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Arylsulfonylation of bilitranslocase in plasma membranes from rat liver enables to discriminate between natural and artificial substrates

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Abstract

The serine protease inhibitor phenylmethylsulfonyl fluoride is shown to cause partial inhibition of bilitranslocase transport activity in rat liver plasma membrane vesicles. This condition can be fully reversed by means of pyridine-2-aldoxime methiodide, indicating that the carrier has undergone sulfonylation. Protection against inactivation is afforded by both bilirubin, the natural substrate, and nicotinic acid, but, unexpectedly, by neither sulfobromophthalein, the chromophoric substrate employed in bilitranslocase transport activity assay, nor rifamycin SV, a competitive inhibitor of sulfobromophthalein transport. From these protection experiments, the K_d for the complex of bilitranslocase with either bilirubin or nicotinic acid has been estimated to be 2.1 and 10.8 nM, respectively. Tentatively, the target for phenylmethylsulfonyl fluoride on bilitranslocase is identified as a recognition site for the physiological substrates.

Keywords: Bilitranslocase; Bilirubin; Transport; Liver; Nicotinic acid; Serine modification

1. Introduction

Bilirubin is a yellow pigment that is found in plasma, mainly as a complex with serum albumin. It is the end product of heme catabolism and is disposed of in the bile. Thus, the liver is the site of its uptake from the circulation, its chemical modification to a polar mono- or diglucuronide derivative, and its excretion into the biliary tract. The first step of bilirubin metabolism in the liver is its transport across the plasma membrane, at the sinusoidal pole of the liver cell. At this level, a 37 kDa membrane protein, called bilitranslocase [1], has been identified as the specific bilirubin carrier. This peptide has been shown to be a necessary and sufficient ingredient to carry out the electrogenic movement of bilirubin analogues in different experimental models (see [2] for a recent review). During the course of a molecular biology study aimed at the elucidation of the primary structure of this protein, a portion of the sequence attracted our attention in that it was characterized by a relative abundance of hydrophilic residues with a high propensity for ß sheet configuration. This suggested that this portion should most probably have an external exposure. In this segment three serine residues were found. On the assumption that this external portion of the protein could have an important functional role, we have undertaken an investiga-

Abbreviations: BSP, sulfobromophthalein; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); 2-ME, 2-mercaptoethanol; 2-PAM, pyridine-2-aldoxime methiodide; PMSF, phenylmethylsulfonyl fluoride.

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tion of the effect of a typical serine-modifier, such as PMSF, on the transport activity of the carrier. Data will be presented in this paper showing that PMSF exerts a dose-dependent inhibitory action on the transport function; the inhibition can be reversed by 2-PAM, a known antidote in organophosphoric poisoning; and, more interestingly, unconjugated bilirubin and nicotinate, presumably the physiological substrates for the carrier, exert a potent protective effect against arylsulfonylation by PMSF. The kinetic analysis of this effect has allowed to derive a K_d value for the complexes bilirubin-bilitranslocase and nicotinate-bilitranslocase of the order of 2 and 10 nM, respectively.

2. Materials and methods

Rat liver plasma membrane vesicles were prepared according to van Amelsvoort et al. [3].

PMSF (Sigma, St. Louis, MO) solutions were in DMSO (Carlo Erba, Milano, Italy).

The effect of PMSF on bilitranslocase transport activity was examined by pre-incubating vesicles (1 mg protein/ml, 10 mM Hepes pH 7.4, 0.25 M sucrose) at 36°C. Reactions were started by the addition of the reagent. DMSO concentration in the suspension did never exceed the value of 1% (v/v).

Measurements of bilitranslocase transport activity were performed by the spectrophotometric technique previously described [4]. The test was carried out by the addition of 10 μl of preincubated vesicles to a cuvette, containing 2 ml assay medium, under stirring conditions, at room temperature. The assay medium composition was 0.1 M potassium phosphate buffer pH 8.0, containing 38 μM BSP. Bilitranslocase transport activity is triggered by the K^+ diffusion potential generated by the addition of 10 μg valinomycin (Sigma, St. Louis, MO), dissolved in methanol. The initial rate of the absorbance drop, monitored at the wavelength-pair 580–514.4 nm, has been shown to be linearly related to bilitranslocase-mediated electrophoretic entry of BSP into vesicles [4,5].

