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Bilitranslocase can exist in two metastable forms with different affinities for the substrates

Evidence from cysteine and arginine modification

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Bilitranslocase is an organic anion carrier involved in bilirubin and phthalein uptake by the liver. In rat liver plasma membranes, its function is assayed by recording the electrogenic sulfobromophthalein movement. This has been found to be inhibited by both cysteine-specific and arginine-specific reagents. Inhibition is both partial and it occurs to the same extent, i.e. approximately 50 %. The effects are not additive. Here we describe the mechanism underlying the above observations. It is concluded that bilitranslocase occurs in two possible states, featured by high and low affinity for the substrates (for sulfobromophthalein, K_m = 5 μM and 37 μM, respectively). Cysteine- or arginine-reactive reagents, by reacting selectively with the low-affinity form, entrap it and shift the equilibrium between the two forms, so that, at completion, only the low-affinity form is present. The substrate concentration in the standard transport assay is 39 μM, a value at which the modified low-affinity form operates in the range of half-maximal velocity. This explains both the apparent half-inhibition measured after the chemical treatments and the lack of additivity. In addition, the substrates are shown to enhance the rate of conversion from the low-affinity to the high-affinity form of the translocator, thus favouring its high-affinity form under physiological conditions.

Keywords: bilitranslocase; bilirubin; carrier protein; transport; liver.

Bilitranslocase is a carrier protein [1] located selectively at the sinusoidal domain of the liver cell plasma membrane [2]. As suggested by an *in vivo* experiment, it is involved in bilirubin uptake by the liver [3]. Both in liposomes [4] and in erythrocyte ghosts [5], the purified protein has been shown to reconstitute an electrogenic sulfobromophthalein transport, of the uniport type. In rat liver plasma membrane vesicles an identical type of transport has been demonstrated [2] and is entirely related to purified bilitranslocase [6]. In this system, sulfobromophthalein transport has been reported, on a number of occasions, to be partially inhibited both by sulfhydryl-specific and arginyl-specific reagents [7−9]. In either case, the same level of inhibition, about 50 %, was observed and the effects were not additive [10]. The conclusion was reached that the modified amino acid residues are closely located on the sulfobromophthalein-carrier protein so that a modification of that particular domain can produce similar or even identical functional effects. Protection experiments [8, 9] carried out with a variety of substrates, including unconjugated bilirubin, conferred specificity to the effects found, in that no indefinite vesicular damage could be called upon to explain the reduced transport activity; even more importantly, these data pointed to a specific bilirubin binding by bilitranslocase. Additionally, the inhibitory effects could be completely reversed upon removal of the chemical modification. Once more, and somewhat surprisingly, the same substrates were shown to accelerate markedly the recovery to the full activity [8, 9], again linking the effect to a specific interaction between bilitranslocase and its ligands, notably bilirubin.

In our attempt to gain a mechanistic view of the partial, non-additive, inhibitory effect brought about by cysteine and arginine modifications, a more stringent kinetic analysis of the native and the modified forms of bilitranslocase in the membrane was undertaken. Results are presented here that allow us to infer the existence of two forms of the carrier, co-existing in equilibrium, whose interconversion is affected by the substrates.

MATERIALS AND METHODS

Rat liver plasma membrane vesicles were obtained as described by van Ameslvoort et al. [11]. Throughout this work a single vesicles pool stored in aliquots under liquid nitrogen was employed. Its qualities were assessed as, and found to be consistent with, those previously described [2].

Stock solutions of the following reagents were used: 2 mM *p*-hydroxymercuribenzoate (Sigma) in 1 mM NaOH; 0.2 M methylglyoxal (Sigma) and 0.1 M *N*-ethylmaleimide (Sigma) in 0.1 M Hepes (Sigma) pH 7.4; mercaptoethanol (BDH); 5 % (mass/vol.) sulfobromophthalein (Merck); 10 mM nicotinic acid (Merck) in 0.1 M Hepes, pH 7.4.

Concentration measurements of the following compounds were carried out spectrophotometrically: sulfobromophthalein ($\varepsilon_{580} = 64\times10^3\ M^{-1}\ cm^{-1}$) [2], *p*-hydroxymercuribenzoate

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Abbreviations. BSP, sulfobromophthalein; C, high-affinity form of bilitranslocase; C*, low-affinity form of bilitranslocase; I, irreversible inhibitor of bilitranslocase, reacting specifically with either cysteines or arginines; Nbs_2, 5,5′-dithio-bis(2-nitrobenzoate); oatp, organic-anion-transporting peptide.

($\varepsilon_{255} = 4.4\times10^3\ M^{-1}\ cm^{-1}$) [12], *N*-ethylmaleimide ($\varepsilon_{305} = 0.62\times10^3\ M^{-1}\ cm^{-1}$) [12], mercaptoethanol [13].

Experiments of bilitranslocase inactivation/reactivation were performed in two-steps: prior incubation and transport assay. Step 1: prior incubation. 1−3 mg protein/ml vesicles, 33 mM Hepes, pH 7.4, 133 mM NaCl; unless otherwise specified in the figure legends, the inhibitor was 0.15 mM *p*-hydroxymercuribenzoate; this reaction mixture was kept at 37 °C; further manipulations are described in the figure legends; aliquots thereof were transferred into the assay system. Step 2: transport assay. Bilitranslocase activity was assayed by the spectrophotometric technique previously described, enabling the continuous recording of sulfobromophthalein movements into vesicles, monitored at the wavelength pair 580 nm−514.4 nm [2]. The test was carried out by the addition of 6−10 µl of previously incubated vesicles to a cuvette, containing 2 ml assay medium (0.1 M potassium phosphate, pH 8.0, 39 µM sulfobromophthalein), under stirring conditions, at room temperature. 4 s later, when no net sulfobromophthalein movement is detected, further sulfobromophthalein entry into vesicles was driven by the K^+ diffusion potential generated by the injection of 10 µg valinomycin (Fluka, dissolved in 10 µl methanol) into the cuvette. The initial, linear absorbance drop lasts nearly 1 s and is the measure of bilitranslocase transport activity [2, 6].

