Biochimica et Biophysica Acta, 685 (1982) 117–122
Elsevier Biomedical Press

BBA 71026

FURTHER STUDIES ON BILITRANSLOCASE, A PLASMA MEMBRANE PROTEIN INVOLVED IN HEPATIC ORGANIC ANION UPTAKE

GIANCARLO LUNAZZI [a], CLAUDIO TIRIBELLI [b], BRUNO GAZZIN [a] and GIANLUIGI SOTTOCASA [a,*]

[a] *Istituto di Chimica Biologica and* [b] *Patologia Speciale Medica, Università di Trieste, Via Valerio 32, 34127 Trieste (Italy)*

(Received June 29th, 1981)

Key words: Bilitranslocase; Sulfobromophthalein-binding protein; Organic anion uptake; (Liver plasma membrane)

Bilitranslocase, a plasma membrane protein involved in bilirubin and other organic anion uptake by the liver, exhibits a high molecular weight (170000) when isolated in the presence of deoxycholate. This value is decreased to approx. 100000 if deoxycholate is not included in the isolation medium. Both preparations can be resolved into two kinds of subunit, α and β, of 37000 and 35500, respectively, by reduction with 2-mercaptoethanol and addition of sodium dodecyl sulfate. Under these conditions the two subunits are still capable of high-affinity sulfobromophthalein binding and, despite the presence of the detergent, may be isolated by preparative polyacrylamide gel electrophoresis still associated with the dye. It may be suggested that the physiological subunit composition of bilitranslocase is α_2-β.

Introduction

In 1978 we reported [1] the isolation and purification of a protein from rat liver plasma membrane. The main feature of this protein was its ability to bind sulfobromophthalein with a dissociation constant identical to that measured in isolated rat liver plasma membrane [2]. This value, in the range of $1 \cdot 10^{-6}$ M, pointed to a physiological role of this compound in hepatic sulfobromophthalein uptake. Subsequently [3,4] we could demonstrate by immunochemical methods that the protein was involved in vivo in hepatic bilirubin uptake. We therefore gave this protein the name bilitranslocase. Distribution studies of the component and experiments carried out either on isolated and perfused liver or isolated hepatocytes pointed to a role of the protein also in fatty acid transport [5]. The isolated compound could bind, in addition to sulfobromophthalein, dibromosulfophthalein, unconjugated bilirubin and a number of dyes such as Indocyanine green and Rifamycin-SV. The molecular weight of bilitranslocase was found to be 170000 [1], when measured in the presence of 1% deoxycholate by gel permeation on Sephadex G-100. This value was not influenced by the addition of high concentration of urea. A molecular weight in the range of $1 \cdot 10^5$ suggests the existence of a quaternary structure in the protein. Independently, and by different techniques, other sulfobromophthalein binding proteins have been isolated from liver plasma membrane and reported to have lower molecular weights [6,7].

This paper collects data of experiments aimed at the resolution of the subunit composition of bilitranslocase.

Methods

Starting material. As starting material a crude preparation of liver plasma membrane was ob-

* To whom correspondence should be addressed.

tained from Wistar albino rats of about 200 g as already described [1].

Sulfobromophthalein binding. The binding capacity at different steps of purification of the protein was evaluated by equilibrium microdialysis according to Demushkin and Shalina [8]. The technique is based on the measurement of the free ligand (sulfobromophthalein) in equilibrium with a half sphere of polyacrylamide gel. The fluid outside the gel contains, in addition to a suitable buffer solution (10 mM phosphate buffer (pH 7.8)/10 mM KCl/1 mM EDTA), 10 μM sulfobromophthalein and 100–200 μg binding protein preparation. The system is incubated at room temperature for 3 h. In our case the volume of the gel and that of the soaking medium were both 0.6 ml. At the equilibrium, aliquots of the external solution of 0.5 ml were added with 2.0 ml 0.1 M NaOH and read at 580 nm using a Pye-Unicam SP6-550 UV/Vis spectrophotometer. The extinction coefficient used was 64.0 $\mu mol^{-1} \cdot cm^2$. In the presence of a binding protein in the outside solution, the gel at the equilibrium contains less sulfobromophthalein than the original 10 μM solution. Knowing the volume and the total amount of sulfobromophthalein present in the system, the concentration free in the gel (and therefore free also outside) may be calculated. By subtracting this value from the concentration of sulfobromophthalein in the outer compartment, the concentration of the dye bound to the protein may be derived.