Protein determination was carried out by the Bradford method [6]. All chemicals were of the highest purity commercially available.

3. Results

The effect of PMSF on the transport activity of bilitranslocase has been tested by pre-incubating rat liver plasma membrane vesicles in the presence of a series of PMSF concentrations. Fig. 1A shows the results of this experiment. A progressive decay of transport activity is observed, whose rate appears to depend on the reagent concentration.

In panel B of Fig. 1 it is shown that the inactivation rate constant is linearly related to PMSF concentration. The derived second-order rate constant is 107.66 M^{-1} min^{-1}.

The deflection from linearity observed above 0.75 mM PMSF could be simply explained on the basis of the inhibitor limited solubility in the aqueous medium

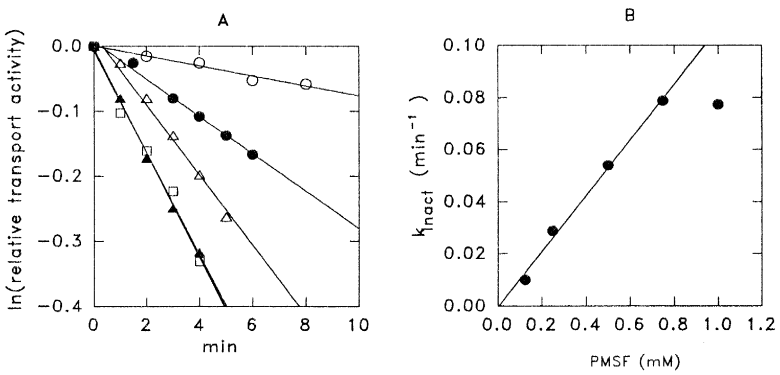

Fig. 1. Effect of PMSF on bilitranslocase transport activity. (A) Kinetics of transport inactivation in the presence of 0.125 (○). 0.25 (●), 0.5 (△), 0.75 (▲) and 1.0 (□) mM PMSF. (B) Inactivation rate constant as a function of reagent concentration. The experimental conditions are described under Section 2.

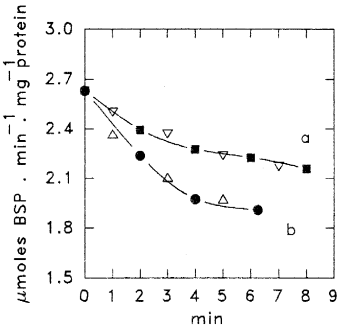

Fig. 2. Kinetics of bilitranslocase inactivation by PMSF. Effect of PMSF in the preincubation and in the assay medium. Experimental conditions: curve a: vesicles have been incubated in the presence of 0.325 mM PMSF and have been assayed either in the absence (■) or in the presence (▽) of 0.325 mM PMSF; curve b: vesicles have been incubated in the presence of 0.65 mM PMSF and have been assayed either in the absence (●) or in the presence (△) of 0.65 mM PMSF.

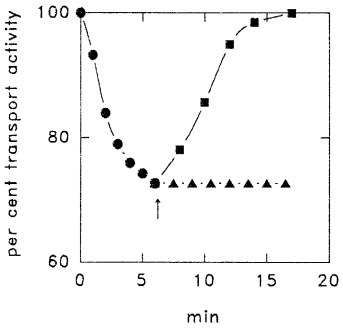

Fig. 3. Bilitranslocase inactivation by PMSF and effects of the subsequent addition of either 2-PAM or 2-ME. Experimental conditions: vesicles have been incubated in the presence of 0.6 mM PMSF. At the time indicated by the arrow either 1.25 mM 2-PAM (■) or 14.2 mM (▲) 2-ME was added.

