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Reconstitution of sulfobromophthalein transport in erythrocyte membranes induced by bilitranslocase

Maddalena Miccio, Gian Carlo Lunazzi, Bruno Gazzin and Gian Luigi Sottocasa

Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, University of Trieste, Trieste (Italy)

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Bilitranslocase, the protein responsible for the anion translocation at the sinusoidal plasma membrane level in liver, was shown to be able to reconstitute the transport of sulfobromophthalein in liposomes in the past. The protein preparation used in those experiments consisted of two subunits of 35.5 and 37 kDa. The isolated 37 kDa protein, when inserted in erythrocyte membrane vesicles, confers to the particles the ability to carry out an electrogenic transport of sulfobromophthalein. The effect is specific and can be inhibited by monospecific polyclonal antibodies raised against the protein. In may be concluded that the 37 kDa protein band, present in previous preparations of bilitranslocase, is not only a necessary but also a sufficient component of the transport system for bilirubin and functional analogues.

Bilitranslocase is a plasma membrane protein isolated in our laboratory from rat liver [1,2]. It may be considered the carrier molecule responsible for the translocation of a number of cholephilic anions including unconjugated bilirubin and functional analogues such as phthalein dyes (sulfobromophthalein) [3,4]. The protein was isolated in the past either in the presence or absence of deoxycholate and displayed a different level of aggregation (molecular masses 170 and 100 kDa, respectively) [1,2]. These preparations could be resolved by SDS-gel electrophoresis into two types of subunits of 37 and 35.5 kDa. More recently, by ion-exchange chromatography, we could obtain a bilitranslocase preparation consisting exclusively of the 37 kDa protein band [4]. This protein was identified as the necessary component of the carrier in that monoclonal antibodies, acting as inhibitors of the transport, recognize specifically only this membrane protein. Conversely, polyclonal monospecific antibodies, raised against this protein, were found to inhibit the transport. Reconstitution of sulfobromophthalein transport in liposomes [5] was successfully carried out in the past using bilitranslocase obtained either in the presence or in the absence of deoxycholate. In view of the fact that the two preparations, in addition to the 37 kDa band contained also a 35.5 kDa subunit, it could not be excluded that the reconstituted transport was to be attributed to this peptide band. On the other hand, erythrocyte ghosts and resealed plasma membrane vesicles obtained therefrom were shown to lack sulfobromophthalein transport [6]. We attempted therefore to insert pure bilitranslocase in erythrocyte membrane to confer sulfobromophthalein transport ability to the system.

Bilitranslocase was prepared according to Miccio et al. [4]. It was electrophoretically homogeneous in the presence of SDS and 2-mercaptoethanol (see Fig. 2).

Human erythrocyte ghosts were obtained according to Fairbanks et al. [7]. The final pellet was diluted 40 times in 5 mM phosphate buffer (pH 8.0) and kept in ice for 4 h. The ghosts, recovered from the incubation mixture by centrifugation at 45 000 × *g* for 15 min, were taken up in 150 mM NaCl, 5 mM phosphate buffer (pH 7.4) and converted to small vesicles by forcing them to pass through a 27-gauge needle, at least three times. The vesicles were either used as such, or mixed (3 : 2) to a bilitranslocase solution (150 μg/ml) in the presence of 0.01% (v/v) Tween 20 [8].

The excess detergent could be removed by overnight dialysis against saline containing 5 mM sodium phosphate buffer (pH 7.4). The latter step may be omitted without effect on the rate of transport measured. Fig. 1 shows an experiment in which the movement of sulfobromophthalein is followed spectrophotometrically at the wavelength pair 580–540 nm [6]. A deflection of the signal indicates the entry of the dye into the more

Correspondence: G.L. Sottocasa, Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, Università di Trieste, via A. Valerio 32, I-34127 Trieste, Italy.

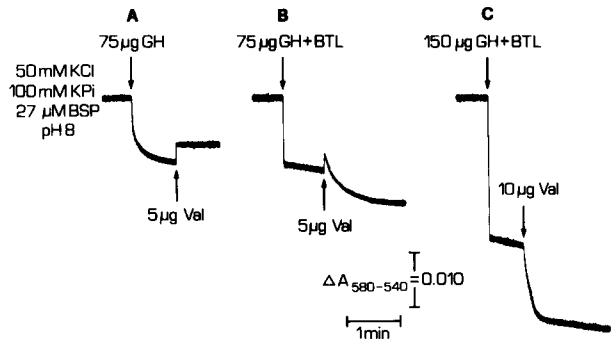

Fig. 1. Sulfobromophthalein transport in erythrocyte ghost vesicles before and after insertion of bilitranslocase. Experimental conditions: room temperature, final volume 2.4 ml; Sigma ZW-II recording dual wavelength spectrophotometer. (A) Control: GH, human erythrocyte ghosts, vesiculated and resealed. (B) and (C). BTL, bilitranslocase. The incorporation of the carrier was performed as described in the text.

