*H*)-quinazolinedione]; DBH, dopamine β-hydroxylase; sulfobetaine 3-12, 3-(dimethyldodecylammonio)propanesulphonate; TFA, trifluoroacetic acid.

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polypeptide observed was a diffuse 70 kDa band corresponding to the vesicular monoamine transporter. In some experiments (Figure 1), a sharp 40 kDa band was also observed, which is interpreted as being non-specific labelling since it was not prevented by the inhibitor tetrabenazine.

PAGE

Samples subjected to SDS/PAGE were solubilised in Laemmli sample buffer, containing 63 mM Tris/HCl, pH 6.8, 10 % (v/v) glycerol, 2 % (w/v) SDS, 5 % (v/v) 2-mercaptoethanol and 30 μM Bromophenol Blue [11]. Unless stated otherwise, 10 % acrylamide gels, with a bisacrylamide/acrylamide ratio of 0.8:30, were used. Molecular-mass standards were phosphorylase *b*, BSA, ovalbumin, carbonic anhydrase, soyabean trypsin inhibitor and lysozyme. Proteins were stained with silver nitrate [12], using sodium thiosulphate to sensitize the gels [13]. Autoradiograms were obtained by exposure of the dried gels to Kodak X-OMAT AR films with Lumix MR 800 screens (Agfa Gevaert) for 2–30 days at −80 °C. When quantitative data were required, the unstained gel was cut into 5 mm slices and the radioactivity in the slices was measured with a γ-counter.

Aggregation and disaggregation of the monoamine transporter

To aggregate the transporter in whole chromaffin granule membranes, the membranes were solubilized at 1 mg of protein/ml in the Laemmli sample buffer or in 100 mM Tris/HCl, pH 6.8, containing 2 % (w/v) SDS and 5 % (v/v) 2-mercaptoethanol, unless stated otherwise, and heated for 5 min at 100 °C in a closed vial.

The aggregates were dissociated using the TFA treatment described by Hennessey and Scarborough [14]. Samples (10 μg of protein in 10 μl) were freeze-dried in 1.5 ml polypropylene Microfuge tubes with a Speed Vac concentrator (Savant; Farmingdale, NY, U.S.A.) and resuspended in 100 μl of anhydrous TFA under a chemical hood. The TFA was then evaporated under a stream of argon. In order to achieve maximal removal of TFA, the tube was inclined and rotated manually during this operation. The dried film on the walls of the tube was then resuspended in 40 μl of Laemmli sample buffer. When samples became slowly acidic because of residual TFA, as indicated by Bromophenol Blue, 0.5 μl aliquots of 2 M Tris/HCl, pH 8.8, were gradually added until the samples turned blue again.

Monoamine transporter purification

All steps were carried out at room temperature. Chromaffin granule membranes (40 mg of protein), to which photolabelled membranes (4 mg of protein) were added, were solubilized in sulfobetaine 3–12 and Nonidet P40 in a sodium acetate buffer, pH 4.5, and fractionated by ion-exchange chromatography on a DEAE-MemSep 1000 cartridge (Millipore, Bedford, MA, U.S.A.) as described in detail previously [6]. The eluate is enriched 10-fold in monoamine transporter. Detergents were removed from the neutralized eluate (2.7 ml) by adsorption on a 2 ml Extracti-Gel column (Pierce), previously equilibrated in water, at a flow rate of 0.4 ml/min. The labelled fractions (4 ml) were concentrated 20-fold by ultrafiltration on a Centricon 30 device (Amicon, Beverly, MA, U.S.A.). The buffer in the concentrate was subsequently changed by 10-fold dilution with 100 mM Tris/HCl, pH 6.8, and a second centrifugation on the Centricon 30. More than 90 % of the radioactivity was recovered in the concentrate (about 200 μl). The concentration of Tris/HCl, pH 6.8, in the sample was raised to 150 mM, and SDS and 2-mercaptoethanol were added at final concentrations of 2 and 5 % respectively. The sample (300 μl) was subsequently heated for 10 min at 100 °C in a closed vial. The heat-induced aggregates were purified by size-exclusion HPLC. The heated sample was filtered on a Durapore 0.45 μm membrane (Millipore) and injected on to a 7.8 mm × 300 mm Protein-Pak 300 SW 10 μm column (Waters), using prefiltered 0.1 % SDS/200 mM sodium phosphate, pH 6.9, as eluent with a flow rate of 0.2 ml/min [15]. The fractions collected (0.2 ml) were analysed by γ-counting and SDS/PAGE. The photolabelled aggregates were eluted in the void volume (5.5 ml). Salt concentrations in the selected fractions were lowered about 100-fold by three cycles of dilution with 0.45 μm filtered water and centrifugation on a Centricon 30. The final concentrate (about 200 μl) was freeze-dried and treated with anhydrous TFA as described above. After evaporation of the TFA, the sample was resuspended in 250 μl of prefiltered 150 mM sodium phosphate, containing 2 % SDS and 5 % 2-mercaptoethanol, and fractionated by size-exclusion HPLC under the same conditions. The TFA-treated sample was eluted as two radioactive peaks, at 5.5 and 6.3 ml, which corresponded to residual aggregates and to the purified disaggregated transporter respectively.

