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Functional reconstitution of carrier proteins by removal of detergent with a hydrophobic ion exchange column

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A method has been developed for the functional reconstitution of membrane proteins in phospholipid vesicles. This method is an extension of a previously published procedure (Ueno, M., Tanford, C. and Reynolds, A. (1984) Biochemistry 23, 3070–3076) for the formation of unilamellar vesicles from mixed micelles of egg phosphatidylcholine and dodecyl octaoxyethylene ether. Mixed micelles are formed from detergent-solubilized protein and egg-yolk phospholipid vesicles. These micelles are subjected to repeated passage through small columns filled with Amberlite XAD-2 beads. Several carrier proteins from the inner mitochondrial membrane have been reconstituted in this way; experimental data are shown for the aspartate/glutamate carrier and the ADP/ATP carrier. Certain parameters proved to be important for optimal efficiency of reconstitution: (a) the ratio of detergent/phospholipid in the mixed micelles, (b) the concentration of phospholipid during the hydrophobic chromatography, (c) the ratio of phospholipid/protein, (d) the ratio of detergent/Amberlite XAD 2 beads, (e) the number of column passages, and (f) the type of detergent. After optimization of these parameters, phospholipid vesicles with a diameter of about 150 nm were obtained. The main advantage of this procedure, however, lies in the fact that high amounts of membrane protein can be incorporated into the phospholipid vesicles, i.e. up to 15% (w/w).

Introduction

There are a variety of methods for the insertion of carrier proteins into phospholipid membranes leading to functional reconstitution of the transport activity (for recent reviews see Refs. 1 and 2). Every single one of the 'classical' methods has its own advantages and drawbacks. Dialysis procedures lead to relatively large and uniformly sized vesicles, but require the use of dialyzable detergents and involve longtime exposure of the solubilized proteins to the detergent micelles. These methods are not applicable to many carrier proteins. On the other hand, nonionic detergents with low critical micellar concentration have been used for the solubilization and purification of the majority of carrier proteins. The most common method for the reconstitution of proteins solubilized by this type of detergent is the freeze/thaw/sonication procedure. This method, however, also has severe disadvantages: (i) in general, the detergent is still present in the proteoliposomes; (ii) in most cases, this method leads to relatively small and heterogeneously sized proteoliposomes; (iii) sonication leads to partial inactivation of a number of reconstituted proteins.

In order to overcome these difficulties, a method

Abbreviations: LAPAO, 3-lauramido-*N*,*N*-dimethylpropylamine oxide; $C_{12}E_8$, dodecyl octaoxyethylene ether.

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was developed for the reconstitution of proteins solubilized by nonionic detergents with low critical micellar concentration. It was directly derived from a procedure described by Ueno et al. [3] for the formation of phospholipid vesicles from mixed micelles of egg phosphatidylcholine and the detergent $C_{12}E_8$. The incentive for extending this method to the functional reconstitution of membrane proteins was the fact that we found $C_{12}E_8$ to be a most suitable detergent for the solubilization of functionally active carrier proteins from the inner mitochondrial membrane [4,5].

The method was further extended to other detergents used for solubilizaiton and purification of membrane proteins, i.e. Triton X-100, Triton X-114 and laurylaminoxide (LAPAO). Thereby the versatility of this procedure, not only in providing unilamellar vesicles of a reasonably large size, but also in creating proteoliposomes with functionally inserted carrier proteins, was demonstrated.

Materials and Methods

The chemicals used in these studies and their sources were as follows: carboxyatractylate, nucleotides, bovine serum albumin (Boehringer Mannheim); radioactive nucleotides and aspartate (Amersham Buchler); Dowex 1X8 (Fluka); Sephadex, PD10 columns (Pharmacia); hydroxyapatite (Bio-Rad); pyridoxalphosphate (Sigma). Dodecyl octaoxyethylene ether ($C_{12}E_8$) was obtained from Nikko Chemicals Co., Tokyo, Triton X-100 and Triton X-114 from Sigma and 3-lauramido-*N*,*N*-dimethylpropylamineoxide (LAPAO) from Th. Goldschmidt AG.