Conditional for the correct understanding of the results is an appreciation that the prior incubation and the transport steps are totally separated. On account of the following facts: (a) 100-fold dilution of all components of the mixture in the assay cuvette; (b) temperature shift; (c) time scale of the second step (< 5 s) compared with the first one (minutes), any chemical reaction occurring in the prior incubation step is halted in the assay step; conversely, the addition of the sample to the assay cuvette virtually does not change the assay-medium composition. Independent, preliminary control experiments (data not shown) were carried out to check the validity of these statements. A systematic control is, however, displayed in the figures as the time zero value, when the transport activity of the complete mixture is assayed, and all other data points obviously refer to variations thereof.

Protein determination was carried out by the Bradford method [14]. All other chemicals were of the highest purity commercially available.

Data were analyzed by the mathematical tools offered by the SigmaPlot program (Jandel Scientific Software GmbH).

RESULTS

Transport kinetics of native and chemically modified bilitranslocase. The concentration dependence of the initial rate of sulfobromophthalein entry into vesicles was examined in the native, the cysteine-modified and the arginine-modified carrier. Selective modification of the 5,5′-dithio-bis(2-nitrobenzoate) (Nbs_2)-sensitive class of cysteines is achieved also with *p*-hydroxymercuribenzoate, as previously reported [7, 8]. Results of this experiment are shown in Fig. 1. Two saturation curves are obtained, one for the control and the other for the modified samples. Both data sets were fitted to the Michaelis-Menten equation. The K_m values were found to be 5.3 µM and 37.3 µM sulfobromophthalein for the native and the modified carrier, respectively. In contrast, identical V_{max} values were found for the three forms of the carrier. The inset in Fig. 1 shows the double-reciprocal plot, for a visual appreciation of this outcome. Kinetically speaking, the chemical modification of these two types of amino acid residues is indistinguishable from typical competitive inhibition. Since we are dealing with irreversible inhibitors

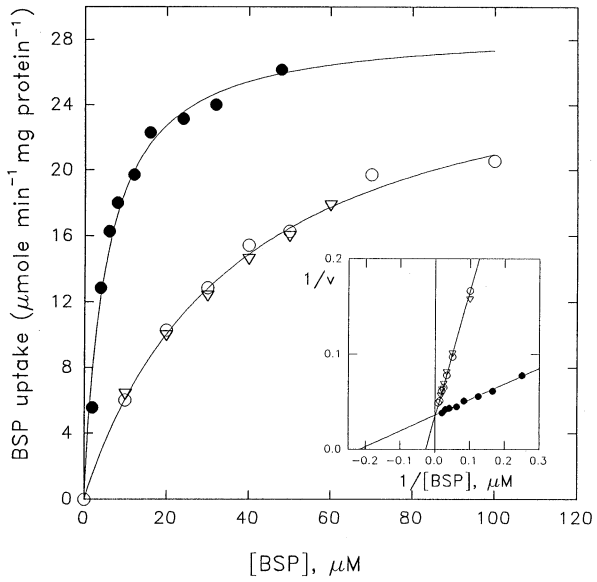

Fig. 1. Substrate concentration dependence of electrogenic sulfobromophthalein uptake rate in plasma membrane vesicles under different conditions. Experimental conditions. Prior incubation: vesicles (3.5 mg protein/ml) were incubated as described in Materials and Methods, in the absence (●) or in the presence of either 0.15 mM *p*-hydroxymercuribenzoate (○) or 10 mM methylglyoxal (▽). Transport assay: 4.3 µl of incubated vesicles were put into 2 ml assay medium containing variable sulfobromophthalein (BSP) concentrations. The assay was carried out as described in Material and Methods. Data were fitted to the Michaelis-Menten equation. Parameters: $V_{max} = 28.8 \pm 0.9$ µmol min^{-1} mg^{-1} protein^{-1}; $K_m = 5.32 \pm 0.63$ µM (filled symbols); $V_{max} = 28.8 \pm 0.8$ µmol min^{-1} mg^{-1} protein; $K_m = 37.35 \pm 2.4$ µM (open symbols). Inset: Double-reciprocal plot of the two data sets. Data were fitted by linear regression analysis.

and, in addition, the system still transports (with unaltered V_{max}), a ternary complex (inhibitor-carrier-sulfobromophthalein) is to be postulated. This is reminiscent of type Ib competitive inhibition, according to Dixon and Webb [15].

These results provide the explanation for the apparent half-maximal inactivation following modification of either the Nbs_2-sensitive cysteine(s) [7, 8] or the arginine(s) [9] previously reported. The routine substrate concentration used in the bilitranslocase transport assay is 39 µM. Under these conditions, the native carrier operates close to V_{max}. In contrast, the data of Fig. 1 show that the modified carrier acts just in the range of its half-maximal velocity. A corollary of this situation is that 50 % inhibition of the carrier activity (under strictly fixed assay conditions) implies that all the carrier molecules in the sample are combined with the inhibitor. This offers an experimental tool to titrate the actual percentage of the modified carrier, C-I (i.e. 50 % transport activity = 100 % [C-I]; 100 % transport activity = 0 % [C-I]).

