Biochimica et Biophysica Acta, 981 (1989) 115–120
Elsevier

BBA 74409

Bilitranslocase is the protein responsible for the electrogenic movement of sulfobromophthalein in plasma membrane vesicles from rat liver: immunochemical evidence using mono- and poly-clonal antibodies

M. Miccio [1], G. Baldini [1], V. Basso [1], B. Gazzin [1], G.C. Lunazzi [1], C. Tiribelli [2] and G.L. Sottocasa [1]

[1] *Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, University of Trieste and* [2] *Istituto di Patologia Speciale Medica, University of Trieste, Trieste (Italy)*

(Received 14 December 1988)

Key words: Bilitranslocase; Sulfobromophthalein; Electrogenic transport; Plasma membrane; (Rat liver)

Monoclonal antibodies raised against bilitranslocase, may display either inhibitory or enhancing activity on the electrogenic transport of sulfobromophthalein, evoked in rat liver plasma-membrane vesicles by the addition of valinomycin in the presence of K^+. In both cases, the target protein is identified with a 37 kDa band in SDS-mercaptoethanol gel electrophoresis of solubilized membranes. The electrophoretically homogeneous protein isolated by ion-exchange chromatography, corresponds in all respects to the 37 kDa protein band of bilitranslocase, obtained in the past by different techniques. Using this protein as antigen, a polyclonal monospecific antibody preparation has been obtained. As expected, the antibody preparation inhibits the electrogenic movement of sulfobromophthalein in plasma membrane vesicles from rat liver. It is concluded that the 37 kDa protein of bilitranslocase is at least a necessary component of the transport system involved in the sulfobromophthalein movement in plasma membrane.

Introduction

Bilitranslocase is a membrane protein isolated in our laboratory from rat liver plasma membrane [1]. Its function is related to the sinusoidal uptake of organic anions, notably bilirubin, and functional analogues. This conclusion has been reached on the basis of different lines of evidence, including in vivo as well as in vitro studies utilizing isolated and perfused liver, isolated hepatocytes and plasma membrane vesicles [2–4]. Particularly significant was the finding that liposomes became selectively permeable to sulfobromophthalein upon addition of purified bilitranslocase [5]. As expected, the protein in solution was found to bind a number of organic anions including dibromo-sulfonphthalein, Indocyanine green, Rifamycin-SV and nicotinate. In line with the finding that the tissue distribution of bilitranslocase indicated the protein to be present in the tissues endowed with an active fatty-acid oxidative metabolism, antibilitranslocase antibodies were found to inhibit the uptake of fatty acids by isolated hepatocytes [6]. Bilitranslocase has been isolated in the past by means of two different techniques in the presence [1] and absence [7] of deoxycholate, respectively. SDS-gel electrophoresis in the presence of 2-mercaptoethanol could resolve both preparations into two bands with mobilities corresponding to 35.5 and 37 kDa [7]. The native molecular mass, measured by gel permeation was different in the two cases (170 and 100 kDa), indicating that a different degree of aggregation was possible in the presence or absence of the detergent.

Despite the evidence collected in support of its function, there is criticism about the origin of the isolated protein which could not be conclusively resolved because of the starting material, which in most cases, was a crude plasma membrane preparation. Constant contaminants of such a preparation are fragments of inner mitochondrial membrane and endoplasmic reticulum.

It became necessary to undertake a systematic study to define unequivocally whether the protein was originally part of the sinusoidal plasma membrane. The

Abbreviation: FPLC, fast protein liquid chromatography.

Correspondence: G.L. Sottocasa, Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole Università di Trieste, 34100 Trieste, Italy.

classical approach to start from highly purified plasma membrane fractions, well-defined from the enzymatic point of view, does not provide convincing evidence in that, even in this case, the degree of purity is such to role out the possibility that minor contaminants may be the origin of the isolated protein. This is particularly true in the case of plasma membrane which is dispersed upon homogenization into a variety of vesicles of different size which may be found in all cell fractions. A completely independent approach may be undertaken if monoclonal antibodies are obtained capable of inhibiting the transport function dependent on the activity of the protein in question. In this paper, we show that monoclonal antibodies selected on the basis of their ability to inhibit sulfobromophthalein uptake by plasma membrane vesicles react selectively with a 37 kDa protein band. The protein may be isolated by a modification of the techniques previously described: it displays unusual characteristics, in that it is a very basic protein and may be bound to a cation exchanger at pH 8.0. It corresponds in all respects to the heaviest subunit of bilitranslocase previously described. Polyclonal monospecific antibodies raised against this protein inhibited the electrogenic movement of sulfobromophthalein in plasma membrane vesicles.