[7]. A less trivial explanation could be invoked, such as the formation of a reversible complex with the reagent, prior to covalent modification [8,9].

This hypothesis has been tested in the next experiment. Vesicles have been incubated with two different concentrations of the reagent. Their transport activity has been measured under two conditions. In the first one, the sample is diluted 200-fold in the assay mixture, so to cause the reagent's dissociation from the hypothetical complex, while in the second one the reagent's concentration is the same both in the pre-incubation and in the transport assay vessels, to avoid any possible dissociation of the complex.

The results in Fig. 2 show that measurements of transport activity, whether performed in the absence or in the presence of the reagent, give data that fit the same curve. It is concluded therefore that PMSF makes no reversible complex with bilitranslocase prior to covalent modification.

In order to obtain information about the kind of covalent bond introduced in the carrier, reactivation experiments have been attempted. Two kinds of reactivators have been used. The first is 2-PAM, a strong nucleophile which reacts with the sulphur atom of sulfonylated proteins and thus displaces the inhibitor from the protein [10]. The second is 2-ME, a nucleophile expected to attack the same atom at the level of a thiosulfonyl ester bond [11]. Their effect on bilitranslocase transport activity is shown in Fig. 3. It can be seen that 2-PAM causes the activity to restore completely, whereas excess 2-ME is ineffective. These data allow to conclude that the covalent modi-

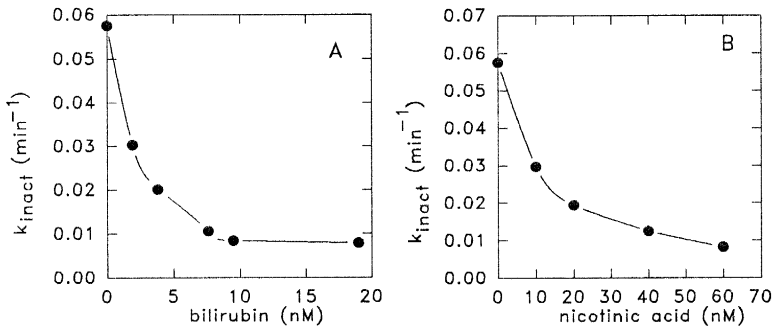

Fig. 4. Effects of either bilirubin or nicotinic acid on the rate constant of bilitranslocase inactivation by PMSF. Experimental conditions: vesicles have been incubated in the presence of 0.51 mM PMSF with increasing concentrations of either bilirubin (A) or nicotinic acid (B).

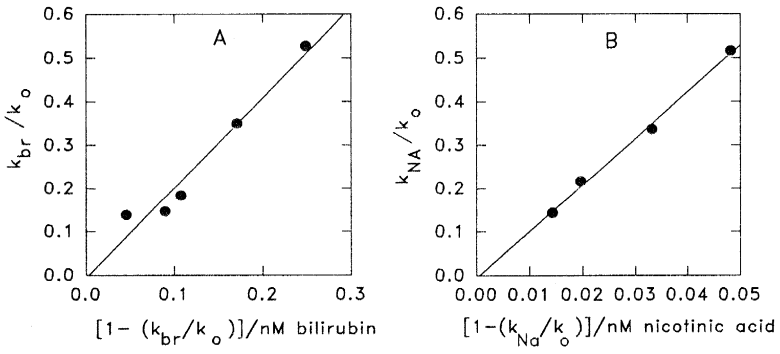

Fig. 5. Scrutton and Utter plot of bilitranslocase inactivation by PMSF in the presence of either bilirubin (A) or nicotinic acid (B). These plots have been constructed with the data of Fig. 4. k_o, k_{br} (A) and k_{NA} (B) refer to the inactivation rate constants obtained either in the absence or in the presence of bilirubin or nicotinic acid, respectively.

fication at the basis of bilitranslocase inactivation is sulfonylation at residues other than cysteines.