The two peaks were quantified by non-linear regression, using the sum of two terms in the form:

$$y = \frac{a}{\sqrt{(\pi c x)}} e^{-\frac{b-x^2}{cx}}.$$

This equation (where x and y are the volume of solvent that has flowed through the column and the concentration of the solute eluted respectively) describes a single peak on the chromatogram [16]. The parameters a, b and c are the area (amount of solute), the abscissa (elution volume) and the width of the peak respectively. The regression yields two area parameters a_{agr} and a_{mon}, for the aggregate and monomer peaks. The disaggregation yield was calculated as the ratio $a_{mon}/(a_{agr}+a_{mon})$.

Protein and [^{125}I]AZIK-labelled transporter assay

Protein was assayed by the method of Schaffner and Weissmann [17], with BSA as standard. The final concentration of SDS in the assay was increased to 1 %, and protein precipitated with trichloroacetic acid was filtered on a 0.45 μm nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The radioactivity of the labelled transporter was determined with a γ-counter, either directly (for purified samples) or after electrophoresis, slicing of the gel and summation of the radioactivity associated with the 70 kDa peak (for less pure samples).

RESULTS

Selective aggregation of the monoamine transporter

When chromaffin granule membranes, solubilized for SDS/PAGE analysis, were boiled for 5 min as recommended [11], the photolabelled transporter was completely aggregated (Figure 1B). The labelled material was found at the bottom of the sample well and mainly at the interface between the stacking and resolving gels, with no radioactivity at the monomer position (70 kDa). Interestingly enough, of the approx. 40 proteins revealed by silver staining, very few were affected by the boiling treatment (Figure 1A). A 27 kDa major band and a 14 kDa polypeptide disappeared from the boiled sample with a concomitant accumulation of silver-stained material at the top of the gel (Figures 1A and 3). The major 27 kDa band, which migrates often as a doublet on storage of the SDS solubilisate (compare

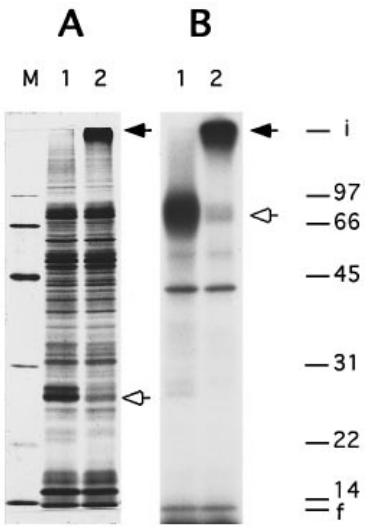

Figure 1 Selective aggregation of the vesicular monoamine transporter

[^{125}]AZIK-labelled chromaffin granule membranes, solubilized in Laemmli sample buffer, were analysed by SDS/PAGE before (lanes 1) or after (lanes 2) heating. (**A**) Silver-stained gel; (**B**) autoradiogram. The aggregates accumulate at the stacking/running gel interface (closed arrows) with concomitant disappearance of monomers of cytochrome *b*-561 and vesicular monoamine transporter (open arrows in **A** and **B** respectively). The position and molecular mass (in kDa) of protein standards are indicated on the right. Abbreviations: i, stacking/running gel interface; f, front.