Materials and Methods

Monoclonal antibodies

The antigen, a bilitranslocase preparation obtained as previously described [7], was used. Two BALB/c mice were injected intraperitoneally with 10 μg protein plus 0.1 ml Freund's complete adjuvant. The treatment was repeated after 15 days, using the same antigen dose plus incomplete adjuvant. A further antigen stimulation was carried out 10 days later by injecting intravenously 5 μg of the protein without adjuvants. After 5 days, the animals were killed and the spleens removed from the preparation of hybridomas according to Kohler and Milstain [8]. A prescreening was performed on the growing clones to select the IgG producers. As antigen a rat liver microsomal preparation was used. This material is routinely produced in the laboratory and it is sufficiently contaminated by plasma membrane fragments for this purpose. IgG were detected on cellulose acetate strips by the classical dot-blot technique with peroxidase-linked anti-mouse antibodies. A second screening was performed on the basis of the effect of the antibody on sulfobromophthalein transport by plasma membrane vesicles [4]. IgG from large-scale cultures of clone number 58 were obtained by sodium sulfate precipitation. The growing medium was dialyzed against 20 vol 18% sodium sulfate water solution for 6 h. The precipitate, collected by centrifugation, was redissolved in a minimum volume of distilled water and dialyzed against isotonic saline solution.

Plasma membrane vesicles

Vesicles were prepared from Wistar albino rat livers according to Van Amelsvoort et al. [9]. Sulfobromophthalein uptake in this material was followed by the spectrophotometric method described by Baldini et al. [4] in the presence of 50 mM KCl and 100 mM potassium phosphate buffer (pH 8.1).

Bilitranslocase preparation

The protein was prepared either from purified plasma membrane fractions according to Ray [10] or from cruder preparations (the fraction indicated as Pellet III by the same author). From this material an acetone powder was obtained and the protein solubilized as already described [1]. At variance with the previously described technique, to the particulate preparation was added 0.1% 2-mercaptoethanol. The extracting medium (100 mM KCl/20 mM Tris-HCl (pH 8.0)/1 mM EDTA) also contained 0.1% 2-mercaptoethanol. When necessary, the extract was stored in liquid nitrogen. The extract was diluted three times with 20 mM Tris-HCl buffer (pH 8.0) containing 0.1% mercaptoethanol. The protein solution, up to 100 ml, was loaded onto a Mono-S column of the FPLC apparatus (Pharmacia, Uppsala, Sweden) preequilibrated with a buffer system consisting of 20 mM Tris-HCl (pH 8.0)/50 mM KCl/0.1% mercaptoethanol. Details of the chromatographic run are given in Fig. 4. On a larger scale, a similar chromatographic procedure has been applied by means of a glass column (2 cm diameter) packed with 10 ml SP-Sephadex C-50 preequilibrated as described above. For details, see legend to Fig. 5.

SDS-gel electrophoresis

Samples, boiled 3 min in 5% 2-mercaptoethanol/1.2% sodium dodecyl sulfate were applied to pre-cast gradient gels (10–15% polyacryl amide) (Phastsystem, Pharmacia). The electrophoresis was performed under standard experimental conditions. Staining was carried out as suggested by the manufacturer with Coomassie brilliant blue. Blotting of the protein bands was carried out by diffusion at 70 °C on cellulose nitrate strips following the method recommended by Pharmacia.

Monospecific polyclonal antibodies. A New Zealand albino rabbit was injected intramuscularly in the leg with 200 μg bilitranslocase dissolved in 1.5 ml 20 mM Tris-HCl/90 mM KCl buffer (pH 8.0) plus an equal volume of incomplete Freund's adjuvant. Bi-monthly booster injections of 200 μg of antigen in incomplete adjuvant were administered until a satisfactory antibody titer was obtained. Blood samples were taken from the marginal vein of the ear before each injection and the serum antibody level was estimated by radial immunodiffusion according to Ouchterlony [11]. From serum, IgG were precipitated as described above.