Amberlite XAD2 was obtained from Serva, Heidelberg. The Amberlite beads were washed three times with methanol and then equilibrated and stored in destilled water. Bongkrekate was a gift from Professor Berends, Delft. All other chemicals were of analytical grade.

Egg-yolk phospholipids were prepared according to Ref. 6. Preformed liposomes were prepared by sonicating egg-yolk phospholipids in the appropriate buffer at a concentration of about 80 mg phospholipid/ml [7].

Isolation of carrier proteins. The aspartate/glutamate carrier from mitochondria was solubilized by $C_{12}E_8$ and purified as described previously [4,5]. In most experiments the eluate of the PD10 columns was used for reconstitution, in some experiments the carrier was further purified by hydroxyapatite-HPLC [5]. The adenine nucleotide carrier from mitochondria was either solubilized by Triton X-100 and purified as described previously [8] or the eluate of the PD10 column which also contained the enriched Asp/Glu carrier (see above) was used, as indicated in the corresponding experiments. The measurement of reconstituted adenine nucleotide exchange [9] and reconstituted aspartate/glutamate exchange [4] has been described previously.

Determinations and calculations. Protein was determined by a modified Lowry method including precipitation of the protein [5,10]. Phosphate was analyzed according to Chen et al. [11]. The internal volume of the liposomes was determined by including 50 mM Na_2HPO_4 in the interior of the liposomes. Phosphate was determined with and without combustion of the sample. The anorganic phosphate measured without combustion corresponds to the internal volume, whereas the difference between the amounts of phosphate measured with and without combustion corresponds to the amount of organic phosphate, i.e., phospholipid. In this way the internal volume per molecule phospholipid can be calculated.

Results and Discussion

1. Basic procedure

As already mentioned in the Introduction, this procedure for reconstituting carrier proteins is derived from a method developed by Ueno et al. [3] for preparing unilamellar vesicles. Before going into the experimental details, the principles of the procedure shall be outlined.

To the solubilized carrier protein first detergent (usually $C_{12}E_8$) is added in concentrated solution (100 mg/ml) in order to provide the appropriate amount of detergent for the following step of micelle formation. Next, phospholipid is added in the form of preformed liposomes. The phospholipid concentration must be checked by phosphate determination, assuming an average molecular weight of 800. Now all further components can be added, i.e. substrates, ions, buffers, etc. The mix-

ture is equilibrated for about 15 min in the cold.

Washed Amberlite is applied to small columns, usually pasteur pipettes, the lower outlets of which are stopped with small pieces of cotton. The Amberlite columns are washed two times with the appropriate reconstitution buffer (without detergent and phospholipid). The buffer is then removed by pressing air through the columns. The micellar soltuion is applied onto the columns and passed through the Amberlite beads. This procedure is repeated 10–15 times. The eluent after the last elution cycle contains functionally reconstituted proteoliposomes.

2. Parameters of the reconstitution procedure

The procedure described here is not only applicable to the nucleotide carrier and the aspartate/glutamate carrier from the inner mitochondrial membrane with which we have dealt here, but also to a variety of other membrane proteins. In order to understand the parameters of this reconstitution procedure, it is necessary to discuss the optimization of the different variables required to obtain maximum carrier activity on the one hand, and appropriate vesicle size on the other. There are a limited number of parameters exerting a predominant influence on the results of the reconstitution procedure: the amount and the kind of detergent, of phospholipid, and of membrane protein; the amount of Amberlite and the number of passages over the hydrophobic column. These parameters shall be discussed in the following.