Polyacrylamide gel electrophoresis. Electrophoretic analysis was performed both in 7% gels at pH 8.9 according to Davis [9] and in the presence of 0.1% sodium dodecyl sulfate (SDS) as well as by slab gel electrophoresis using a concentration gradient of acrylamide from 10 to 20% according to Laemmli [10]. Staining was performed in both cases by using standard techniques with Coomassie brilliant blue.

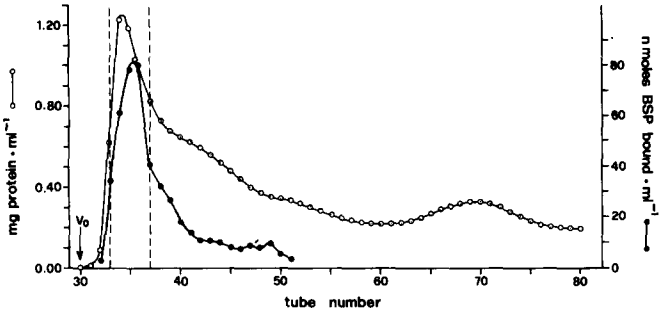
Determination of molecular weights. The molecular weight of the purified protein was estimated either by gel filtration on Sephadex G-100 essentially according to Andrews [11], or by relative electrophoretic mobility using as standard proteins bovine serum albumin (Sigma), ovalbumin, chymotrypsinogen (Boehringer) and whale myoglobin (Sigma). Protein elution patterns from column were monitored with Uvicord LKB at 280 nm.

Deoxycholate determination. The bile salt has been determined at 385 nm in 65% sulfuric acid according to Mosbach et al. [12].

Protein determination. Protein content of the different fractions was determined according to Waddel [13] in the presence of 3 mM NaOH.

Results

Fig. 1 shows the elution pattern obtained from a Sephadex G-100 column of an extract prepared from acetone powder as described by Tiribelli et al. [1]. Clearly, and in agreement with previous data, the bulk of sulfobromophthalein binding activity is eluted from the column immediately following the void volume. When the deoxycholate concentration is decreased to 0.05% during gel filtration the elution pattern is somewhat changed with the appearance of a second peak well after the void volume, as shown in Fig. 2. This result suggests that, at a low concentration of deoxycholate, at least two distinct states of aggregation of the binding protein appear with a clear resolution in gel permeation. A third type of experiment has also been performed in the total absence of deoxycholate. The solubilization step was carried out using a salt solution comprising 10 mM phosphate buffer (pH 7.8)/100 mM KCl/1 mM EDTA. The extraction was performed in ice, homogenizing in a Potter homogenizer equipped with a Teflon pestle. The suspension was centrifuged at $43000 \times g$ for 5 min. The pellet, after separation, was resuspended in the same volume of the same buffer and the operation was repeated twice. The three combined supernatants have been concentrated under N_2 pressure using a XM-100 Diaflo membrane in the cold room. The concentrated extract was loaded onto a Sephadex G-100 column (2×83 cm) preequilibrated with the same buffer. The effluent from the column was monitored at 280 nm for protein. Essentially, therefore, the medium employed in this experiment, in contrast to that published previously [1], does not contain deoxycholate and has a higher ionic strength; in addition, all operations are carried out at 0–4°C. Experiments with deoxycholate, in contrast, have always been run at room temperature to avoid clogging of