Immunoblotting. Immuno dot-binding assay of bilitra...slocase by the supernatants of different hybridoma

cultures was carried out according to Herbrink [12]. Immunodetection on dots was performed by the biotin-streptavidin system (Amersham, U.K.).

Results

Starting from two BALB/c mice injected with a bilitranslocase-enriched protein fraction, 144 clones have been grown. Only 58 of them were IgG producers. The growth media of these clones have been tested for their ability to inhibit sulfobromophthalein transport induced in plasma membrane vesicles, by the addition of valinomycin in the presence of K^+ [4]. Fig. 1 collates the results of this screening. 25 media were completely ineffective on the transport activity measured; 8 showed an enhancing activity and the others inhibited the transport to variable extents. On the basis of these results, we decided to investigate further clone number 58, which showed the highest inhibitory activity. The clone was therefore grown on a large scale, the growth medium collected and immunoglobulins precipitated therefrom. The purified IgG were tested for their ability to inhibit the electrogenic transport of sulfobromophthalein by plasma membrane vesicles. The results are given in Fig. 2. Already at 8 μl, the activity is depressed by 50%. The antibody solution contained 8 mg protein/ml. A more precise titer on a protein basis cannot be reliably calculated in view of the fact that the medium contained horse serum and it is therefore expected that the antibody solution contained horse immunoglobulins. The latter have, however, no effect on the activity tested, as shown by the results obtained with inactive clones (Fig. 1). It is expected that the effect of the monoclonal IgG is the result of the specific binding to an essential part of the protein involved in the transport. It was relevant, therefore, to identify the membrane protein present in the vesicle which could interact with the immunoglobulin produced by clone number 58.

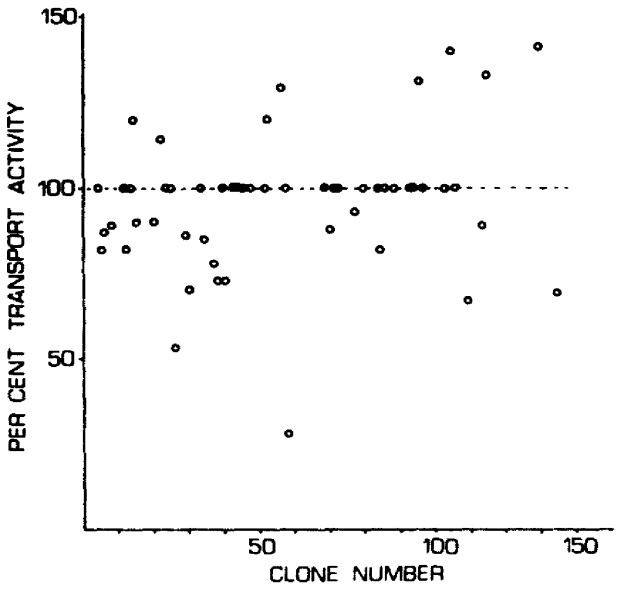

Fig. 1. Effect of culture media on the electrogenic sulfobromophthalein transport by plasma membrane vesicles. Experimental conditions: 25 μl plasma membrane vesicles (13 mg protein/ml) were preincubated in ice with an equal volume growth medium of the different clones for 10 min. The suspension was then transferred to 2.4 ml assay medium (100 mM potassium phosphate buffer (pH 8.1)/0.018 mM sulfobromophthalein/50 mM KCl) in a 3 ml cuvette as described in Ref. 4. The reaction was started by addition of 3 μg valinomycin dissolved in 3 μl methanol. Control experiments were run preincubating the vesicles with fresh medium.

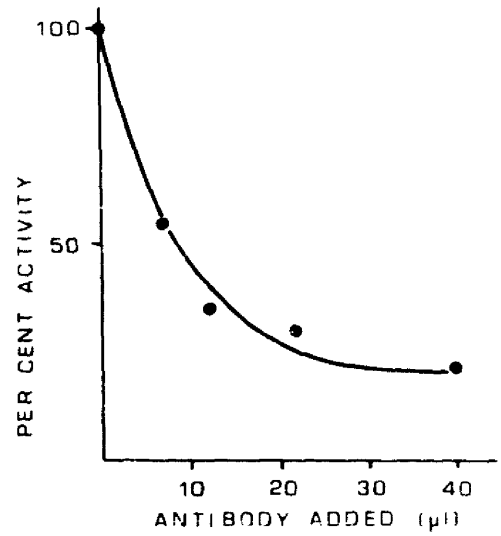

Fig. 2. Inhibitory effect of IgG purified from growth medium of clone number 58 on sulfobromophthalein transport by plasma membrane vesicles from rat liver. Experimental conditions: the experiment was performed as in the legend to Fig. 1. The blank consisted of vesicles preincubated with an equal volume of dialyzing solution.