(a) Detergent/phospholipid ratio. Fig. 1 shows the influence of the detergent ($C_{12}E_8$)/phospholipid ratio on the internal volume and on the transport activity of the reconstituted proteoliposomes containing Asp/Glu carrier from mitochondria. After an initial increase the internal volume remains more or less constant under these experimental conditions. The optimum ratio for functional reconstitution is found to be in the range of 1.2–2.0 mg $C_{12}E_8$/mg phospholipid, however, ratios below 1.0 have to be avoided. The vesicles obtained under these conditions reach an internal volume of about 5 μl/mg phospholipid, which corresponds to a liposome diameter of 150 nm when assuming a homogeneous unilamellar vesicle population.

(b) Phospholipid concentration. Unfortunately

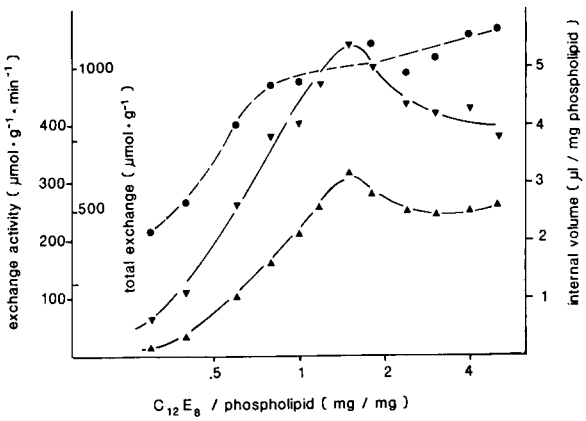

Fig. 1. Reconstitution of the Asp/Glu carrier: variation of the detergent/phospholipid ratio. The constant reconstitution parameters were: phospholipid, 5 mg/ml; protein/lipid, 0.05 g/g; $C_{12}E_8$/Amberlite, 25 mg/g; number of column passages, 15. Enriched Asp/Glu carrier protein after elution from PD10 columns [4,5] was used for the reconstitution. The exchange was measured under the following conditions: external aspartate: 0.1 mM. internal aspartate: 20 mM, pH 6.5, 21°C. The exchange activity (▲) was calculated from initial velocity measurements [14], whereas the total amount of labelled aspartate taken up ('total exchange' (▼)) was calculated from the exchange equilibrium after 20 min [14]. The internal volume (●) was calculated by phosphate determination.

the optimization of the detergent/phospholipid ratio as shown in Fig. 1 is not independent of the absolute concentration of phospholipid and detergent. However, under the experimental conditions described here, phospholipid concentrations between 4 and 15 mg/ml lead to results comparable to those described in Fig. 1. When lower concentrations of phospholipid are used, difficulties arise sometimes with respect to the Amberlite columns, which become very small under these conditions. When phospholipid concentrations higher than 15 mg/ml are used, the specific internal volume decreases (not shown).

(c) Phospholipid/protein ratio. This parameter is believed to be strongly dependent on the type of membrane protein used for the reconstitution experiment. The results for reconstituted Asp/Glu carrier from beef heart are shown in Fig. 2. It is seen that the absolute carrier activity increases with the amount of carrier protein incorporated, up to rather high levels, where the inserted protein represents about 10% of the total weight of the proteoliposomes. The specific transport activity,