The unavoidable conclusion to be drawn from the data above is that, irrespective of the amino acid targeted (cysteine or arginine) and also of the chemical nature of the reagents employed (mercurials, disulfides, maleimides, dicarbonyls, etc.), only one kinetic species appears, featured by a sevenfold higher K_m value.

The electrogenic sulfobromophthalein entry into vesicles has been shown to be partially inhibited also by the serine-specific reagent phenylmethylsulfonyl fluoride [16]. In this case, maximal inhibition is only by about 30 %. A kinetic investigation of the same kind as that shown in Fig. 1 revealed that phenylmethylsulfonyl fluoride affected only the maximal rate of transport (19.9 µmol min^{-1} mg protein versus 28.8 µmol min^{-1} mg

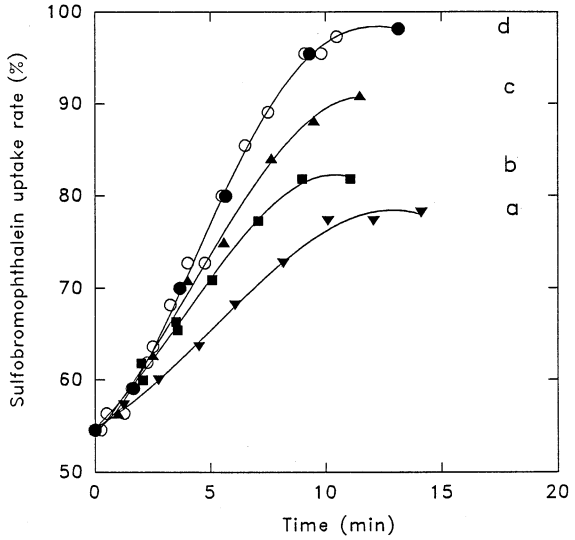

Fig. 2. Bilitranslocase reactivation induced by mercaptoethanol: effect of early removal of the reactivator. Experimental conditions. Prior incubation: vesicles were incubated with 0.15 mM *p*-hydroxymercuribenzoate for 8 min before the addition of 0.417 mM mercaptoethanol (○); 0.417 mM *N*-ethylmaleimide was added 20 (▼), 30 (■), 40 (▲) and 80 (●) s later. Transport assay: as in Fig. 1; see also Materials and Methods. The reactivation curves were obtained by fitting data to third-order regression.

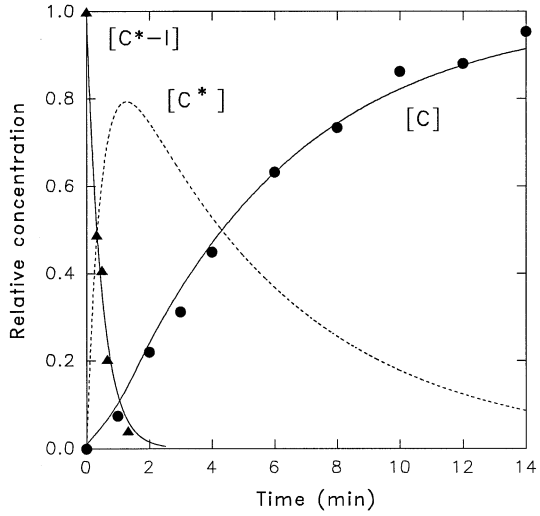

Fig. 3. Plot of C*-I, C* and C concentrations versus time. [C*-I] data points (▲) were obtained from the last data points of curves a, b, c and d in Fig. 2. These were fitted to a single exponential decay. [C] data points (●) were obtained with 0.417 mM mercaptoethanol, as described in Fig. 2; they were fitted to Eqn (2) in the text. The curve describing [C*] (dashed line) was computed as described in the text. Parameters: $k_1 = 2.109 \pm 0.1689$ min^{-1}; $k_2 = 0.179 \pm 0.006$ min^{-1}.

protein^{-1}), whereas the K_m values were left much the same (5.0 μM and 5.32 μM). It can therefore be concluded that phenylmethylsulfonyl fluoride inhibits bilitranslocase transport activity by a different mechanism than that found with cysteine and arginine reagents.

Complex bilitranslocase reactivation kinetics. Fig. 2 shows the kinetics of reactivation induced by mercaptoethanol to vesicles pretreated with *p*-hydroxymercuribenzoate. Clearly, reactivation occurs sigmoidally, as previously reported [8, 9]. This is the typical time-course of a two-step, irreversible reaction, which implies the formation of an intermediate before the appearance of the final product [17]. It may be reasonably assumed therefore that such an intermediate is the free carrier, still displaying a high K_m for transport. We could denote this compound as C*. This mechanism can be so described:

$$C^* - I \xrightarrow{k_1} C^* + I \xrightarrow{k_2} C + I\,. \qquad (1)$$

If we call the two rate constants k_1 and k_2, the kinetics of C appearance can be described by the following equation [17]:

$$[C] = [C^* - I]\,\{1 + [1/(k_1 - k_2)]\,[k_2 e^{(-k_1 t)} - k_1\, e^{(-k_2 t)}]\}\,. \qquad (2)$$

Kinetics of mercurial displacement from bilitranslocase. The other curves of Fig. 2 derive from an experiment designed to measure the rate of C*-I disappearance. Mercaptoethanol was added to vesicles previously incubated with the mercurial, in order to displace the inhibitor from the carrier and so induce its reactivation. Curve a was obtained by adding a titrated amount of *N*-ethylmaleimide 20 s after the addition of mercaptoethanol. The addition results in the removal of mercaptoethanol, thus stopping the first of the two consecutive reactions written in Eqn (1). Since, under these conditions, only a fraction of C*-I could be converted to C*, only an equivalent fraction of C could be recovered at the end. Curves b, c and d were obtained in the same way, but *N*-ethylmaleimide was added at later times, i.e. at 30, 40 and 80 s, respectively. Clearly, the later mercaptoethanol was removed, the higher the ultimate recovery of C. Particularly meaningful is curve d, which superposed with the control one, indicating that *N*-ethylmaleimide was not in excess, having reacted only with free mercaptoethanol while not with bilitranslocase cysteines. Another control (data not shown) established that *N*-ethylmaleimide was not defective, since its addition before mercaptoethanol, as expected, completely prevented reactivation.