Fig. 3, panel A, shows an SDS-PAGE pattern obtained from solubilized vesicles stained with Coomassie brilliant blue. Panel B in the same figure shows the corresponding immunodecorated pattern after immunoblotting. The only positive band which can be detected in the pattern corresponds to a molecular mass of 37 kDa. We decided, therefore, to purify bilitranslocase by a modification of the techniques previously described [1,7].

Two modifications were introduced: (1) whenever possible, the protein solutions were maintained in reduced form by addition of 2-mercaptoethanol during processing and (2) the final step was performed by ion-exchange chromatography using an FPLC Mono-S column. The former was suggested by the observation in previous experiments showing that the protein was unstable on aging and could lose either the binding capacity or the ability to reconstitute the transport in liposomes. On the other hand, preparations stored in the presence of 2-mercaptoethanol for 2 weeks were still

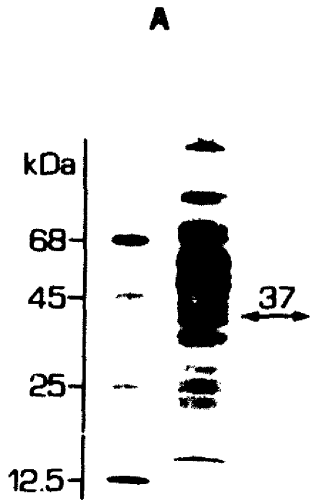

Fig. 3. SDS-polyacrylamide gel electrophoresis of isolated plasma membrane vesicles. Panel A: staining with Coomassie brilliant blue; panel B: after blotting and immunodecoration using purified IgG from clone number 58. Experimental conditions: 250 μg vesicles dissolved in 2.5% SDS and 5% 2-mercaptoethanol boiled for 3 min and applied to pre-cast gradient gels (10–15%) (Phastsystem, Pharmacia, Sweden). As standard proteins, first lane, cytochrome *c*, chymotrypsinogen, ovalbumin and serum albumin were used. Immunoblotting as described under Materials and Methods.

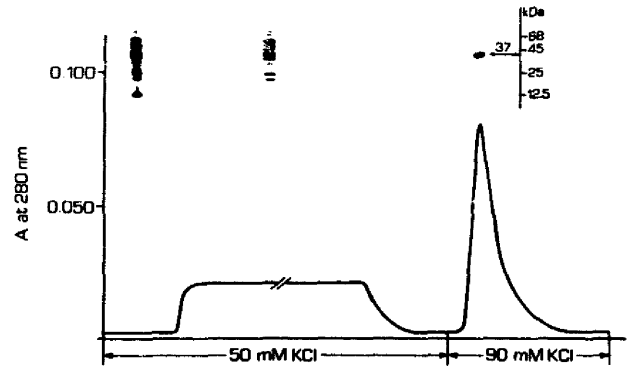

Fig. 5. Ion-exchange chromatography of the protein extract on SP-Sephadex C-50. Experimental conditions: protein load 420 mg; buffer system as in legend to Fig. 4.

fully active in the reconstitution experiments. The latter modification was introduced in view of the high resolution power of the technique.