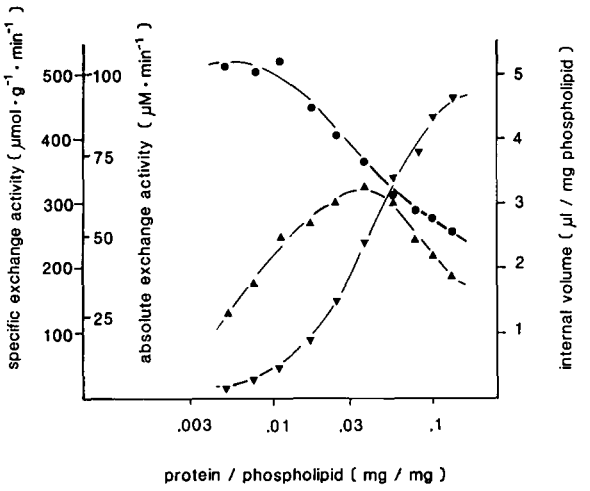

Fig. 2. Reconstitution of the Asp/Glu carrier: variation of the protein/phospholipid ratio. The $C_{12}E_8$/lipid ratio was 1.5 (g/g), all other conditions were the same as in Fig. 1. 'Specific exchange activity' (▲) corresponds to the 'exchange activity' as described in Fig. 1, wheras 'absolute exchange activity' (▼) is the total amount of substrate transported by a definite volume of the reconstituted vesicles. The internal volume (●) was calculated by phosphate determination.

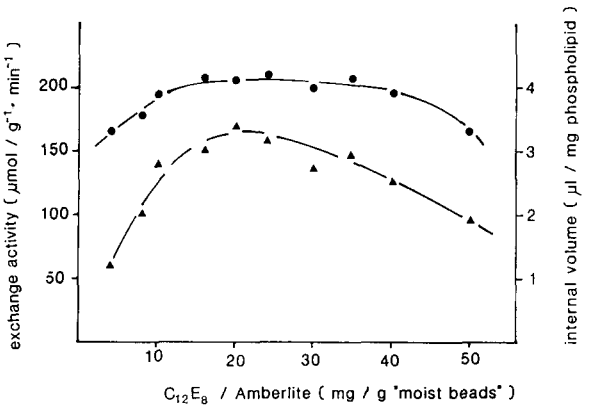

Fig. 3. Reconstitution of Asp/Glu carrier: variation of the $C_{12}E_8$/Amberlite ratio. The constant reconstitution parameters were: phospholipid concentration, 8 mg/ml; $C_{12}E_8$/lipid ratio, 1.5; protein/lipid ratio, 0.05; number of column passages, 15. All other conditions as in Fig. 1. The internal volume (●) and the exchange activity (▲) were determined.

on the other hand, already decreases at moderate concentrations of incorporated protein. The vesicle size is concomitantly reduced when high amounts of protein are inserted. The amount of carrier protein which can be incorporated by this procedure is at least one order of magnitude higher as compared to the amount incorporated in freeze/thaw/sonication procedure [4,8] and is comparable to amounts reached in detergent dialysis procedures [12,13].

(d) Detergent/Amberlite ratio. This parameter is of course closely connected with the following one, the number of column passages. However, if the standard number of 15 elution cycles is applied, the dependence of vesicle size and carrier function on the detergent/Amberlite ratio can be described a shown in Fig. 3. In this case, too, as in Fig. 1, the vesicle size is not as sensitive as the transport function of the reconstituted carrier protein. Thus, correct insertion of a functionally active transport protein seems to be a more critical process than the simple formation of unilamellar vesicles.

(e) Number of column passages. A much higher number of elution cycles was found to be appropriate for functional reconstitution of carrier proteins, compared to that required for the formation of liposomes alone [3]. Fig. 4 demonstrates that at least 8-9 cycles are necessary in order to obtain an active carrier protein. The small decrease during the last few cycles is due to loss of protein (and phospholipid) on the hydrophobic column. Furthermore, it is shown that the correct number of elution cycles needed for the formation of appropriate proteoliposomes is very much dependent on the ratio of detergent/Amberlite. Thus, also the number of column passages has to be optimized in connection with the appropriate detergent/Amberlite ratio.

