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The sulfhydryl groups responsible for bilitranslocase transport activity respond to the interaction of the carrier with bilirubin and functional analogues

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Both inactivation of sulfobromophthalein transport in rat liver plasma membrane vesicles by sulfhydryl group reagents and subsequent reactivation by 2-mercaptoethanol are shown to be modulated by ligands to bilitranslocase. In particular, bilirubin, sulfobromophthalein and Thymol blue behave as negative effectors in the inactivation reaction and as positive effectors in the reactivation reaction. Kinetic data provide further evidence of the existence of two classes of sulfhydryl groups involved in transport activity. The effect brought about by remarkably low concentrations of bilirubin is in line with the physiological function of bilitranslocase as a bilirubin carrier.

Introduction

Bilitranslocase is a plasma membrane carrier protein localized at the sinusoidal pole of the liver cell, where it performs the translocation of bilirubin and other organic anions from the blood into the cell [1–3].

Bilitranslocase transport function may be assayed in vitro as the rate of electrogenic BSP uptake by rat liver plasma membrane vesicles [2]. BSP is a functional analogue of bilibrubin and is chosen for its solubility, chromophoric and pH-indicator properties.

Recently, using sulfhydryl group reagents, the electrogenic BSP transport activity in liver plasma membrane vesicles has been shown to depend on the reduced state of two classes of thiol groups [4], on the basis of their different reactivity towards specific reagents. Both classes appear to belong to the same carrier system.

In this work, the reactivity of the thiol groups has been studied in more depth, with particular attention to the possible influence exerted by the interaction of bilirubin and the two phthaleins BSP and Thymol blue [5] with bilitranslocase.

Data presented in this paper show that bilitranslocase interaction with the organic anions both prevents the carrier from being inactivated by sulfhydryl group reagents and accelerates the rate of its reactivation by 2-mercaptoethanol. The data, in addition, supply further evidence of the existence of two distinct classes of thiol groups involved in transport activity.

Materials and Methods

Stock solutions of the reagents used were: 0.1 M NEM (Sigma, St. Louis, MO, U.S.A.), dissolved in water immediately before use; 2-ME (BDH, Poole, U.K.), dissolved in water. The stock solutions of the other sulfhydryl group reagents were prepared as in Ref. 4. 1 mM bilirubin (Sigma, St. Louis, MO, U.S.A.), dissolved in 0.1 M NaOH and then diluted with water to final concentration of 100–300 nM in 0.5 mM NaOH immediately before use. All other reagents were the same as those used in previous work [2,4,5]. Vesicles were prepared, stored and utilized as described in Ref. 5.

Inactivations of electrogenic BSP transport by sulfhydryl group reagents were obtained by reaction of the latter with vesicles. The inactivation reactions were started by the addition of 3 μl reagent to a tube containing 3 μl BSP, Thymol blue or bilirubin and 24 μl (= 0.5 mg protein) vesicles, already equilibrated at 36 °C. The time zero test was started by the addition of 3 μl solution without reagent. Additions of 0.3 μl 2-ME

Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoate); 2-ME, 2-mercaptoethanol; NEM, *N*-ethylmaleimide; pHMB, *p*-hydroxymercuribenzoate; BSP, sulfobromophthalein.

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either without or with one of the organic anions were made with a microsyringe. Reactions were stopped by diluting 25 μl samples in the transport assay medium.

Measurements of BSP electrogenic transport activity were carried out by the spectrophotometric technique described in Ref. 2 as applied in Ref. 5. The test was started by adding 25 μl of pre-incubated vesicles to 1.975 ml transport assay medium, composed of 0.1 M potassium phosphate buffer (pH 8.1) and 10–30 μM BSP. The valinomycin-induced uptake phase was started by adding 5 μg of the ionophore dissolved in methanol [5]. The wavelength pair was 580 − 514.4 nm. In view of the fact that the electrogenic transport of BSP in vesicles has been shown to be measured by the initial rate of uptake of the dye induced by addition of valinomycin in the presence of potassium [2], all the data presented in this paper are expressed as change of this parameter.

Protein determination was performed by the Bio-Rad protein assay, taking γ-globulin (standard I) as the standard.

Results

Fig. 1 shows the effect of BSP, Thymol blue and bilirubin on the rate of inactivation of BSP transport activity in rat liver plasma membrane vesicles by DTNB. Data are plotted as log of percent initial rate versus time. All of these compounds, which have been shown to be translocated by bilitranslocase [2,3,5], decrease the rate of inactivation by DTNB. As shown in the figure, the effect is concentration dependent. Remarkably low (10 nM) is the concentration of bilirubin that makes the system refractory to inactivation, suggesting the specificity of this effect.