The technique has been applied both to purified plasma membrane fractions and to crude Pellet III fractions according to Ray [10]. The elution pattern from a Mono-S column is presented in panel (a) of Fig. 4. Under the experimental conditions employed (low ionic strength and pH 8.0), the bulk of the protein is not retained on the column and may be found in the effluent during input of the sample. After washing, a KCl gradient is started. Three major peaks are eluted at relatively low ionic strength. The first contains bilitranslocase, as assessed by binding studies of sulfobromophthalein, and the third may be easily recognized as cytochrome *c*. The presence of this pigment may vary from one preparation to another in relation to the extent of contamination of the starting material by inner mitochondrial membrane. When purified plasma membranes are used, the peak-height ratio is largely in favor of bilitranslocase (data not shown). Panel (b) in Fig. 4 shows the elution patterns obtained from re-chromatography of the fraction included between the two vertical lines of the previous panel. It should be noted that during loading of the sample, a trace amount of protein is not retained by the exchanger. This presumably corresponds to acidic proteins copurified with bilitranslocase in the previous chromatographic run. In this second chromatographic run the detachment is carried out by an abrupt increase of ionic strength. The

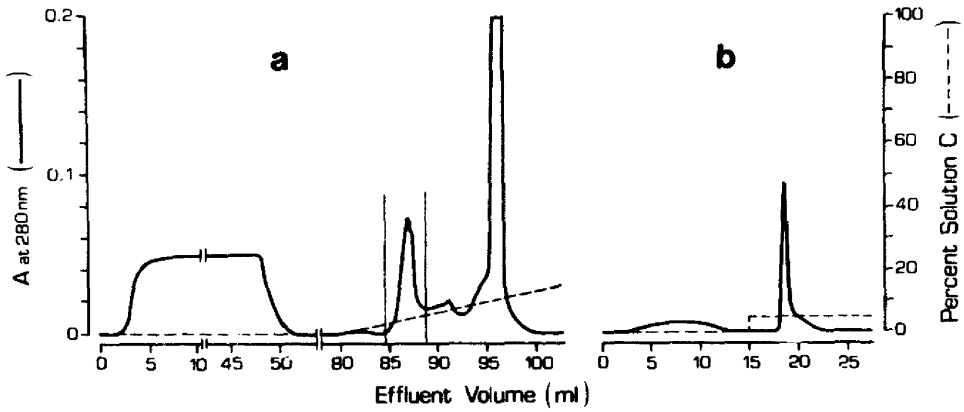

Fig. 4. FPLC of protein extract from acetone powder of plasma membranes. For experimental conditions, see text. Protein load in panel (a) 120 mg extract from purified plasma membrane fraction according to Ray [10]. Dashed line: elution gradient; Solution C consisted of 1.3 M KCl in 0.02 M Tris-HCl buffer (pH 8.0) and 0.1% (v/v) 2-mercaptoethanol. In panel (b) the pooled fractions collected between the two vertical lines of the previous chromatographic run were diluted three times with 20 mM Tris-HCl (pH 8.0) 0.1% 2-mercaptoethanol and loaded onto the same chromatographic system.

peak obtained consists of a homogeneous protein band in SDS-gel electrophoresis. By comparison with the appropriate protein standards its mobility corresponds to a molecular mass of 37 kDa.

In order to be able to scale up the preparation procedure, we decided to apply the technique developed for FPLC to a traditional column chromatography under comparable experimental conditions. The ion exchanger used was SP-Sephadex C-50. The elution pattern of this column is presented in Fig. 5. In the upper part of the panel three electrophoretic patterns are also presented, corresponding to the input (upper left), the bulk of the protein not retained by the exchanger (middle) and the protein eluted at 90 mM KCl (upper right). Obviously, the results are superimposable to those obtained by FPLC, with the advantage that the whole procedure may be applied to a large sample and may be completed in a shorter time. Under these conditions, contaminating cytochrome *c* remains on the exchanger at the end of the run.

It was interesting at this point to check whether the isolated protein was recognized by the monoclonal antibody number 58, which was previously shown to inhibit the electrogenic movement of sulfobromophthalein in plasma membrane vesicles. This is shown in Fig. 6, were in panel A an SDS-gel electrophoresis is presented. The first lane includes four protein standards and the second the protein peak eluted from the ion exchanger. Clearly the band at 37 kDa gives a net positive response.