3. Reconstitution of different carrier proteins

As another representative example, the adenine nucleotide carrier has been reconstituted from an enriched preparation [5]. All parameters described above turned out to have a very similar influence on the two carriers, with the exception of one variable, i.e. the phospholipid/protein ratio. In Fig. 5 the results are given for the ATP/ADP carrier and can be compared with the data for the Asp/Glu carrier from Fig. 2. In Fig. 5 another interesting result is presented in order to elucidate the advantages of the Amberlite procedure. The internal volume cannot be determined only by phosphate analysis, but can also be calculated based on the exchange equilibrium, where the

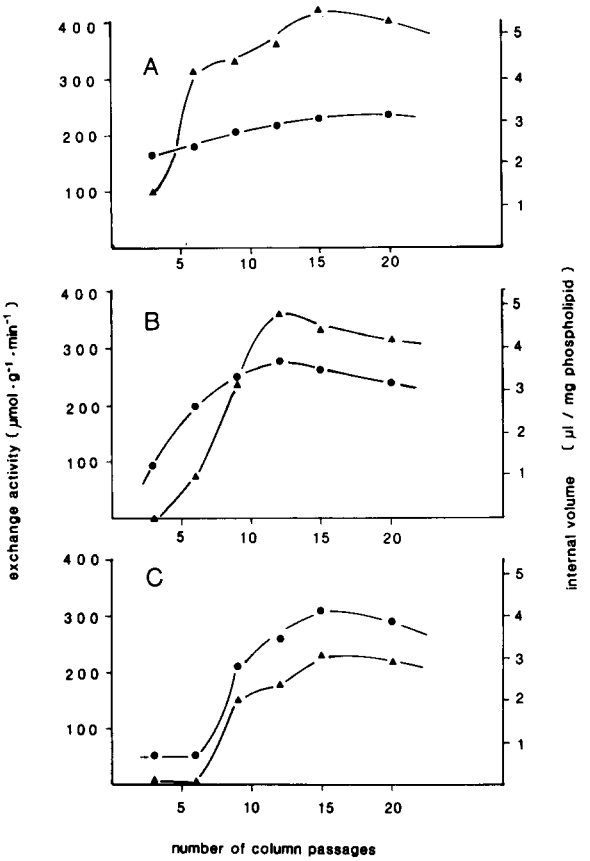

Fig. 4. Reconstitution of Asp/Glu carrier: variation of the number of Amberlite column passages. The $C_{12}E_8$/lipid ratio was 1.3, the phospholipid concentration was 4.5 mg/ml. The $C_{12}E_8$/Amberlite ratio was 10 mg/g in (A), 20 mg/g in (B) and 40 mg/g in (C). The protein/phospholipid ratio was 0.033 g/g in (A), 0.09 g/g in (B) and 0.011 g/g in (C). The internal volume (●) and the exchange activity (▲) were determined.

specific radioactivity of internal and external substrates has become equal, as shown in the following equation [14]

$$\left(\frac{\text{Internal}}{\text{volume}}\right) = \left(\frac{\text{external}}{\text{volume}}\right) \times \frac{\text{internal radioactivity (dpm)}}{\text{external radioactivity (dpm)}}$$

$$\times \frac{\text{external substrate concentration}}{\text{internal substrate concentration}}$$

If all vesicles are unilamellar and carry functionally active transport proteins, the internal volume measured by phosphate determination should be equal to the 'active' internal volume as calculated above.