Fig. 2 shows that the slow reversal of DTNB-induced inactivation by 10-fold molar excess 2-ME may be strongly accelerated if the thiol is added along with one of the three 'substrates'. The effect is unexpectedly large and particularly evident for bilirubin and BSP.

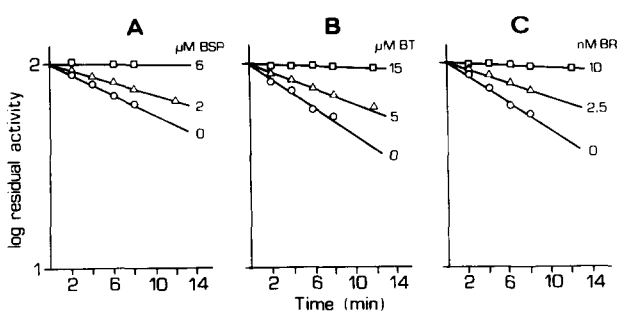

Fig. 1. Effect of organic anions on the rate of inactivation by DTNB of BSP electrogenic transport in rat liver plasma membrane vesicles. Experimental conditions: 40 mM Hepes (pH 7.4), 60 mM NaCl, 10 mg protein/ml, 1.5 mM DTNB, $t = 36\,^{\circ}C$; panel A: 0 (○), 2 (△) and 6 (□) μM BSP; panel B: 0 (○), 5 (△) and 15 (□) μM Thymol blue (BT); panel C: 0 (○), 2.5 (△) and 10 (□) nM bilirubin (BR).

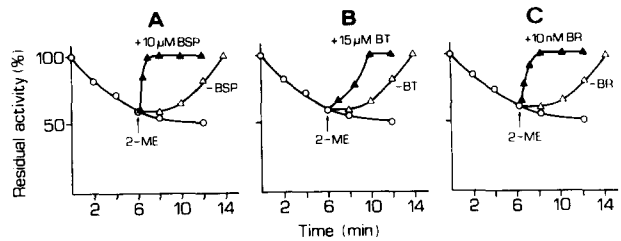

Fig. 2. Time-course of inactivation by DTNB of electrogenic BSP transport in rat liver plasma membrane vesicles and of reactivation by 2-mercaptoethanol without and with organic anions. Experimental conditions: 1.5 mM (DTNB) (○); at 6 min (arrow), addition of 15 mM 2-ME without (△) and with (▲) 10 μM BSP (panel A), 15 μM Thymol blue (BT, panel B) or 10 nM bilirubin (BR, panel C); $t = 36\,^{\circ}C$; further details as in Fig. 1.

It has been shown [4] that inactivation of transport activity by DTNB is due to modification of just one class of thiols, without affecting the second one. The latter, in turn, can react with other reagents, such as pHMB and NEM. These reagents can react with both the DTNB-sensitive and the DTNB-insensitive classes. Moreover, they have been shown to discriminate between the two classes as well. In fact, when used at concentrations causing 50% inhibition of transport, the modification involves just the DTNB-sensitive class of thiols. For instance, by pretreating the vesicles with 1.2 mM NEM, the DTNB-sensitive class of thiols is irreversibly derivatized, thus leaving only the other class available for modification by pHMB. Data in Fig. 3 illustrate the inactivation due to pHMB modification of thiols either sensitive to DTNB (panel A) or insensitive to it (panel B). In both cases, BSP virtually prevents the reaction of pHMB with each class of thiols, but, surprisingly, the DTNB-insensitive one is protected by a concentration which is ten times lower than that protecting the other class.

In Fig. 4 the 2-ME-induced reversal of BSP transport inhibition by pHMB is shown. When DTNB-sensitive thiols are modified by reaction with 0.3 mM pHMB (panel A), a 3-fold molar excess of 2-ME (1 mM) causes

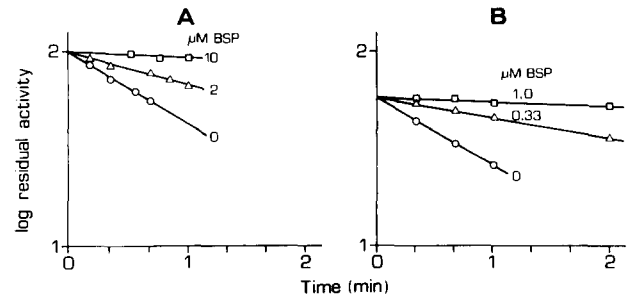

Fig. 3. Effect of BSP on the rate of inactivation by pHMB of electrogenic BSP transport in rat liver plasma membrane vesicles. Experimental conditions: 0.3 mM pHMB with 0 (○), 2 (△) and 10 (□) μM BSP (panel A); after 2-min preincubation with 1.2 mM NEM, 0.3 mM pHMB with 0 (○), 0.33 (△) and 1 μM BSP (□) (panel B); $t = 36\,^{\circ}C$; further details as in Fig. 1.