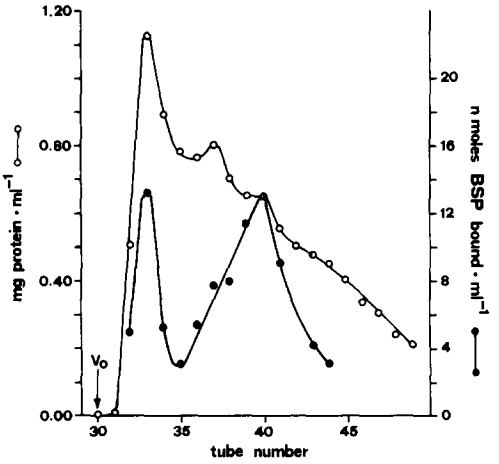


Fig. 1. (Left.) Elution profile from Sephadex G-100 column in the presence of 0.4% (w/v) sodium deoxycholate. Experimental conditions: the acetone powder, prepared as described [1], was extracted and treated as reported in that paper. The column (2×83 cm) was pre-equilibrated with a buffer solution containing 10 mM phosphate buffer (pH 7.8)/10 mM KCl/10 mM EDTA/0.4% deoxycholate. The protein load on the column was 90 mg in 5 ml. The elution has been carried out at room temperature. Volume of each fraction, 3.0 ml. O——O, protein content; ●——●, sulfobromophthalein (BSP) bound/ml.

Fig. 2. (Right.) Elution profile from Sephadex G-100 column in the presence of 0.05% (w/v) sodium deoxycholate. Experimental conditions as in Fig. 1 with the exception of deoxycholate concentration (see text). Protein load, 87 mg in 5 ml.

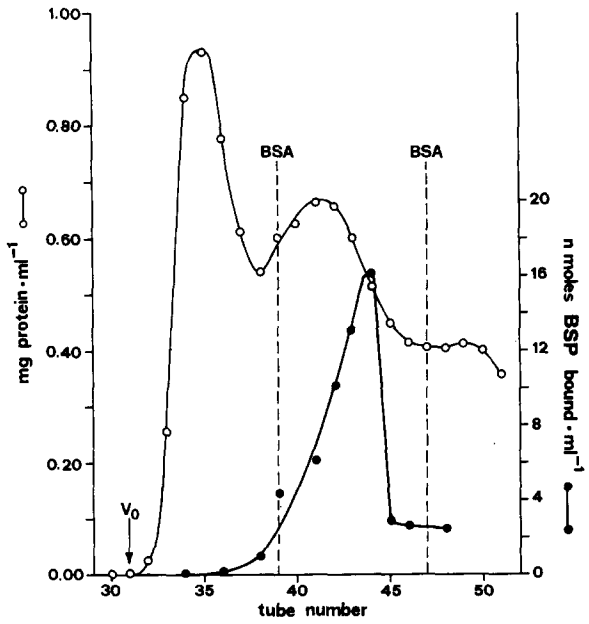


Fig. 3. Elution profile from Sephadex G-100 column in the absence of sodium deoxycholate. For experimental details see text. Protein load, 86 mg in 5 ml. BSA, bovine serum albumin; BSP, sulfobromophthalein.

the column. The elution profile from the Sephadex G-100 column is presented in Fig. 3. The graph shows that virtually all the binding capacity is confined in a single peak largely included in the internal volume. The figure shows also the position in the elution pattern of bovine serum albumin monomer and dimer respectively run on the same column in a separate experiment. The maximal binding activity is eluted with a volume corresponding to an M_r in the vicinity of 100000.

From the three experimental conditions used and from the three results obtained, it may be therefore concluded that the protein aggregates in the presence of deoxycholate to form a higher molecular weight complex. In the absence of the detergent, the molecular weight measured is still in the range of $1 \cdot 10^5$, however, suggesting the possible existence of a quaternary structure. In the attempt to resolve the molecule in its monomeric components, we have proceded to the identification of sulfobromophthalein binding peptides after treatment with 2-mercaptoethanol.

The electrophoretic analysis of the fraction

showing the highest binding activity after such a treatment is presented in Fig. 4. Samples were first added with sulfobromophthalein 1 mM and then treated at 100°C for 3 min in the presence of 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol. During the run, two distinct colored bands could be seen moving with different mobilities. One, very fast and rather diffuse, corresponded to the free dye and the second, very sharp, was associated with the protein. Both bands disappeared upon acidification with 12.5% trichloroacetic acid used as a fixative. If this step was prolonged for several hours, the first, fast-moving band diffused out so that, upon realkalinization with 0.1 M borate buffer (pH 10.5), only the protein-bound sulfobromophthalein was visualized. If the sample was kept for hours at alkaline pH, the coloured band faded away, indicating that the association of the dye to the protein is not maintained by a covalent bond.