The last data points of each curve are a measure of the concentration of the activated carrier, [C], at equilibrium. Since, at those times of the experiment, $[C_{tot}] = 1 = [C^*\text{-}I] + [C]$, C concentration could be calculated from these data and plotted versus the time of *N*-ethylmaleimide addition. They fitted an exponential decay equation, with a rate constant $k_1 = 2.1$ min^{-1}.

Disclosure of an intermediate C* in the bilitranslocase-reactivation reaction. Taking $k_1 = 2.1$ and fitting the reactivation data points to Eqn (2), k_2 was found to be 0.18 min^{-1}. Then, given that at any time $[C_{tot}] = 1 = [C^*\text{-}I] + [C^*] + [C]$, by difference [C*] can be calculated [17].

Fig. 3 collates the time-course of the concentration changes of all three forms of the carrier, i.e. C*-I, C* and C. C* appears as a transient intermediate, that rises steeply to a considerably high concentration and then slowly decays to C.

Effect of substrates on the rate of C*-to-C conversion. Our next target was to characterize the reported rate-enhancing effect of bilitranslocase ligands on the reactivation kinetics [8, 9]. The effect of sulfobromophthalein on the reactivation rate was therefore examined. The conditions of the experiment were the same as those of Fig. 2. It was chosen to add the substrate 1.5 min after the addition of mercaptoethanol, because at that time the prevailing species of the carrier is C*, and the appearance of C is obviously related to only k_2. Fig. 4A shows that sulfobromophthalein enhances the reactivation rate in a concentration-dependent manner. Fig. 4B is a plot of k_2 versus sulfobromophthalein concentration. The data fit a hyperbolic function, with a limiting value of 1.9 min^{-1} and a half-saturation value of

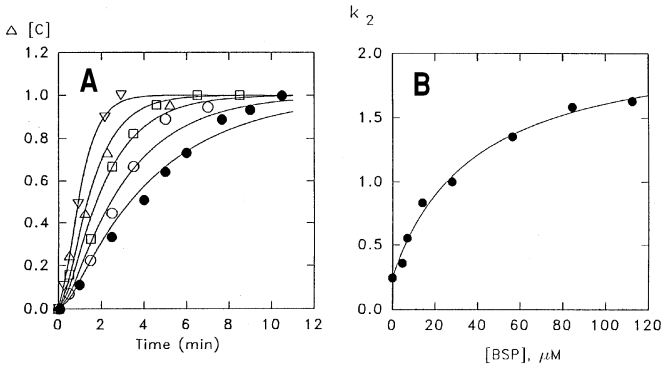

Fig. 4. Effect of sulfobromophthalein on the rate of mercaptoethanol-induced conversion to the high-affinity conformer of bilitranslocase. (A) Time-course of the conversion. (B) Dependence of the C*-to-C conversion rate constant (k_2) upon sulfobromophthalein concentration. (A) Experimental conditions. Prior incubation: vesicles were incubated with 0.15 mM *p*-hydroxymercuribenzoate for 8 min before the addition of 0.417 mM mercaptoethanol; increasing sulfobromophthalein concentrations were added 1.5 min later; for clarity, reactivation time-courses are shown for only the following conditions: control (●), 4.69 (○), 7.03 (□), 14.06 (△) and 112.5 (▽) μM sulfobromophthalein. Transport assay: see Materials and Methods. Curves were obtained by taking $k_1 = 2.1$ min^{-1} and fitting data to Eqn (2) in the text and respective k_2 values were found. (B) Plot of k_2 versus [sulfobromophthalein]; data were fitted to the equation: $k_2 = [k_{2max}[\text{sulfobromophthalein}]/(K_d + [\text{sulfobromophthalein}])] + k_0$, where k_0 is the conversion rate constant in the absence of substrates (0.2450 ± 0.0157 min^{-1}); parameters: $k_{2max} = 1.877 \pm 0.122$ min^{-1}; $K_d = 37.68 \pm 6.18$ μM sulfobromophthalein.

37.7 μM. From all of these data it can be concluded that the enhancing effect of sulfobromophthalein on the reactivation rate is certainly due to a specific acceleration of the transition from the low-affinity to the high-affinity state of the carrier. The half-saturation value is a measure of the dissociation constant of the C*-sulfobromophthalein complex, which is virtually the same as the K_m value of the modified carrier (37.3 μM) already found.

The effect of nicotinic acid on the reactivation rate was examined in an experiment designed exactly as the previous one. This ligand was chosen because, like sulfobromophthalein, it is freely soluble in water. Based on previous research [16], nicotinate is to be regarded as the strictest and the simplest functional analogue of bilirubin. From the data obtained (not shown), the dissociation constant of the C*-nicotinate complex was found to be 78.6 nM, which is higher than that of the C-nicotinate complex (11 nM, [16]) by, again, a factor of about seven.