To confirm that the 37 kDa protein was in fact the site of blockade of sulfobromophthalein transport, we

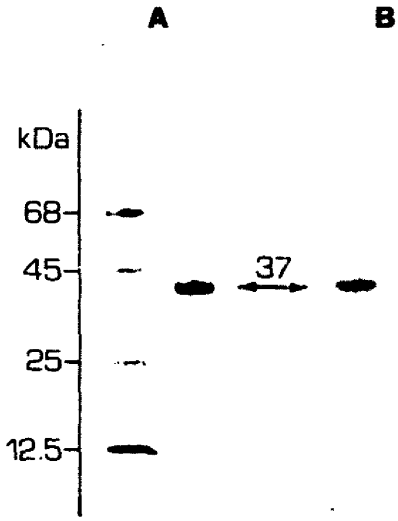

Fig. 6. SDS-gel electrophoresis of the isolated protein. Experimental conditions as in legend to Fig. 3. Protein load 0.2 μg of the protein present in the peak obtained from the chromatographic step in the legend to Fig. 5. Panel A: staining with Coomassie brilliant blue; panel B: immunodecorated pattern with IgG from clone number 58.

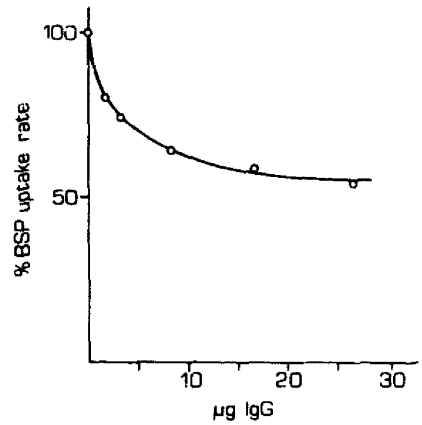

Fig. 7. Inhibition of sulfobromophthalein uptake by polyclonal monospecific antibilitranslocase IgG in plasma membrane vesicles. Experimental conditions as in legend to Fig. 2.

have evoked the production of polyclonal monospecific antibodies in the rabbit injected with the protein peak. As expected immunoglobulins derived from this serum inhibit sulfobromophthalein transport in plasma membrane vesicles as shown in Fig. 7. The inhibition is not as efficient as in the case of clone number 58, presumably from the fact that the antibody preparation is expected to contain both inhibitory and enhancing IgGs.

If the assumption is that bilitranslocase is indeed the protein acting as carrier at the plasma membrane level to promote the movement of sulfobromophthalein, all monoclonal IgGs acting on this process should recognize this protein component, irrespective of their inhibitory or enhancing activity. A number of these immunoglobulins have been tested in a dot-blot system against pure bilitranslocase. It is worth mentioning that the most evident positive response was found with the supernatant of clone number 104 which displayed a marked activating effect on the transport function in vesicles (see Fig. 1).

Discussion

Although the evidence collected in the past in favor of a carrier function of bilitranslocase was rather convincing, doubts have been raised about the cellular origin of the protein and the specificity of bilitranslocase for its ligands [12]. Data presented in this paper confirm and extend the conclusion that: (1) the electrogenic movement of sulfobromophthalein in plasma membrane vesicles is mediated by bilitranslocase and (2) bilitranslocase originates, during preparation, from the sinusoidal portion of this membrane. In addition, it may be stated that the 37 kDa band of bilitranslocase, shown in previous studies to bind sulfobromophthalein, is the protein molecule responsible for the translocation of the dye by plasma membrane vesicles.

The conclusion that bilitranslocase is originally localized in the sinusoidal plasma membrane of liver is

supported by the following findings: (1) the yield of the protein during purification corresponds directly with the degree of purity of the plasma membrane fraction used as starting material; (2) the chromatographic system used for the isolation of bilitranslocase allows it to separate simultaneously also cytochrome *c*. The latter component is a quantitative indication of the degree of contamination by inner mitochondrial membrane fragments. The ratio between the amount of cytochrome *c* and the amount of bilitranslocase varies in different preparations.

A further argument supporting this view comes from the experimental design. We are studying an exclusive plasma membrane vesicle activity, such as the electrogenic dye movement [4]. The inhibition of this activity is taken as a selective criterion to screen the antibodies. It is reasonable to assume that the effect brought about by the addition of these antibodies is attributed to the interaction with a membrane protein localized at the plasma membrane level. In addition, monoclonal immunoglobulins showing an enhancing effect on the transport activity interact with the pure protein. In turn, monospecific polyclonal antibodies raised pure bilitranslocase exert an inhibitory action on sulfobromophthalein transport by plasma membrane vesicles.