Fig. 5 shows that the peptide(s) with sulfobromophthalein binding activity migrates in the gel together with the major protein component of the analyzed fraction.

It is worth mentioning that this strong binding capacity persists in spite of the heat pretreatment in the presence of the detergent, reduction by mercaptoethanol and polyacrylamide gel-electrophoresis. This finding is interesting, in addition, because it allows identification of the subunit(s) responsible for sulfobromophthalein binding as a peptide(s) exhibiting a molecular weight in the range of 35000–37000. Interestingly the binding capacity in the gel is preserved only if sulfobromophthalein is added before sodium dodecyl sulfate. From these experiments, we had the impression that the component was not a single disc in gel electrophoresis but rather two very close-moving protein bands. Preliminary experiments carried out with a sulfobromophthalein affinity chromatography column similar to that reported by Reichen et al. [6] indicated that the binding peptide(s) is (are) the only component(s) of bilitranslocase prepared in the absence of deoxycholate. The conclusion at this point was that the low molecular form of bilitranslocase obtained in the absence of deoxycholate, could be resolved in one or two subunits still capable of high affinity sulfobromophthalein binding with a molecular weight in the vicinity of 35000. It was therefore necessary, at this point, to try to convert the high molecular form of bilitranslocase (170000) obtained in the presence of deoxycholate into the same sulfobromophthalein-binding subunits as in the preparation without detergent.

With this aim, we have carried out a classical

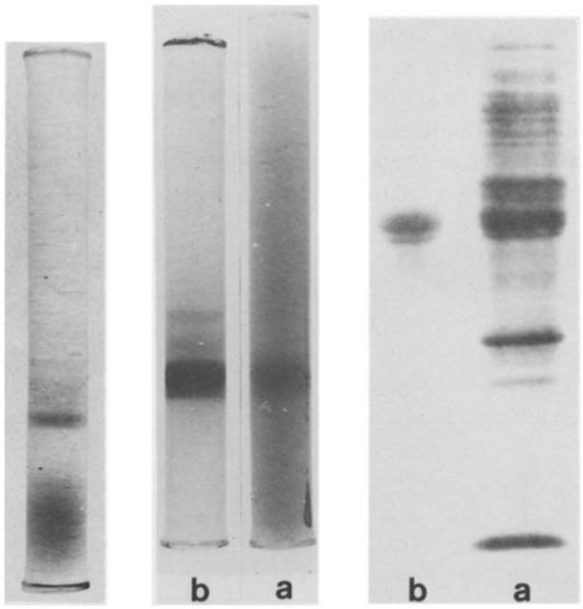


Fig. 4. (Left-hand gel.) Sodium dodecyl sulfate polyacrylamide gel electrophoresis of fraction No. 44 in Fig. 3. The material has been treated as described in the text and not stained for protein. Note the diffused free sulfobromophthalein still present in the lower part of the gel. Total protein per gel 120 μg.

Fig. 5. (Center two gels.) Sodium dodecyl sulfate polyacrylamide gel electrophoresis of fraction No. 44 in Fig. 3. Experimental conditions as in Fig. 4 except that gel a has been stained with Coomassie brilliant blue, whereas gel b has meanwhile been kept in 12.5% trichloroacetic acid. At the time of the photographic recording gel b was soaked in 0.1 M borate buffer (pH 10.5) and the picture was taken as soon as the purple blue band reappeared in the gel. Note that the lower diffuse-sulfobromophthalein region has disappeared during the treatment.