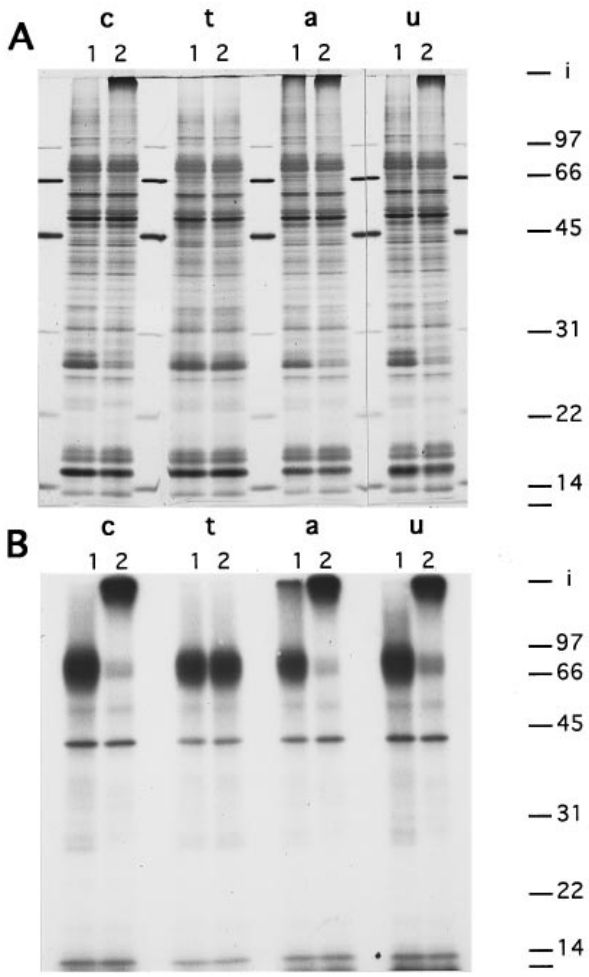

Figure 2 Reversal of the aggregation by treatment with anhydrous TFA

Photolabelled membranes, solubilized in 2% SDS/5% 2-mercaptoethanol/100 mM Tris/HCl, pH 6.8, were boiled (lanes 2) or not (lanes 1) and subjected to the following treatments before SDS/PAGE on a 12% gel: (c) dilution in Laemmli sample buffer; (t) and (a), samples were freeze-dried, resuspended in 100 μl of anhydrous TFA or acetonitrile respectively and, after evaporation of the solvent under a stream of argon, resuspended in sample buffer; (u) dilution in sample buffer containing 7 M urea. (**A**) Silver-stained gel; (**B**) autoradiogram.

Figures 1A and 2A), is likely to be cytochrome *b*-561 [18], a polytopic membrane protein [19]. The 14 kDa polypeptide was tentatively identified as the H^+-ATPase proteolipid [20]. A careful examination of the silver-stained profiles reveals also a 34 kDa band lost after boiling, as well as two faint bands of 23 and 31 kDa. The monoamine transporter, which represents only 0.2–0.4% of the membrane protein [21], could not be identified on the silver-stained profile.

These observations show that the aggregative behaviour of the vesicular monoamine transporter is shared by few proteins. Because of the remarkable stability of the aggregates (see below), this 'selective' aggregation will be referred to as SDS-resistant. The heat-induced aggregation was also observed in other detergents, such as Nonidet P40 and sulfobetaine 3–12 (results not shown), but since all the analyses were performed by SDS/PAGE, our study was restricted to SDS.

The control of aggregation

When the boiling treatment was applied to chromaffin granule membranes solubilized in a simple SDS/Hepes buffer instead of the classical Laemmli sample buffer, no aggregation occurred (results not shown). This observation led us to investigate the effect of the components of the sample buffer, the composition of which is indicated in the Experimental section. It should be noted that, in these experiments, only the composition of the boiling medium was varied. After the boiling step, all samples were supplemented with concentrated Laemmli sample buffer and run under the same conditions.

When 2-mercaptoethanol was added to the SDS/Hepes buffer, thermal aggregation of the vesicular transporter was restored (results not shown). The 2-mercaptoethanol effect suggested the involvement of thiols in the aggregation process. However, thermal aggregation was not observed after addition of dithiothreitol in the SDS/Hepes buffer. An alternative explanation is that 2-mercaptoethanol, which is used at a higher concentration (5%, v/v) than dithiothreitol, was acting as an organic solvent. To test this, alcohols were substituted for 2-mercaptoethanol at the same volume ratio. Methanol, ethanol, propan-2-ol and butan-2-ol mimicked the effect of 2-mercaptoethanol on the aggregation of the photolabelled transporter (results not shown). In all cases, aggregation was complete since the monomer band was absent and the profile of the high-molecular-mass smear indicated that the size of the aggregates increased with the length of the alcohol. In the case of butanol, the monoamine transporter aggregated completely without heating. Observation of the silver-stained gel indicated that 2-mercaptoethanol and alcohols had similar effects on cytochrome *b*-561.