Testing the effect sulfobromophthalein on the rate of mercurial displacement from bilitranslocase. To establish whether the substrates enhance the reactivation rate also by modulating k_1, the rate of C*-I disappearance in the presence of sulfobromophthalein was measured. The experimental design was the same as that reported in Fig. 2, but mercaptoethanol addition was preceded by the addition of 26.2 μM sulfobromophthalein. Fig. 5 is the plot of the pooled data obtained either in the presence or in the absence of sulfobromophthalein. Under both conditions, C*-I disappearance can be described by a common, single exponential, with much the same rate constant as determined before (2.05 min^{-1}). These data enable us to conclude that the substrate rate-enhancing effect is solely due to the modulation of k_2.

Pathways to bilitranslocase inhibition. The data shown so far provide a mechanism for the reactivation of bilitranslocase following cysteine (or arginine) restoration. As to the mechanism of the carrier inhibition, the above results allow to envisage two alternative pathways:

(A) $\quad C + I \rightarrow C - I \rightarrow C^*\text{-}I$

(B) $\quad C \leftrightarrow C^*;\ C^* + I \rightarrow C^*\text{-}I\ .$

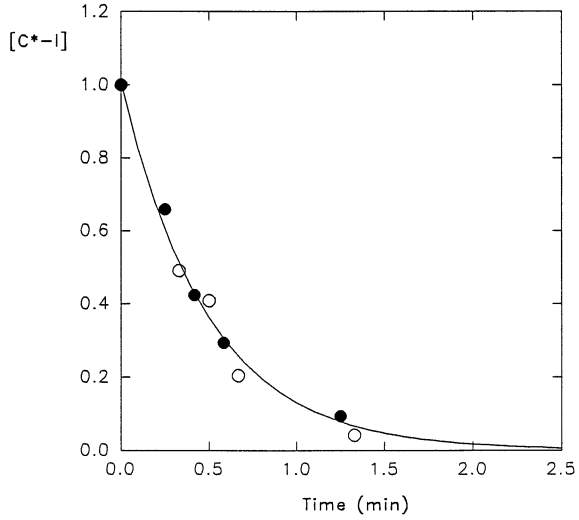

Fig. 5. Time-course of C*-I disappearance induced by mercaptoethanol: effect of sulfobromophthalein. C*-I concentrations in the absence of sulfobromophthalein (○) were obtained from the last data points of curves a, b, c and d in Fig. 3. Corresponding values in the presence of 26.2 μM sulfobromophthalein (●) were obtained as described in the text. The pooled data were fitted to a single exponential decay ($k_1 = 2.050 \pm 0.0951$ min^{-1}). Other details as in Fig. 2.

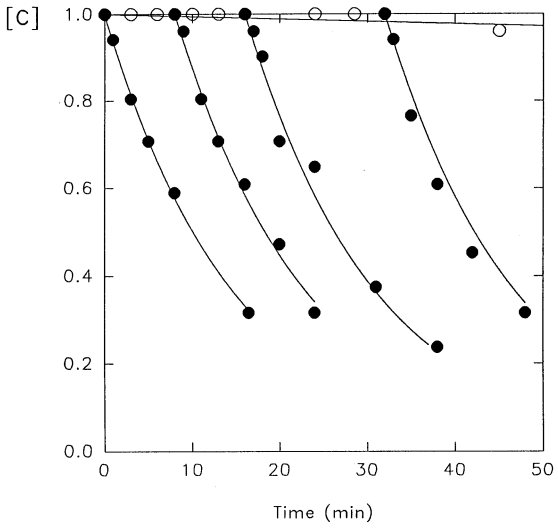

Fig. 6. Time-course of the bilitranslocase high-affinity form disappearance induced by *p*-hydroxymercuribenzoate at 0 °C and effect of subsequent temperature jumps. Experimental conditions. Prior incubation: vesicles (2.82 mg protein/ml) were incubated in ice with 0.15 mM *p*-hydroxymercuribenzoate; samples (3 μl) were then assayed for transport activity (○); aliquots were diluted with 5 volumes of buffer (33 mM Hepes, pH 7.4, 135 mM NaCl) at 37 °C and incubated at this temperature later on. Samples (18 μl, 0.47 mg protein/ml, 0.025 mM *p*-hydroxymercuribenzoate) were then assayed for transport activity (●). Transport assay: see Materials and Methods. Data were fitted to single exponential decays: inactivation rate constant at 0 °C and 0.15 mM *p*-hydroxymercuribenzoate $= 0.565 \times 10^{-3}$ min^{-1}; inactivation rate constants at 37 °C and 0.025 mM *p*-hydroxymercuribenzoate (pooled data) = 0.0677 min^{-1}.