On these grounds, we believe we are providing evidence that the 37 kDa subunit of bilitranslocase obtained by the previous techniques [1,7] is involved as a necessary component in the translocation of the anion in plasma membrane vesicles. The finding that the presence of thiols during the preparation of bilitranslocase preserves the characteristics of the protein and improves the yield of the technique may suggest that reduced -SH groups may be important both for the structure and function of the translocator. The involvement of these groups in the transport function has been investigated separately [13]. The behavior of the protein in solution deserves some explanation. Presumably, we are dealing with an intrinsic membrane protein which, however, can be isolated as a water-soluble compound. The water-solubility properties are perhaps to be attributed to a relatively high density of positive charges (the estimate p*I* is higher than 9.0). The protein associates spontaneously with liposomes and makes them selectively permeable to sulfobromophthalein [5]. It is difficult to understand the mechanism by which a water-soluble protein is easily inserted into a lipid bilayer, unless one assumes that the protein may have two meta-stable conformations, one, in water, with a number of positive charges exposed and the other, in lipid, where the hydrophilic groups may perhaps delimit the hydrophilic channel.

One point which remains to be clarified concerns the beta subunit (35.5 kDa) constantly copurified by previous techniques both in the presence and absence of deoxycholate. The importance of this subunit in bilitranslocase function was inferred in the past on the basis of the observation that the dye could be found associated to the protein even after electrophoretic purification. Two possibilities for this exist: (a) we are dealing with a degradation product of the native 37 kDa protein; (b) this component copurifies with the heaviest one, being acidic in nature and interacting electrostatically with the basic 37 kDa subunit. To accept explanation (b) we have to assume also that the dye found associated with it is bound covalently, which may occur by photoreaction of it with the protein during processing. Other explanations are also possible.

We are inclined to conclude, however, that bilitranslocase, when prepared in the presence of a reductant and isolated by ion-exchange chromatography, consists of a single molecular species with an apparent molecular mass of 37 kDa.

The primary structure and the existence of possible different conformers of bilitranslocase are currently under study in our laboratory.

Acknowledgments

This work was supported by grants from Ministero della Pubblica Istruzione and Fondo per lo Studio delle Malattie del Fegato.

References

1 Tiribelli, C., Lunazzi, G.C., Luciani, M., Panfili, E., Gazzin, B., Liut, G.F., Sandri, G. and Sottocasa, G.L. (1978) Biochim. Biophys. Acta 532, 105–112.
2 Sottocasa, G.L., Tiribelli, C., Luciani, M., Lunazzi, G.C. and Gazzin, B. (1979) in Function and Molecular Aspects of Biomembrane Transport (Quagliariello, E., Palmieri, F., Papa and Klingenberg, M., eds.), pp. 451–458, Elsevier/North Holland Biomedical Press, Amsterdam.
3 Persico, M. and Sottocasa, G.L. (1987) Biochim. Biophys. Acta 930, 129–134.
4 Baldini, G., Passamonti, S., Lunazzi, G.C., Tiribelli, C. and Sottocasa, G.L. (1986) Biochim. Biophys. Acta 856, 1–10.
5 Sottocasa, G.L., Baldini, G., Sandri, G., Lunazzi, G.C. and Tiribelli, C. (1982) Biochem. Biophys. Acta 685, 123–128.
6 Tiribelli, C., Luciani, M., Sandri, G., Lunazzi, G., Gazzin, B., Renaud, G., Infante, R. and Sottocasa, G.L. (1981) in Familial Hyperbilirubinaemia (Okolicsany, L., ed.), pp. 43–49, John Wiley & Sons, Chichester.
7 Lunazzi, G.C., Tiribelli, C., Gazzin, B. and Sottocasa, G.L. (1982) Biochim. Biophys. Acta 685, 117–122.
8 Kohler, G.E. and Milstain, C. (1975) Nature 256, 495–497.
9 Van Amelsvoort, Y.M.M., Sips, H.J. and Van Dam, K. (1978) Biochem. J. 174, 1083–1086.
10 Ray, T.K. (1970) Biochim. Biophys. Acta 196, 1–9.
11 Ouchterlony, O.E. (1948) Acta Pathol. Microbiol. Scand. 26, 507–511.
12 Frimmer, M. and Ziegler, K. (1988) Biochim. Biophys. Acta 947, 75–99.
13 Passamonti, S. and Sottocasa, G.L. (1989) Biochim. Biophys. Acta 979, 294–298.