The SDS-resistant thermic aggregation was also influenced by pH. Decreasing the pH by the use of sodium phosphate buffers in the boiling medium increased the size of the aggregates (results not shown). This observation might explain why the transporter was more prone to heat-induced aggregation in Tris than in

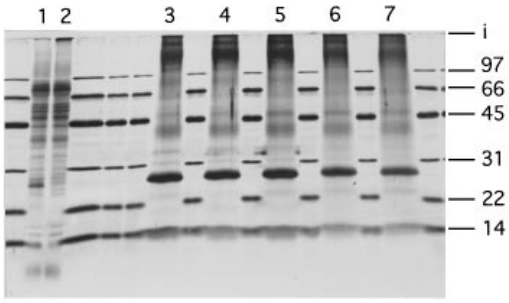

Figure 3 Composition of the aggregates isolated from whole chromaffin granule membranes

Solubilized membranes were boiled for 5 min and fractionated by SDS/PAGE on a 2 mm-thick, 16-cm-wide 10% slab gel. The top 5 mm of the resolving gel was cut away to recover the aggregates by electroelution. Aliquots of the electroeluate were freeze-dried, incubated with anhydrous TFA for increasing periods of time in vials sealed with Teflon caps and analysed by SDS/PAGE on a 13% gel. Lane 1, untreated membranes (4 μg of protein); lane 2, boiled membranes (4 μg of protein); lanes 3–7, aggregates incubated for 0.25, 1, 3, 6 and 20 h in TFA (each lane was derived from 400 μg of starting protein).

Hepes buffer, since the two buffers have quite different temperature-dependences [22].

From these observations, a maximal SDS-resistant aggregation appears to require several cumulative conditions, which all individually have a denaturing effect: high temperature, organic solvents and acidic pH. It should be noted that purification steps in which the protein is submitted to one or more of these conditions result in a more or less rapid aggregation.

How to disperse the aggregates

The heat-induced aggregates are very stable. After the boiling step, prolonged incubation with SDS or treatment with 7 M urea did not reverse significantly the aggregation (Figure 2, lanes *u*). To test the effect of organic solvents, the boiled aqueous medium was freeze-dried, resuspended in the solvent and analysed by SDS/PAGE after chasing the solvent with a stream of argon (Figure 2, lanes *t* and *a*). Most of the solvents tested did not dissociate the aggregates, but rather promoted the aggregation of the transporter in the unboiled sample. This effect was particularly pronounced with 60% formic acid, a classical solubilizer in peptide chemistry, which fully aggregated the transporter and cytochrome *b*-561 of the unboiled solution (results not shown). Hennessey and Scarborough [14] described anhydrous TFA as a powerful agent for disaggregating peptides purified by reverse-phase HPLC, for SDS/PAGE. When this treatment was applied to the boiled membranes, the aggregation of the labelled monoamine transporter and cytochrome *b*-561 was completely reversed (Figure 2, lanes *t*). The TFA treatment was also able to dissociate the aggregates formed by a previous treatment with formic acid (not shown).

The experiment was repeated on aggregates isolated by electroelution from the top of an SDS/polyacrylamide gel (Figure 3). Cytochrome *b*-561 and the 14 kDa polypeptide were clearly seen among the limited number of proteins present in the disaggregated material. Their high relative enrichment indicated that an aggregation/disaggregation cycle might be an efficient purification step. Silver staining also revealed in the purified preparation a diffuse 40 kDa unidentified component, as well as minor bands of 23, 31 and 34 kDa. Above 50 kDa, a high background prevented the identification of the monoamine transporter. TFA is known to promote breakage of polypeptide