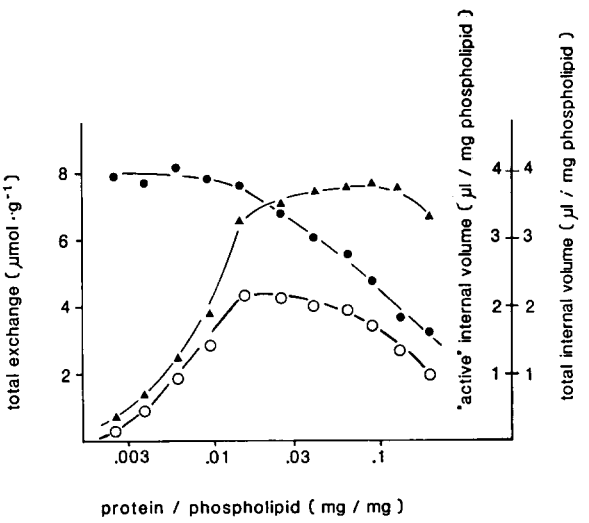

Fig. 5. Reconstitution of ADP/ATP carrier: variation of the protein/phospholipid ratio. Enriched ADP/ATP carrier protein was used after elution from PD10 columns [4,5]. The phospholipid concentration was 5.0 mg/ml, the $C_{12}E_8$/lipid ratio varied between 1.8 and 2.5 g/g. The total amount of exchanged substrate in the exchange equilibrium (▲) was determined [14]. The total internal volume (●) was calculated by phosphate determination, for calculation of the active internal volume (○) see text.

It is seen that in the case of the ADP/ATP carrier (Fig. 5) incorporation of high amounts of protein leads to a vesicle preparation consisting predominantly of proteoliposomes which are capable of active nucleotide transport. In the case of the Asp/Glu carrier (Fig. 2), the optimum activity is reached already at lower levels of incorporated protein.

This result can be generalized. In experiments with other mitochondrial carriers (not shown) it has been observed repeatedly that especially the optimum phospholipid/protein ratio is the most sensitive parameter when varying the type of inserted protein. Therefore, this ratio should always be carefully optimized for every carrier protein to be reconstituted by the method described here.

4. Variation of detergent

The original procedure for the formation of pure phosholipid vesicles used $C_{12}E_8$ as detergent [3]. In the present experiments, too, $C_{12}E_8$ was predominantly used as detergent, since the Asp/Glu carrier can only be purified with satisfy-