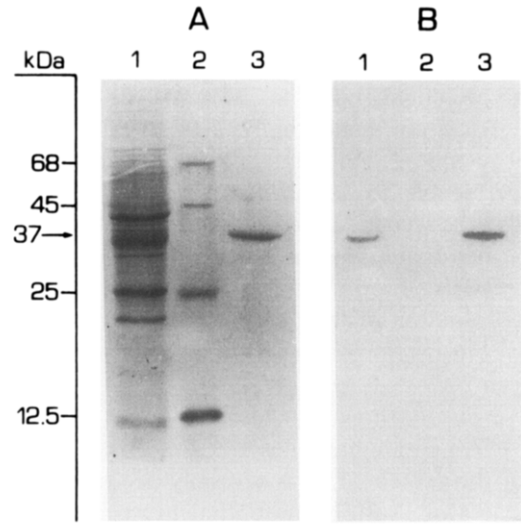

Fig. 2. SDS-PAGE of bilitranslocase preparation and corresponding immuno-blot. (Panel A) Lane 1, crude extract from acetone powder [1]; lane 2, protein standards (serum albumin, chymotrypsinogen, ovalbumin and cytochrome *c*); lane 3, purified bilitranslocase. 10% polyacrylamide, staining with Coomassie brilliant blue. (Panel B) Electroimmuno-blot using antibilitranslocase as primary antibody. Development by biotin-streptavidin kit supplied by Amersham, U.K. Lane 1, extract; Lane 2, protein standards; Lane 3, bilitranslocase.

acidic internal compartment of the vesicles. Upon addition of the vesicles a first drop in absorbance is recorded. As discussed elsewhere [6], this may be attributed, in addition to entry of the dye, to a combination of effects such as light scattering, absorption of the compound to the surface of the membrane etc. Addition of valinomycin results in the creation of a potential across the membrane which may induce an electrogenic movement of the dye. In liver plasma membrane vesicles, the initial rate of this absorbance change was shown to be linearly related to the amount of bilitranslocase present in the assay [6]. Trace A shows that the control vesicles do not respond, as expected, to the addition of valinomycin. Trace B and C display a different morphology in comparison with trace A: (1) the change in absorbance induced by the addition of the vesicles stabilizes almost immediately, indicating that the equilibrium between the two compartments may be reached instantaneously; (2) the addition of valinomycin is followed by a further deflection of the signal, indicating the existence of an electrogenic movement of the dye. It may be observed that the rate of electrogenic entry is doubled by doubling the amount of vesicles added to the sample (trace C). Were the effect observed specifically induced by the insertion of bilitranslocase into the plasma membrane, it was expected that specific antibodies to bilitranslocase could abolish it. To check this, rabbits were immunized using 0.3 mg purified bilitranslocase, obtained as previously described [4]. Antigen plus incomplete Freund's adjuvant was given in four injections into the four legs. The operation was repeated a month later. A secondary immuno response was evoked by intramuscular administration of 0.2 mg antigen and, at 15-day intervals, 30 ml blood samples were taken from the marginal vein of the ear. The serum IgG fraction was obtained by Keckwick's method [9]. Fig. 2 (panel A) shows the electrophoretic pattern of bilitranslocase (lane 3) and acetone powder extract of rat liver plasma membranes (lane 1). For comparison, in lane 2 appropriate protein standards are also included. In panel B the corresponding immuno-decorated patterns, after electro-blotting are presented. Clearly, the antibody preparation reacts exclusively with bilitranslocase even in the crude extract. From these data it may be concluded therefore that: (1) the bilitranslocase preparation consists of a single, electrophoretically homogeneous protein and (2) the antibody preparation is mono-specific. The effect of the antibody was tested on the reconstituted transport activity. Fig. 3 shows that incubation of bilitranslocase-containing vesicles with the IgG preparation completely abolished the transport activity. In agreement with previous data [4] the assay was not influenced by pretreatment of the vesicles with

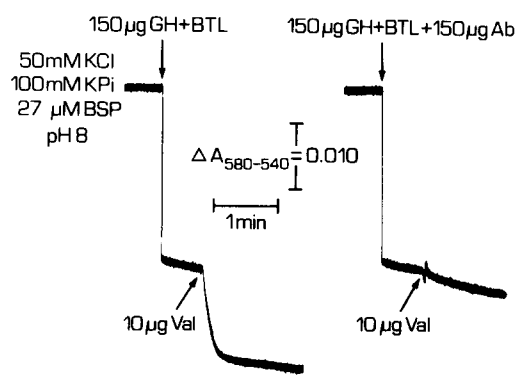

Fig. 3. Effect of antibilitranslocase antibodies on sulfobromophthalein transport by reconstituted human erythrocyte ghosts vesicles. Experimental conditions as in Fig. 1; for further details see text.

either serum from non immunized rabbits nor by IgG derived therefrom.

Data presented show that Bilitranslocase isolated by ion-exchange chromatography may be inserted into a natural membrane, thus making the membrane specifically permeable to sulfobromophthalein. Phenomenologically the reconstituted system resembles a liver plasma membrane vesicles preparation [6]. At variance with previous experiments with pure phospholipids [5], the incorporation of the protein does not occur spontaneously, but it requires the addition of a detergent such as Tween 20.

These results show that bilitranslocase, identified as the 37 kDa peptide band described in previous preparations of the protein [1,2], is not only necessary but also sufficient for sulfobromophthalein transmembrane transport.

On the basis of the previous data, the possibility was considered that the 37 kDa protein band could be the recognition site for cholephilic anions. Presumably such a recognition site was located on the outer surface of the membrane and could therefore be accessible to specific antibodies [10]. Theoretically, the 35.5 kDa protein, co-purified with bilitranslocase with previous methods, could have acted in series with the other subunit in making the membrane permeable to the transported species. Such a hypothesis is no longer tenable in view of the present data and we are forced to concluded that, in the natural membrane, the only protein involved in the transport function for cholephilic anions is the 37 kDa protein band. As for the meaning of the 35.5 kDa peptide previously described [2], it may be regarded either as a degradation product of the heaviest one or as an acidic protein co-purified with bilitranslocase for electrostatic reasons. It may be recalled here that the 37 kDa protein has a p*I* higher than 9.0 [4]. We propose to limit the term bilitranslocase to this basic protein which, on the basis of the experimental evidence collected, is to be regarded as the carrier for cholephilic anions at the sinusoidal pole of hepatocytes.

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