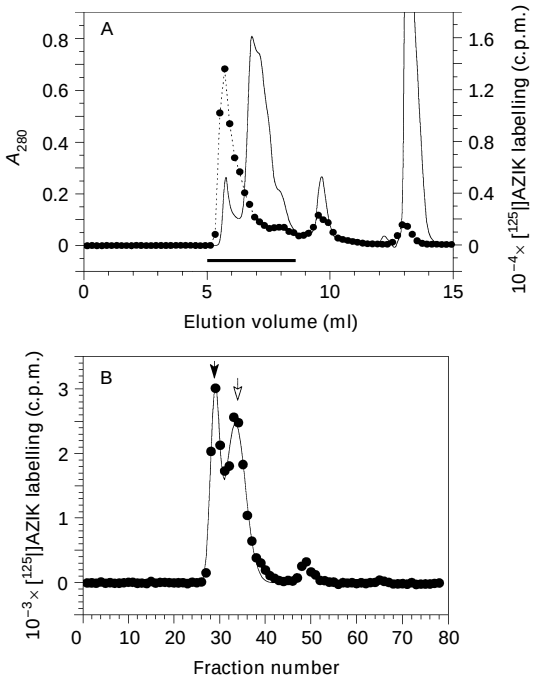

Figure 4 Size-exclusion HPLC

(**A**) Purification of the photolabelled aggregates. An extract enriched in photolabelled transporter by DEAE-cellulose chromatography was heated in 2% SDS/5% 2-mercaptoethanol/100 mM Tris/HCl, pH 6.8, and fractionated by HPLC on a Protein-Pak 300 SW column. ——, A_{280} ●, ^{125}I radioactivity. All polypeptides were eluted in the underlined region of the chromatogram, as shown by the injection of protein-free controls. The peaks of 9.8 and 13.4 ml correspond to micelles and free solutes respectively (**B**) Analysis of the TFA-treated aggregates. The purified photolabelled aggregates were treated with TFA and subjected to a second HPLC on the same column. The two major peaks correspond to aggregates (closed arrow) and transporter monomer (open arrow), as shown by SDS/PAGE analysis of the fractions. The peaks were quantified by non-linear regression (——), as described in the Experimental section. In this experiment, 66% of the aggregates were dissociated.

chains, especially at Asp-Pro linkages [23]. However, when anhydrous conditions were carefully observed, the apparent molecular mass of cytochrome *b*-561 and the 14 and 40 kDa polypeptides did not change after incubation for up to 20 h in TFA (Figure 3).

Purification of the vesicular monoamine transporter by an aggregation/disaggregation strategy

The aggregation/disaggregation procedure was applied to a fraction enriched in vesicular monoamine transporter. Chromaffin granule membranes photolabelled with AZIK were solubilized with a mixture of neutral and zwitterionic detergents, and fractionated on a DEAE-cellulose matrix as previously described [6]. The detergent in the eluate was removed by adsorption on an Extracti-Gel column, and the buffer was exchanged for Tris/HCl, pH 7.5. After addition of SDS and 2-mercaptoethanol, the concentrate was heated for 5 min at 100 °C in a closed vial and fractionated by size-exclusion HPLC. As shown in Figures 4(A) and 5, the photolabelled aggregates were eluted in the void volume.

The purified aggregates were desalted by ultrafiltration, freeze-dried and solubilized in anhydrous TFA for 5 min. The TFA was evaporated under a stream of argon and the sample was resuspended in an SDS/phosphate buffer. This desalting step

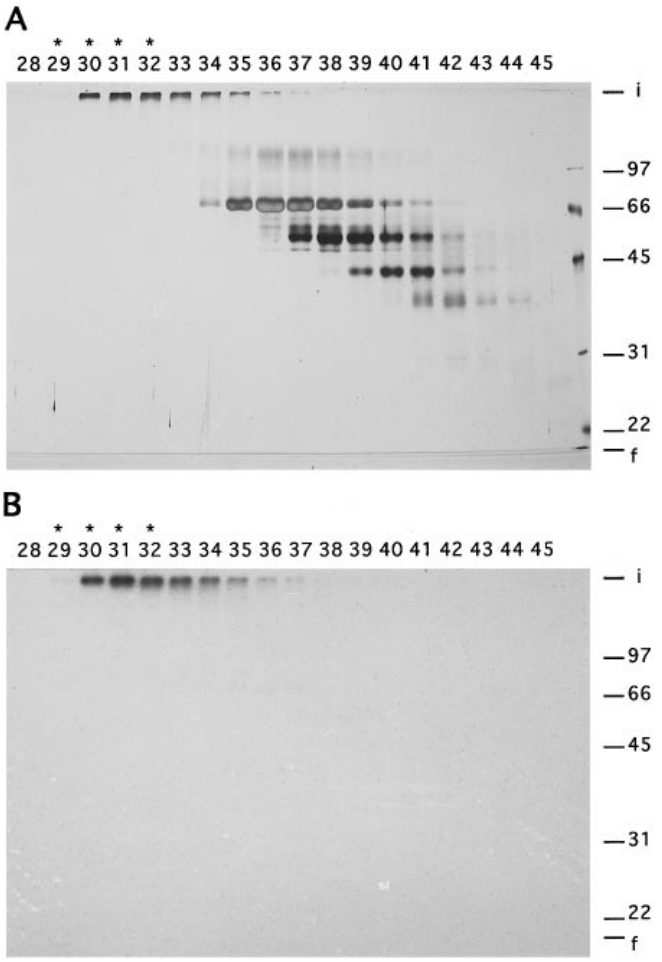

Figure 5 SDS/PAGE analysis of the aggregated material fractionated by size-exclusion HPLC