Fig. 6. (Right-hand two gels.) Polyacrylamide slab electrophoresis of fractions derived by deoxycholate treatment before and after preparative SDS gel electrophoresis. Sample a corresponds to pooled fractions as indicated in Fig. 1 between the two dashed lines. Sample b corresponds to the fraction included between the two dashed lines in Fig. 7. For experimental details see text.

deoxycholate extraction and purification [1] omitting only the last step which removes low molecular weight contaminants. The sulfobromophthalein binding fractions eluted from the Sephadex G-100 column have been depleted of their deoxycholate content by gel-filtration on a Biogel P2 column (2×100 cm) pre-equilibrated with a buffer solution consisting of 10 mM phosphate buffer (pH 7.8)/10 mM KCl/1 mM EDTA/10 μM sulfobromophthalein. Addition of sulfobromophthalein during gel-filtration resulted in a bilitranslocase preparation still fully capable of dye binding and water soluble despite the removal of the detergent. This experiment had been suggested by the observation that when bilitranslocase binding capacity is measured with a Biogel P2 column in the presence of 10 μM sulfobromophthalein, the protein is recovered still in a water-soluble form and totally depleted of its deoxycholate content. It was therefore concluded that binding of sulfobromophthalein by the protein could help in maintaining this membrane protein in solution even in the absence of the detergent. Obviously the material eluted from the column contained sulfobromophthalein in excess with respect to the original 10 μM concentration. From the measurement of this value, the binding capacity of the preparation may be calculated, with great accuracy in view of the high amount of protein in the eluate. In good agreement with the data already published [1], the binding capacity at this step was found to be in the range of 55–65 nmol sulfobromophthalein bound $\times$ mg protein.

When such a preparation, concentrated under N_2 pressure on a PM-10 Diaflo membrane, is analyzed by gradient slab electrophoresis in the presence of sodium dodecylsulphate and 2-mercaptoethanol, a number of peptides ranging from 90000 to 13000 are resolved (Fig. 6a). The most abundant components are two peptides (α, β) which visually bind sulfobromophthalein and of which the molecular weights may be calculated by comparison with standard proteins mobility as 37000 and 35000, respectively. Further purification of the two sulfobromophthalein binding subunits has been achieved by preparative gel electrophoresis carried out with an apparatus already described [14] in the presence of sodium dodecyl sulphate, 2-mercaptoethanol and sulfobromophtha-lein as mentioned for analytical procedure. Fig. 7 shows the elution pattern from the electrophoretic column. The two dashed lines indicate the fraction which contains the binding peptides. This fraction has been analyzed by slab electrophoresis and the results are present in Fig. 6b. The fraction contains virtually only the two binding components and the position is consistent with the two molecular weight values mentioned above.

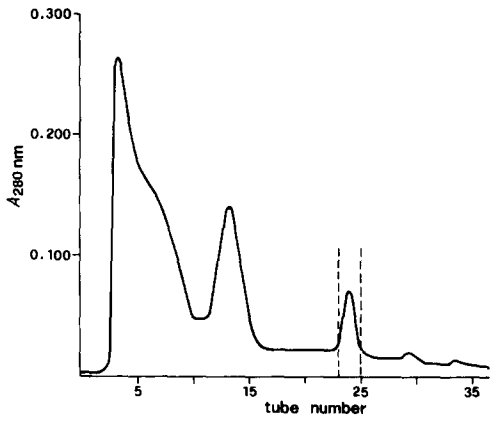


Fig. 7. Elution pattern from a preparative sodium dodecyl sulfate gel electrophoresis. The apparatus used has already been described in [14]. Experimental conditions: pooled fractions included between the two dashed lines in Fig. 1 were treated with 1 mM sulfobromophthalein and heated at 100°C for 3 min in the presence of 1% SDS and 1% 2-mercaptoethanol. The electrophoretic system consisted of two layers of gel of 5 ml each at 3 and 12% acrylamide, respectively. The buffer used for the run and for the elution was 50 mM Tris-glycine (pH 8.3)/1% SDS.

Discussion

Data presented in a previous paper [1] showed that bilitranslocase, the plasma membrane protein involved in hepatic uptake of sulfobromophthalein and other organic anions, shows an apparent molecular weight of 170000 in the presence of the detergent deoxycholate. This value corresponds, however, to an aggregated form which depends on the presence of the bile salt. In the absence of the detergent a more native form of the protein exhibits a molecular weight in the vicinity of 100000. Both forms, by reduction by 2-mercaptoethanol and in presence of sulfobromophthalein and sodium dodecyl sulfate, are resolved into two distinct subunits α and β with molecular weights of