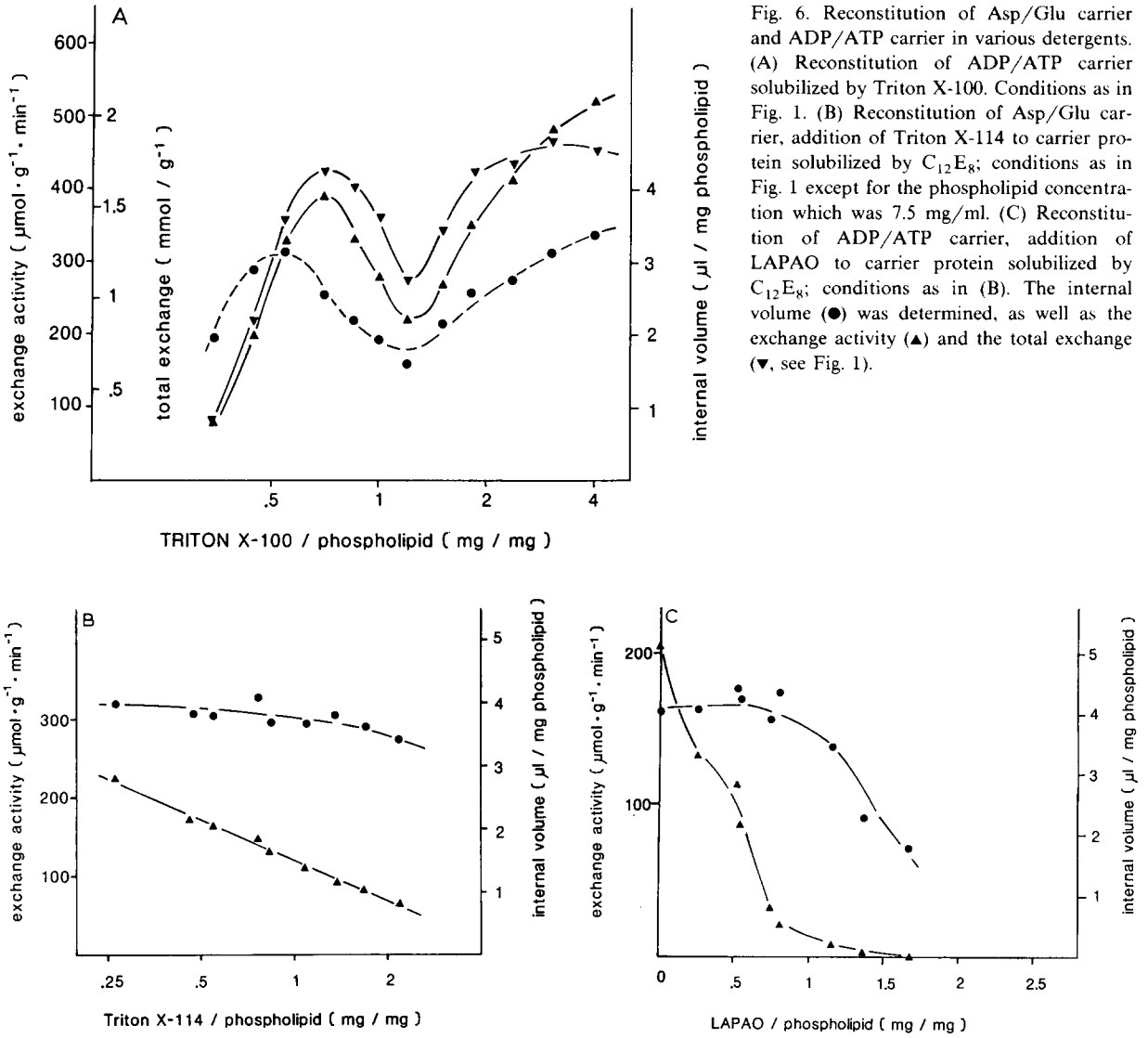

Fig. 6. Reconstitution of Asp/Glu carrier and ADP/ATP carrier in various detergents. (A) Reconstitution of ADP/ATP carrier solubilized by Triton X-100. Conditions as in Fig. 1. (B) Reconstitution of Asp/Glu carrier, addition of Triton X-114 to carrier protein solubilized by $C_{12}E_8$; conditions as in Fig. 1 except for the phospholipid concentration which was 7.5 mg/ml. (C) Reconstitution of ADP/ATP carrier, addition of LAPAO to carrier protein solubilized by $C_{12}E_8$; conditions as in (B). The internal volume (●) was determined, as well as the exchange activity (▲) and the total exchange (▼, see Fig. 1).

ing activity by using $C_{12}E_8$ [4,5] and since the ADP/ATP carier also proved to be very stable when purified in this detergent. However, the method would not be so very valuable if it could only be applied to $C_{12}E_8$/phospholipid micelles, since many other carriers have been isolated in other detergents. Therefore, in addition to $C_{12}E_8$, the detergents Triton X-100, Triton X-114 and LAPAO were tested, which have been used for the isolation of various carriers.

Some of the data we obtained with these detergents are shown in Fig. 6. Fig. 6A shows the results of an experiment in which the ADP/ATP carrier was purified in Triton X-100 [8] and functionally reconstituted with the Amberlite column method. Apart from the reconstitution optimum at higher ratios of detergent/phospholipid, already known from Fig. 1, an additional optimum range at a Triton/phospholipid ratio of 0.7 appears. This is also reflected by the dependence of the vesicle size (internal volume) on the detergent/lipid ratio. Compared to Fig. 3, the activity of the ADP/ATP carrier was lower, presumably because the exposure to Triton X-100 in the mixed

micelles leads to an inactivation of the protein [8]. Also in several repeat experiments the specific internal volume of the vesicles turned out to be lower, compared to the method using $C_{12}E_8$.

The detergents Triton X-114 and LAPAO were tested by adding them to $C_{12}E_8$-solubilized protein during the reconstitution procedure (Figs. 6B and 6C). Both detergents led to rapid inactivation of the reconstituted ADP/ATP carrier and Asp/Glu carrier. Nevertheless, substantial amounts of these detergents could be added before affecting the formation of large unilamellar vesicles. They may therefore also be applicable and suitable when dealing with other carriers.