Fractions 28–45, corresponding to the underlined region of the chromatogram in Figure 4(A), were analysed. (**A**) Silver-stained gel; (**B**) autoradiogram. Asterisks show the fractions pooled for further purification.

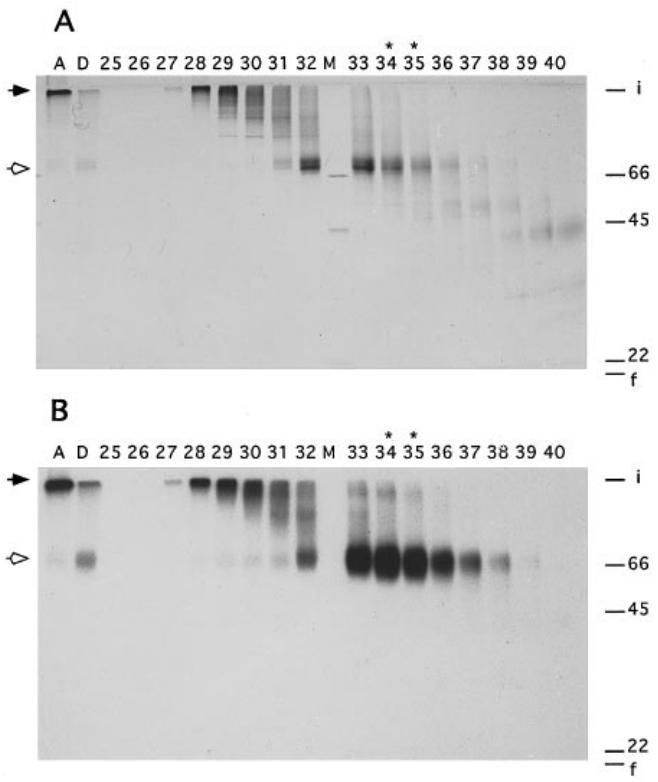

Figure 6 SDS/PAGE analysis of the aggregates after TFA treatment and size-exclusion HPLC

(**A**) Silver-stained gel; (**B**) autoradiogram. Fractions were analysed, and compared with unfractionated aggregates, before (lane A) or after (lane D) the TFA treatment. Molecular-mass standards were inserted between fractions 33 and 34 to show the lack of coincidence between protein and radioactivity, which is due to the presence of dopamine β-hydroxylase (DBH) in fractions 32 and 33. The pooled fractions are labelled with an asterisk.

was introduced because an excess of salts in the sample prevented complete evaporation of TFA, resulting in a strong acidification of the sample resuspended in the aqueous buffer. The TFA-treated material was subjected to a second size-exclusion HPLC, under the same conditions. The radioactivity was eluted in two major peaks (Figure 4B), which corresponded to residual aggregates and monoamine transporter monomers, as shown by SDS/PAGE of the fractions (Figure 6). The two minor peaks with longer elution times (Figure 4B) represented less than 10% of the recovered radioactivity, and they were not associated with proteins or peptides. The disaggregation yield, calculated from the areas of the major peaks as indicated in the Experimental section, had a mean value of 70 ± 12% (mean $\pm$ S.D.; 14 experiments), with variations between 47 and 93%. Such variations contrast with the experiments on solubilized chromaffin granule membranes, where full disaggregation was reproducibly observed (Figure 2). Despite many attempts, the disaggregation yield of purified aggregates could not be reproducibly improved.