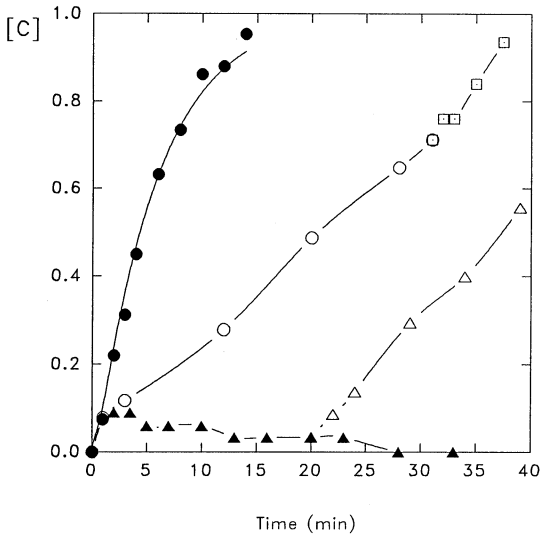

Fig. 7. Time-course of the bilitranslocase high-affinity form appearance induced by mercaptoethanol and effect of early addition of *p*-hydroxymercuribenzoate. Experimental conditions. Prior incubation: vesicles (2.82 mg protein/ml) were incubated with 0.15 mM *p*-hydroxymercuribenzoate at 37 °C for 10 min; the experiment starts with the addition of 0.41 mM mercaptoethanol; samples (3 µl) were assayed for transport activity (●); after 1 min at 37°C, two aliquots were withdrawn: aliquot A was diluted with 3 vol. of ice-cold buffer (33 mM Hepes pH 7.4, 135 mM NaCl), kept in ice and samples (12 µl, 0.7 mg protein/ml, 0.065 mM excess mercaptoethanol) tested for transport activity (○); at 41 min, this aliquot was transferred to 37 °C (□); aliquot B was treated like aliquot A, but the ice-cold dilution buffer was supplemented with 0.287 mM *p*-hydroxymercuribenzoate; samples (12 µl, 0.7 mg protein/ml, 0.15 mM excess *p*-hydroxymercuribenzoate) were tested for transport activity (▲); aliquot B′ was withdrawn from aliquot B, added with 0.41 mM mercaptoethanol and tested for transport activity (△).

Pathway (A) implies that the conversion to C* is necessarily preceded by either cysteine or arginine derivatization. Alternatively, pathway (B) implies both that C* is the specific target of the inhibitors and that it occurs in the native membrane in equilibrium with C. To discriminate between the two models, experiments reported below have been carried out.

Testing the reaction of *p*-hydroxymercuribenzoate with C. Fig. 6 shows the results of an experiment in which plasma membrane vesicles were incubated at 0 °C with *p*-hydroxymercuribenzoate. At different time intervals samples from the suspension were brought to 37 °C. Such a temperature jump was attained by sixfold dilution of the samples with pre-heated buffer. It should be stressed that in this way the inhibitor concentration was lowered accordingly. Open circles indicate that C concentration did not appreciably decline during 45 min prior incubation in ice. Two possible explanations are conceivable: either the mercurial did not appreciably react with cysteine(s), or, alternatively, the conformational change converting C-I to C*-I (see pathway A) did not take place at all. However, raising the temperature at any time resulted in the disappearance of C at a rate independent of the length of the prior incubation time. Note that this rate was exactly the same as that found at time 0. Had C-I concentration increased along the time, a proportional increase of the rates should have been detected. The latter supports the concept that C does not react with the mercurial at an any appreciable rate at 0 °C.

Testing the reaction of *p*-hydroxymercuribenzoate with C*. The results of a reactivation experiment are shown in Fig. 7. Vesicles treated with *p*-hydroxymercuribenzoate, were exposed to mercaptoethanol under a number of experimental conditions. At the onset of the experiment, C concentration was close to 0, i.e. all the carrier was in the C*-I form. Upon addition of mercaptoethanol, C appeared. Filled circles describe the rate of C formation at 37 °C. All other curves start 1 min after the addition of the thiol. At this time, it was experimentally measured (data not shown) that the carrier forms were: C*-I = 15 %; C*= 75 %; and C = 10 %. An aliquot was chilled with 3 vol. of ice-cold buffer and kept in ice thereafter. The open circles (Fig. 7) describe the rate of C formation under this condition (at later times, the reaction was speeded up by heating the sample at 37 °C, open squares). The filled triangles (Fig. 7) show that *p*-hydroxymercuribenzoate added to an identical sample blocked the conversion to C. As expected, mercaptoethanol, added later, induced C formation (Fig. 7, open triangles). The specific inhibitory effect of the mercurial observed at 0 °C shows that C*, the prevailing form in this experiment, can react at a significant rate at a low temperature. Note that C does not have the same property, as shown by the experiment reported in Fig. 6.

Taken together, these results (Figs 6 and 7) allow us to conclude that the most reactive species towards the sulfhydryl reagent is C* rather than C. It follows reasonably that both C and C* co-exist in the membrane and the latter is trapped by covalent modification, thus shifting the equilibrium to the right. These data show, in addition, that the covalent modifications on the cysteine(s) or the arginine(s), *per se*, are devoid of functional effects on bilitranslocase.

DISCUSSION

There is little doubt that sulfobromophthalein moves in isolated, intact hepatocytes in a rheogenic manner [18]. This conclusion has been reached on the basis of the observation that the distribution of the anion across the cell is influenced by the presence or absence of a physiological membrane potential. It should be concluded therefore that an electrogenic carrier has to play a key role in the process. A substantial amount of experimental data has been collected, demonstrating that bilitranslocase can be such a carrier: firstly, the reconstituted system obtained with proteoliposomes containing purified bilitranslocase operates electrogenically, in that the movement of the dye can be efficiently driven by a membrane potential [4]; secondly, in plasma membrane vesicles, electrogenic sulfobromophthalein transport can be demonstrated with a sensitive spectrophotometric assay [2] and immunochemical studies have allowed the identification of the carrier responsible for this electrogenic movement with bilitranslocase [6].

This assay has been so designed that the measurement is taken after the system has spontaneously equilibrated, so that the phthalein has reached its electrochemical equilibrium before the imposition of a membrane potential. The latter represents therefore the only driving force to the transport. It follows that, under these conditions, electroneutral movements cannot be detected, by definition. This eliminates the possible contribution to the measure of other sulfobromophthalein carriers, responsible for this type of transport, notably organic-anion-transporting polypeptide (oatp) [19]. This carrier has been claimed to carry out electroneutral organic anion transport [20, 21]. However, the physiological substrate of oatp is taurocholate, which, in this case, is transported by a sodium-independent mechanism [22]. A consistent observation is that taurocholate, either in the presence or in the absence of sodium, exerts no inhibition at all on the spectrophotometrically recorded electrogenic sulfobromophthalein transport in rat liver plasma membrane vesicles [23],

clearly indicating that bile acid carriers take no part in the measurement. The involvement of other electrogenic sulfobromophthalein carriers cannot, in principle, be discounted. Evidence in this sense is, however, still missing. An additional point to be raised concerns the type of the physical measurement carried out. Spectrophotometry offers the advantage over other techniques usually employed in the study of membrane transports, of being a continuous one, allowing us to follow rapid kinetics and giving a complete picture of events rather than discrete points at different times [24]. Only under these conditions can a sound kinetic analysis be performed with acceptable experimental confidence.