The other parameters influencing the reconstitution procedure described here, i.e. the absolute phospholipid concentration, the detergent/Amberlite ratio, and the number of Amberlite column cycles, were also tested with these detergents (not shown). The results turned out ot be more or less similar to those obtained with $C_{12}E_8$.

5. Further experimental details

Every method, especially in the field of reconstitution procedures, has its own difficulties and therefore also its own 'tricks'.

(a) Addition of phospholipid. Many experiments have shown that it is important in what state the phospholipids are added during the reconstitution procedure. We always had the best reproducible results when the phospholipids were added as sonicated liposomes. Addition in undispersed form led to unsatisfactory results.

(b) Surprisingly, also the order of addition is important. The order described here, i.e. first addition of concentrated detergent to the solubilized protein, then addition of liposomes, and finally addition of all further components, proved to be superior to other alternatives. Especially the addition of the total amount of detergent before the phospholipid was important for optimal results.

(c) As already seen in Fig. 6, optimum formation of liposomes is not necessarily correlated with optimum activity of the reconstituted carrier protein. This holds true also for the size of the Amberlite column. Whereas the formation of liposomes was found to be dependent only on the detergent/Amberlite ratio and relatively independent of the size (length) of the column, this is not so in the case of the reconstituted carrier activity. Long columns always lead to unsatisfactory reconstitution results; the best activity is obtained with short columns, such as Pasteur pipettes.

(d) In order to quantitate both the yield of liposomes and the extent of reconstitution, it is necessary to measure the degree of dilution caused by the passages over the Amberlite column. When using the 'dry' column, i.e. pressing out excess buffer before applying the detergent/phospholipid micelles, the remaining buffer (inside the beads) reproducibly amounts to about 50% of the weight of the 'moist Amberlite' originally applied onto the column.

Conclusion

A method is presented which reproducibly leads to the formation of large unilamellar vesicles as well as functionally reconstituted carrier proteins. The original procedure described by Ueno et al. [3] for the formation of unilamellar liposomes from mixed micelles of $C_{12}E_8$ and egg-yolk phosphatidylcholine had to be adapted in some respects to achieve a functional reconstitution of carrier proteins. The proteoliposomes obtained can be optimized with respect to their size (Figs. 1 and 2), reaching internal volumes of about 5 μl/mg phospholipid, which corresponds to vesicles of about 150 nm diameter. Furthermore, the method can be optimized with respect to maximum amount of functionally active carrier protein (Figs. 2 and 5). In the case of the ADP/ATP carrier from mitochondria, the proteoliposomes, still tight and stable, can consist of more than 10% (weight) protein. In this case, every proteoliposome carries about 100 nucleotide carrier molecules, however, only a part of them functionally active [8].

The activity of the carrier proteins tested is good, in general better than that observed in experiments where reconstitution is carried out by freeze/thaw/sonication procedures. In any case, disregarding the absolute transport activity, the proteoliposomes obtained by the method described here are definitely more suitable for kinetic studies than freeze/thaw/sonication vesicles, since they are much larger. Thus the useful kinetic resolution time before isotopic equilibrium is reached [14] is much longer than than obtained in

sonicated proteoliposomes. This is especially an advantage for carriers like the ADP/ATP carrier, the Asp/Glu carrier, the citrate carrier or the oxoglutarate carrier from mitochondria, which cannot be reconstituted by dialysis methods [8,4,15,16], which, if applicable, would also lead to large liposomes. The carrier proteins mentioned above, as well as many other membrane proteins, cannot withstand dialyzable detergents, i.e. detergents with high critical micellar concentrations. They are thus ideal candidates for treatment with the Amberlite procedure for functional reconstitution.

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