In Figure 6(A), the comparison of the position of the radioactive and silver-stained materials indicates that the labelled protein has not been homogeneously purified over the whole radioactivity peak, since protein and radioactivity are not exactly coincident. Careful observation of the stained proteins shows that the transporter, which appears as a homogeneous broad band, was eluted from the size-exclusion column just after a 70 kDa doublet, which was identified by immunoblotting as

Table 1 Purification of the [^{125}I]AZIK-labelled transporter by aggregation

Protein was assayed as described by Schaffner & Weissmann [17]. The amount of photolabelled transporter present in the starting membranes and in the DEAE-cellulose eluate was determined by SDS/PAGE, slicing of the gel and γ-counting of the 70 kDa peak. The amount of transporter present in the HPLC eluates was determined by γ-counting of the selected fractions

Purification step	Protein (mg)	$10^{-3} \times$ Labelled transporter (c.p.m.)	Yield (%)	Purification factor
Membranes	43.3	103	100	1
DEAE-cellulose eluate	1.47	35.9	35	10.2
First HPLC eluate	0.060	23.8	23	166
Second HPLC eluate	0.0041	2.7	2.6	274

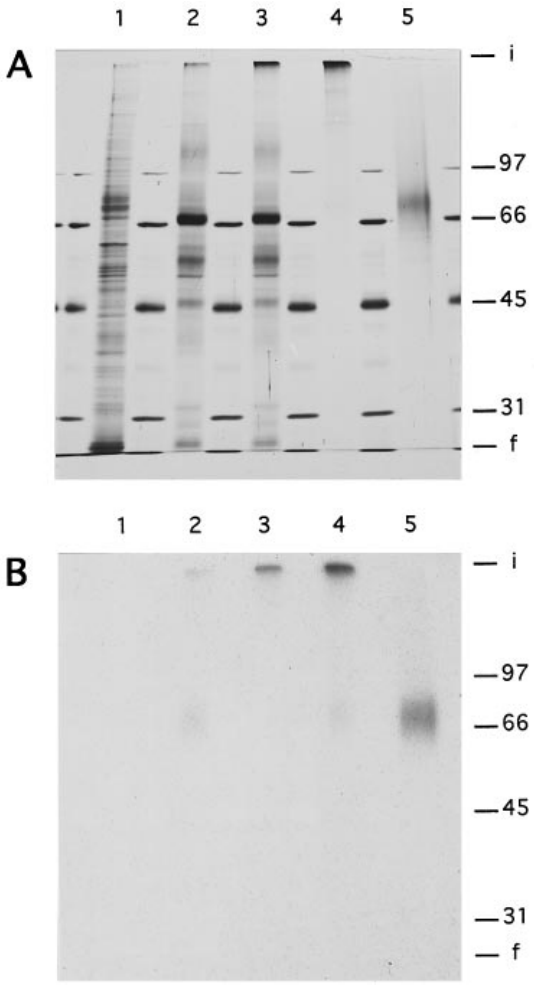

Figure 7 SDS/PAGE analysis of the purification

The preparation described in Table 1 was analysed. (**A**) Silver-stained gel; (**B**) autoradiogram. Lane 1, chromaffin granule membranes (1.2 μg of protein); lanes 2 and 3, DEAE-cellulose eluate (1.0 μg) before and after heating respectively; lane 4, purified aggregates (0.2 μg); lane 5, purified disaggregated transporter (0.2 μg).

DBH, a major protein of the chromaffin granule membrane (results not shown). It may be noted, however, that immunoblotting analysis of control and boiled SDS-solubilized membranes did not reveal any loss of monomeric DBH by aggregation (results not shown). Therefore the traces of DBH observed might result from a secondary association with the aggregated proteins, rather than from a real aggregation or co-aggregation. The silver staining revealed also the presence in the purified aggregates of the diffuse 40 kDa polypeptide mentioned above (Figure 3), which was eluted in the last fractions of the size-exclusion HPLC (Figure 6A, lanes 39 and 40). By contrast, cytochrome *b*-561 was absent. Because of its higher pI [24], it had been removed during the first ion-exchange chromatographic step.

The purification procedure is summarized in Table 1 and illustrated in Figure 7. Only fractions almost devoid of DBH were pooled after the second HPLC, and this purified material appeared to be homogeneous (lane 5). The corresponding overall purification factor was 274, which is consistent with a purification of the transporter to homogeneity since this protein represents 0.2–0.4 % of the chromaffin granule membrane proteins [21]. The aggregation is the most efficient step of the purification, with a 16-fold purification factor and a 66 % yield. However, the yield falls dramatically in the last step (about 10 %), because of the incomplete disaggregation and the overlap of transporter and DBH peaks.