Further support for the concept that only one carrier, i.e. bilitranslocase, is being measured by the spectrophotometric assay is provided by the data here reported: the partial inhibition of sulfobromophthalein transport by cysteine-modifying or arginine-modifying reagents can be perfectly accounted for by the conversion of bilitranslocase from a high- to a low-affinity form. Were another carrier, with very close K_m value, involved in the process measured, it should be postulated that the chemical modification would have affected this carrier too, and with identical effects. The coincidental occurrence of all such events looks highly improbable.

On the basis of what is reported here, the high-affinity form, characterised by a K_m value of about 5 μM, prevails in standard vesicle preparations. The chemical derivatization causes the low-affinity form (K_m = 37 μM) to appear as the prevailing one. We denote the two forms as C and C*, respectively.

The substrates may greatly accelerate the C* to C conversion and it may therefore be inferred that, in the membrane, these two forms coexist in equilibrium, certainly modulated by the substrate concentration. Conversely, at least two classes of common inhibitors (cysteine-specific and arginine-specific) operate in the opposite direction, by entrapping C* in the form of C*-I. C*, being the preferred target of the inhibitors, reveals a major structural feature, not shared by its high-affinity companion, i.e. the exposure of relevant cysteine(s) and arginine(s) to their reagents. We have observed that a polymer-linked disulfide can inhibit electrogenic sulfobromophthalein transport in vesicles exactly like Nbs_2 (unpublished results), suggesting that the cysteine is exposed at the surface of the membrane.

These conclusions shed light on previous work carried out on this matter. Based on experiments of sequential modification of cysteines and arginines, aimed at testing the possible additiveness of the two kinds of inhibitions, the two amino acid residues were said to be closely located on the carrier protein and to belong to a common functional domain [10]. Data in this paper confirm those early conclusions and allow us to understand that the common functional domain is that specific region of the carrier, which is readily available for modification only in the C* form.

It is tempting at this point to foresee the effects produced *in vivo* by a shift of the equilibrium between the two forms of the translocator, related to the appearance or disappearance of metabolites endowed with allosteric effector function. As far as we know, the only physiological compounds with this property are the substrates, which would tend to increase the high-affinity C form. It is easy to imagine that a shift in this direction would end up with just a transient, lower steady-state concentration of all the substrates in plasma. This, in turn, may be followed by a relatively slow back-shift in the C↔C* equilibrium to reach the original situation. Adjustments on an oscillatory basis are very common in physiological systems, however.

A completely different situation may be conceived if a mutated form of bilitranslocase had a higher than normal propensity to occur as C*. Were this the case, the possibility exists that, under different metabolic conditions, normally producing no appreciable change in bilirubinemia, jaundice may appear. Typically, such a situation is found in Gilbert's subjects [25]. On a number of occasions, it has been pointed out that the syndrome is accompanied by an impairment of the clearance of a number of organic anions [26]. Most of these, though, are not glucuronoconjugated, which rules out the direct involvement of the demonstrated genetic defect of glucuronyl transferase [27], as the basis of this additional feature of Gilibert's syndrome. It has been proposed that bilitranslocase could also be defective in Gilbert's subjects [28, 29] and the pathogenetic mechanism could be that suggested above. These possibilities are currently under investigation by our group.

The obvious possibility is still open that C* and C may even be components along the translocation pathway. A quite different experimental set-up is needed to test this point [30].