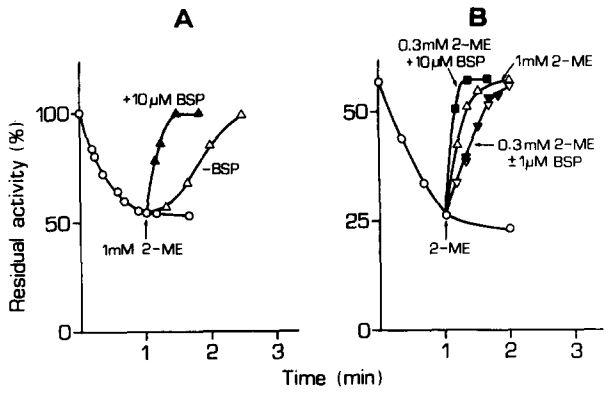

Fig. 4. Time-course of inactivation by pHMB of electrogenic BSP transport in rat liver plasma membrane vesicles and of reactivation by 2-mercaptoethanol without and with BSP. Experimental conditions: 0.3 mM pHMB (○) without (panel A) and with (panel B) 2′ pre-incubation with 1.2 mM NEM; at 1 min (arrow), addition of 1 mM 2-ME without (△) and with 10 μM BSP (▲) (panel A); at the same time (arrow), addition of either 1 mM (△) or 0.3 mM 2-ME without (▽) and with 1 μM (▼) or 10 μM (■) BSP (panel B); $t = 36\,^{\circ}C$; further details as in Fig. 1.

transport activity to undergo recovery at a low rate, that 10 μM BSP makes at least 5-fold faster, disregarding the shapes of the curves. On the other hand, when DTNB-insensitive thiols are modified with 0.3 mM pHMB (panel B), both a 3-fold molar excess 2-ME and a concentration of the thiol even equimolar with the inhibitor allow recovery to occur very fast, a behavior that diverges from that seen in panel A. This rate can be further accelerated by the addition of 10 μM BSP. Surprisingly, 1 μM BSP, a concentration which was completely effective in protecting against pHMB inhibition, failed to accelerate the process.

The effect of BSP and bilirubin concentration on transport activity inhibition measured after 5 min incubation with a series of NEM concentrations is shown in Fig. 5. This experiment, though not enabling a quantitative evaluation of the rate constants, brings to evidence the different pattern of protection against different concentrations of NEM.

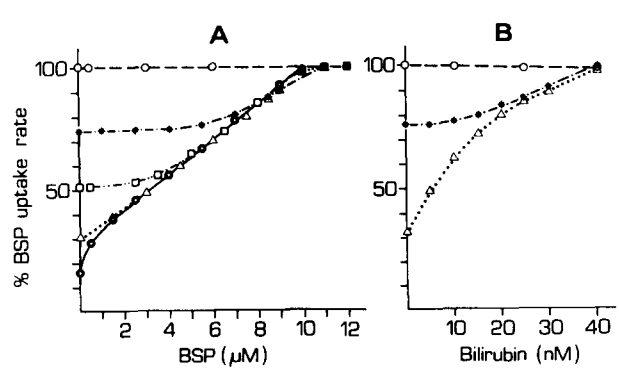

Fig. 5. Effect of organic anions on inactivation by NEM of electrogenic BSP transport in rat liver plasma membrane vesicles. Experimental conditions: panel A: 0 (○), 0.6 (★), 1.2 (□), 3.0 (△) and 10.0 (○) mM NEM for 5 min in the presence of BSP; panel B: 0 (○), 0.6 (★) and 3.0 (△) mM NEM for 5 min in the presence of bilirubin; $t = 36\,^{\circ}C$; further details as in Fig. 1.