DISCUSSION

Mechanism of SDS-resistant aggregation and TFA-induced disaggregation

Our analysis of the induction of aggregation indicates that it required conditions known to disrupt protein structure: heating, organic solvents and acidic pH. We thus propose that the proteins susceptible to SDS-resistant aggregation are those that retain a significant level of structure in the presence of SDS. Such residual secondary structure has been described for polytopic membrane proteins such as lactose permease [25] and bacteriorhodopsin [26]. Accordingly, aggregation would be triggered by a partial unfolding of the residual structures, as depicted in the model of Figure 8. In a similar way, the aggregation of soluble proteins has been shown to derive from folding or unfolding intermediates rather than from native or fully unfolded proteins, and similar models of aggregation have been proposed [27].

Only anhydrous TFA was able to dissociate the SDS-resistant aggregates. It has also been reported to solubilize efficiently hydrophobic peptides [14] or small hydrophobic proteins, such as myelin [28] and the fungal wall protein hydrophobin [29]. Interestingly, NMR studies have shown that anhydrous TFA provides conditions, difficult to achieve otherwise, for complete denaturation of bacteriorhodopsin [26] and crambin, a hyperstable protein [30]. This complete unfolding, which might result from the hydrogen-bonding of TFA with the carbonyl groups of the peptide backbone [31], suggests a possible mechanism for the dispersion of the aggregates (Figure 8) and, more generally, for the exceptional solubilizing power of TFA. After evaporation of TFA and resuspension in aqueous medium, the absence of reaggregation suggests that polypeptides remained fully unfolded or, more speculatively, that SDS-resistant structures have been regenerated.

Its solubilizing power makes anhydrous TFA a potentially interesting solvent for the chromatography of large hydrophobic

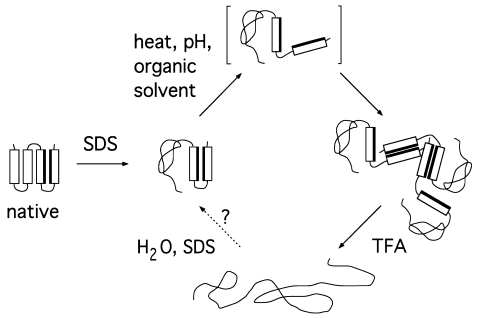

Figure 8 A model of the SDS-resistant aggregation of proteins

Whereas most proteins completely lose their native structure in SDS, some intrinsic proteins retain a significant level of structure. The residual structure is symbolized by two boxes interacting along their shadowed sides. Conditions weakening these interactions, such as heat, organic solvents or low pH, allow intermolecular interactions of the complementary boxes and result in the formation of aggregates. The protein is completely denatured when transferred to anhydrous TFA, thus resulting in the dissociation of the aggregates.

polypeptides. Furthermore, this solvent contrasts with detergent-based aqueous solvents, in which the embedding of polypeptides in detergent micelles attenuate their size differences. Therefore better resolutions might be expected using size-exclusion chromatography in anhydrous TFA. We explored this approach for fractionating the proteins of the purified aggregates, and encouraging results were obtained (B. Gasnier and C. Sagné, unpublished work).

Aggregation as a purification procedure for membrane proteins

The vesicular monoamine transporter purified by the new method has been sequenced both directly and after CNBr fragmentation. Sequences identical with those derived from bovine $VMAT_2$ cDNA were obtained [32], demonstrating that the primary structure had not been altered by the treatment used. The experiments reported here indicate that the strategy might be successfully applied to the purification of other proteins from the chromaffin granule membrane, e.g. cytochrome *b*-561, the 14 kDa polypeptide and a diffuse 40 kDa protein. The last of these needs to be characterized further. The procedure might also be useful for proteins from other membranes, since aggregation is a known characteristic of several polytopic membrane proteins. The aggregation step might be introduced at different stages of the purification protocol. Interestingly, it can even be used as a final step, without further disaggregation, since we have observed that aggregates of the vesicular monoamine transporter can be directly cleaved, chemically or enzymically, for amino acid sequencing analysis (C. Sagné, unpublished work). Therefore the phenomenon investigated here might be of general interest for the purification and structural characterization of membrane proteins.

We are grateful to the *Service Vétérinaire des Abattoirs de Mantes* for collecting bovine adrenals. This work was supported by the *Centre National de la Recherche Scientifique* (URA 1112) and by the BIOMED Programme of the European Community (contract no. BMH 1 CT 93110 to J.-P. H). C. S. is supported by a fellowship from the *Ministère de la Recherche et de la Technologie.*

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Received 15 January 1996; accepted 20 February 1996