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REFERENCES

1. Sottocasa, G. L., Lunazzi, G. C. & Tiribelli, C. (1989) Isolation of bilitranslocase, the anion transporter from liver plasma membranes for bilirubin and other organic anions, *Methods Enzymol. 174*, 50−57.
2. Baldini, G., Passamonti, S., Lunazzi, G. C., Tiribelli, C., Sottocasa, G. L. (1986) Cellular localization of sulfobromophthalein transport activity in rat liver, *Biochim. Biophys. Acta 856*, 1−10.
3. Tiribelli, C., Lunazzi, G. C., Luciani, M., Gazzin, B. & Sottocasa, G. L. (1978) Carriers and specific hepatocellular receptors for endogenous and exogenous anions, in *Problems of intrahepatic cholestasis* (Gentilini, P., Popper, H., Sherlock, S. & Teodori, U., eds) pp. 111−116, U. Karger AG, Basel.
4. Sottocasa, G. L., Baldini, G., Sandri, G., Lunazzi, G. C. & Tiribelli, C. (1982) Reconstitution *in vitro* of sulfobromophthalein transport by bilitranslocase, *Biochim. Biophys. Acta 685*, 123−128.
5. Miccio, M., Lunazzi, G. C., Gazzin, B. & Sottocasa, G. L. (1990) Reconstitution of sulfobromophthalein transport in erythrocyte membranes induced by bilitranslocase, *Biochim. Biophys. Acta 1023*, 140−142.
6. Miccio, M., Baldini, G., Basso, V., Gazzin, B., Lunazzi, G. C., Tiribelli, C. & Sottocasa, G. L. (1989) Bilitranslocase is the protein responsible for the electrogenic movement of sulfobromophthalein in plasma membrane vesicles from rat liver: evidence using mono- and poly-clonal antibodies, *Biochim. Biophys. Acta 981*, 115−120.
7. Passamonti, S. & Sottocasa, G. L. (1989) The role of sulfhydryl groups in sulfobromophthalein transport in rat liver plasma membrane vesicles, *Biochim. Biophys. Acta 979*, 294−298.
8. Passamonti, S. & Sottocasa, G. L. (1990) The sulfhydryl groups responsible for bilitranslocase transport activity respond to the interaction of the carrier with bilirubin and functional analogues, *Biochim. Biophys. Acta 1021*, 9−12.
9. Passamonti, S., Battiston, L. & Sottocasa, G. L. (1990) Arginine residues are involved in the transport function of bilitranslocase, *Biochim. Biophys. Acta 1025*, 122−126.
10. Passamonti, S. & Sottocasa, G. L. (1990) Organization of functional groups of liver bilitranslocase, *Biochim. Biophys. Acta 1041*, 195−200.
11. Van Amelsvoort, J. M. M., Sips, H. J. & Van Dam, K. (1978) Sodium-dependent alanine transport in plasma-membrane vesicles from rat liver, *Biochem. J. 174*, 1083−1086.
12. Jocelyn, P. C. (1987) Spectrophotometric assay of thiols, *Methods Enzymol. 143*, 44−67.
13. Ellman, G. L. (1959) Tissue sulfhydryl groups, *Arch. Biochem. Biophys. 82*, 70.
14. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of micrograms quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem. 72*, 248−254.
15. Dixon, M. & Webb, E. C. (1964) *Enzymes*, Longmans, London.

16. Passamonti, S., Battiston, L. & Sottocasa, G. L. (1997) Arylsulfonylation of bilitranslocase in plasma membranes from rat liver enables to discriminate between natural and artificial substrates, *Biochim. Biophys. Acta 1323*, 130−136.
17. Fersht, A. (1985) *Enzyme structure and mechanism*, W. H. Freeman and Company, New York.
18. Persico, M. & Sottocasa, G. L. (1987) Measurement of sulfobromophthalein uptake in isolated rat hepatocytes by a direct spectrophotometric method, *Biochim. Biophys. Acta 930*, 129−134.
19. Jaquemin, E., Hagenbuch, B., Stieger, B., Wolkoff, A. W. & Meier, P. J. (1994) Expression cloning of a rat liver Na+-independent organic anion transporter, *Proc. Natl Acad. Sci. USA 91*, 133−137.
20. Shi, X. Y., Bai, S., Ford, A. C., Burk, R. D., Jaquemin, E., Hagenbuch, B., Meier, P. J. & Wolkoff, A. W. (1995) Stable inducible expression of a functional rat liver organic anion transport protein (oatp) in rat liver and kidney, *J. Biol. Chem. 270*, 25591−25595.
21. Meier, P. J. (1996) Hepatocellular transport systems: from carrier identification in membrane vesicles to cloned proteins, *J. Hepatol. 24* (Suppl. 1), 29−35.
22. Hagenbuch, B., Scharschmidt, B. F. & Meier, P. J. (1996) Effect of antisense oligonucleotides on the expression of hepatocellular bile acid and organic anion uptake systems in *Xenopous laevis* oocytes, *Biochem. J. 316*, 901−904.
23. Passamonti, S. & Sottocasa, G. L. (1988) The quinoid structure is the molecular requirement for recognition of phthaleins by the organic anion carrier at the sinusoidal plasma membrane level in the liver, *Biochim. Biophys. Acta 943*, 119−125.
24. Tipton, K. F. (1993) Principles of enzyme assays and kinetic studies, in *Enzyme assays: a practical approach* (Eisenthal, R. & Danson, M. J., eds) p. 37, IRL Press, Oxford.
25. Fevery, J. (1981) Pathogenesis of Gilbert syndrome, *Eur. J. Clin. Invest. 11*, 417−418.
26. Gentile, S., Saponara, A., Foglia, F., Grigni, N., Garofano, M. L., Porcellini, M. & Coltorti, M. (1981) Impaired plasma disappearance of nicotinic acid and interference with rifamycin SV in Gilbert's syndrome − a defect in hepatic uptake of organic anions? *Ital. J. Gastroenterol. 13*, 94−99.
27. Bosma, P. J., Chowdhury, J. R., Bakker, C., Gantla, S., de Boer, A., Oostra, B. A., Lindhout, D., Tytgat, G. N. J., Jansen, P. L. M., Oude Elferink, R. P. J. & Chowdhury, N. R. (1995) The genetic basis of the reduced expression of bilirubin UDP-glucuronosyltransferase 1 in Gilbert's syndrome, *N. Engl. J. Med. 333*, 1171−1175.
28. Gentile, S., Tiribelli, C., Baldini, G., Lunazzi, G. C. & Sottocasa, G. L. (1985) Sex differences of nicotinate-induced hyperbilirubinemia in Gilbert's syndrome: implication of bilitranslocase function, *J. Hepatol. 1*, 417−429.
29. Gentile, S., Persico, M., Baldini, G., Lunazzi, G. C., Tiribelli, C. & Sottocasa, G. L. (1985) The implication of bilitranslocase function in the impaired rifamycin SV metabolism in Gilbert's syndrome, *Clin. Sci. 68*, 675−680.
30. Stein, W. D. (1990) Carrier-mediated transport: facilitate diffusion, in *Channels, carriers and pumps: an introduction to membrane transport*, pp. 127−171, Academic Press Inc., San Diego.