37000 and 35500, respectively. The β subunit seems to be less abundant and it is possible that the physiological membrane component consists of an α_2-β structure. Similarly, the deoxycholate complex could consist of five subunits. A point which emerges from this experimentation concerns the very high affinity shown by the dissociated subunits for sulfobromophthalein in absence of deoxycholate. Although direct measurement of sulfobromophthalein binding on the isolated peptides is still difficult due to the presence of the dye during the purification procedure, there are indications that the dissociation constant of the complex is even lower than 5 μM, the value found for the polymeric structure. This finding was expected from the observation that sulfobromophthalein is not detached during electrophoresis. The observation that the molecular weight of 170000 derives presumably from the aggregation of five peptides implies a much more physiological ratio in terms of mol dye bound per mol protein. A question which remains to be solved concerns the relation between the subunits composition of bilitranslocase resulted from this study and other membrane sulfobromophthalein-binding proteins reported in literature [6,7]. Recently, in fact, Reichen and Berk [6] reported the isolation of an organic anion-binding protein from liver plasma membrane capable of high affinity sulfobromophthalein binding and displaying a molecular weight of 60000. Similarly, Wolkoff and Chung [7] have isolated by affinity chromatography a sulfobromophthalein-binding component with molecular weight of 55000. We are not in a position to offer an explanation capable of reconciling the diversity of these figures which, in view of the experimental conditions employed, should all refer to monomeric structures. The truth will probably be reached by direct immunological and chemical comparison of the three protein preparations.

At the functional level our bilitranslocase preparation has been proven capable of reconstituing sulfobromophthalein transport in a model system, as already reported briefly [15] and published in extenso in the accompanying paper [16].

Acknowledgements

This work has been supported by grants form the Consiglio Nazionale delle Ricerche (Rome) and by a special grant from University of Trieste. The Authors are deeply indebted to Miss Luisella Vici for excellent typing of the manuscript.

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 Tiribelli, C., Panfili, E., Sandri, G., Frezza, M. and Sottocasa, G.L. (1976) in Diseases of the Liver and Biliary Tract (Leevy, C.M., ed.), pp. 55–59, Karger, Basel
3 Sottocasa, G.L., Panfili, E., Sandri, G., Liut, G.F., Tiribelli, C., Luciani, M. and Lunazzi, G.C. (1979) in Macromolecules in the Functioning Cell (Salvatore, F., Marino, G. and Volpe, P., eds.), pp. 205–218, Plenum Press, New York
4 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, S. and Klingenberg, M., eds.), pp. 451–458, Elsevier/North-Holland Biomedical Press, Amsterdam
5 Tiribelli, C., Luciani, M., Lunazzi, G.C., Gazzin, B., Renaud, G., Infante, R. and Sottocasa, G.L. (1981) in Familial Hyperbilirubinemia (Okolicsanyi, L., ed.), pp. 43–49, John Wiley&Sons, Chichester
6 Reichen, J. and Berk, P.D. (1979) Biochem. Biophys. Res. Commun. 91, 484–489
7 Wolkoff, A.W. and Chung, C.T. (1980) J. Clin. Invest. 65, 1152–1161
8 Demuskin, V.P. and Shalina, N.M. (1980) Anal. Biochem. 105, 230–232
9 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404–427
10 Laemmli, U.K. (1970) Nature 227, 680–685
11 Andrews, P. (1964) Biochem. J. 91, 222–233
12 Mosbach, E.H., Helen, J., Halpern, E. and Kendall, F.E. (1954) Arch. Biochem. Biophys. 51, 402–410
13 Waddel, W.J. (1956) J. Lab. Clin. Med. 48, 311–316
14 Sottocasa, G.L., Sandri, G., Panfili, E., de Bernard, B., Gazzotti, P., Washington, F.D. and Carafoli, E. (1972) Biochem. Biophys. Res. Commun. 47, 808–813
15 Sottocasa, G.L., Sandri, G., Baldini, G., Lunazzi, G.C., Gazzin, B. and Tiribelli, C. (1980) Gastroenterol. 79, 1125
16 Sottocasa, G.L., Baldini, G., Sandri, G., Lunazzi, G.L. and Tiribelli, C. (1982) Biochim. Biophys. Acta, 685, 123–128