As expected, at low NEM concentrations, the effect of BSP (panel A) appears only above a threshold value. This is, however, progressively reduced to zero. To understand this result, one should keep in mind that the incubation time in the absence and in the presence of different BSP concentrations is the same at all concentrations of NEM. Under these conditions, within the time of the reaction of the sulfhydryl-group reagent with the carrier, the antagonising effect of BSP may become apparent only if the plateau of the reaction has not been attained before sampling. Conversely, any depression of the rate of reaction between NEM and the sulfhydryl groups cannot be detected. At 3 mM NEM, protection against inhibition appears to be linearly related to BSP concentration. Above 3 mM NEM, the experimental points diverge from linearity, suggesting a hyperbolic relation between BSP concentration and inactivation by NEM.

The picture given by bilirubin (panel B) is only quantitatively different. Also with NEM, bilirubin displays its protective effect at very low concentrations. The linear relation between protection against transport inactivation and bilirubin concentration appears at NEM concentrations well above 3 mM. This could be the reflection of the very high affinity of the translocator for bilirubin.

Discussion

The results presented above show that bilitranslocase inactivation by sulfhydryl group reagents can be prevented by three different organic anions that interact with the carrier. The effect of both BSP and Thymol blue is obtained at concentrations which are peculiar to each of them and seem to be related to the K_m values for their electrogenic transport in the system (about 5 and 20 μM, respectively). On the contrary, the effective concentration range of bilirubin lies below 30 nM, an indication of very high affinity of bilitranslocase for the bile pigment. This conclusion is not surprising, in view of the fact that in plasma, in the presence of albumin, the free concentration of bilirubin is extremely low, both for its association with albumin and liver plasma membranes [6] and for its limited solubility in water at pH 7.4 [7,8]. Nevertheless, the uptake of the pigment occurs efficiently at the plasma membrane level. The direct measurement of the dissociation constant of the bilitranslocase-bilirubin complex has never been carried out.

The specificity of the effect brought about by the three 'substrates' speaks against unspecific mechanisms of action, related, for instance, to the charged nature of the compounds, particularly since the reaction mixtures are buffered at a relatively high ionic strength.

Rather, the effect seems to be related to a modification of physico-chemical parameters [9–13] of the microenvironment of the thiol groups of bilitranslocase, occuring when the protein performs transport of the anions. It follows that the thiol groups involved in transport are not likely to be exposed to the medium, whose influence could override that of the low concentrations of the organic anions.

The effect of the specific organic anions on the rate of both inactivation and reactivation of transport is not readily explained. One of the straightest explanations would be that organic anions do interact directly with the thiol groups of bilitranslocase, thus competing with the reagents for occupying a site of the protein where the thiol groups are situated. None of our data, however, can rule out the possibility that the modification of the thiol groups reactivity might result from interactions of the organic anions with other aminoacyl residues exerting a more or less mediated influence over the thiol groups. The only meaningful conclusion one can draw from the data shown is that interaction of the organic anions with the protein help thiol groups keeping their reduced state. It is expected that bilirubin at physiological concentrations may exert a protective effect on the carrier against sinusoidal blood oxygen.

The diversity of the two classes of thiols present in bilitranslocase [4] is confirmed by the following findings: (a) BSP protects DTNB-insensitive thiols from pHMB modification at concentrations that are one order of magnitude lower than those protecting DTNB-sensitive thiols; (b) after the treatment with pHMB, the rate of reactivation by 2-ME of the DTNB-sensitive class of thiols is different from that of the DTNB-sensitive one. It should be borne in mind, however, that the behavior of the DTNB-insensitive class of thiols may be dictated by the preexisting NEM-substitution of the other class. Perhaps this particular behavior might have no physiological meaning, but still indicates once more that the two classes of thiols have distinct chemical properties; (c) protection against NEM inactivation afforded by both BSP and bilirubin is described by two kinds of functions, a straight line and a hyperbolic curve. Phenomenologically, this would mean that the lowest concentrations of NEM react at a higher rate than the highest concentrations, a paradox that can be solved only by postulating the existence of two different classes of thiols, the more so since the K_m for BSP electrogenic transport has been shown to be a single value. Thiols reacting at a high rate with low concentrations of NEM, which were shown to be entirely DTNB-sensitive, are not easily protected by either BSP or bilirubin, whereas thiols that react only with concentrations of NEM above 1.2 mM are readily protected by the anions.

The facts that, in the protein, each of them has its own reactivity and accounts for a fraction of transport activity suggest that they are located in different domains of the protein. Further work on the amino acid sequence and on the arrangement of bilitranslocase within the membrane will answer some of the questions still open.

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