The target of the PMSF effect has been investigated through protection experiments. The kinetics of transport inactivation could, in fact, be different when observed either in the absence or in the presence of ligands to bilitranslocase, as already observed with other functional group-reagents [12,13]. Vesicles have therefore been incubated with PMSF in the presence of increasing concentrations of the following ligands: bilirubin, nicotinic acid, BSP and rifamycin SV.

Fig. 4 collates the data obtained with bilirubin and nicotinic acid, showing the ligand concentration-dependence of the inactivation rate constant decrease. At the highest concentrations tested, protection is nearly complete. The data of Fig. 4 have been plotted according to the equation of Scrutton and Utter [14]:

$$(k_a/k_o) = (k_2/k_1) + K_d[1 - (k_a/k_o)]/[a]$$

where k_a and k_o are the inactivation rate constants obtained in the presence and in the absence of the anionic ligand, respectively; k_2/k_1 is the ratio of the rate constants for the reaction of the bilitranslocase-ligand complex (k_2) and free bilitranslocase (k_1) with the reagent, K_d is the dissociation constant for the ligand and [a] is the ligand concentration.

The plots are shown in Fig. 5. With both ligands, a straight line is obtained, passing through the origin. This indicates that the rate constant for the reaction of the carrier-ligand complex is zero and makes it clear that the complex is not involved in the reaction. From the slopes, the values of the dissociation constants of the two complexes are obtained. These are 2.1 nM and 10.8 nM, for bilirubin and nicotinic acid, respectively.

The same experiments, performed with either BSP or rifamycin SV, have revealed no protection effect at all (data not shown).

Finally, the effect of ligands has been tested also on the kinetics of reactivation. As a matter of fact, when bilitranslocase was inactivated with both cysteine- and arginine-specific reagents, reactivation could be accelerated by the presence of these ligands [12,13]. Therefore, vesicles have been exposed to 1.25 mM 2-PAM (Fig. 6, squares), either in the absence or in the presence of 20 nM bilirubin. It is seen that the reactivation kinetics is not affected by bilirubin. Under these conditions, the reactivation rate

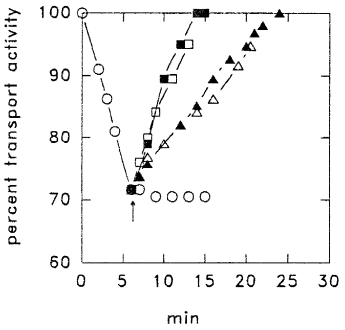

Fig. 6. Time course of bilitranslocase inactivation by PMSF and of reactivation induced by 2-PAM either in the absence or in the presence of bilirubin. Experimental conditions: vesicles have been incubated with 0.6 mM PMSF. At the time indicated by the arrow, 1.25 mM without (□) or with (■) 20 nM bilirubin has been added; the same experiment has been performed also with 0.25 mM 2-PAM without (△) and with (▲) 20 nM bilirubin.

might be maximal, so that the bilirubin effect could pass unnoticed. However, this is missing also when reactivation, induced by 0.25 mM 2-PAM, proceeds at a remarkably lower pace (Fig. 6, triangles).

4. Discussion

The data presented above show that PMSF acts as an inhibitor of bilitranslocase transport activity in rat liver plasma membrane vesicles. The mechanism of inactivation does not include the formation of a reversible bilitranslocase-PMSF complex.

Inactivation can be fully reversed by a nucleophile, such as 2-PAM, allowing to conclude that a sulfonyl ester bond has been formed on the carrier protein [9]. However, S-sulfonylation is ruled out by the fact that 2-ME fails to reactivate [11].

The protective effect of both bilirubin and nicotinic acid permit to infer that the site of sulfonylation has an important, physiological role. This is corroborated by the very low bilirubin concentration, about 10 nM, affording complete protection and the derived K_d value (2.1 nM) for the carrier-bilirubin complex, at the level of the sulfonylation site. Attention may be drawn on the fact that the bilirubin effect is observed within the pigment's solubility limit in water at pH 7.4 [15]. Therefore, there is no need to supply the system with albumin and the interpretation of the results is straightforward.

The results obtained with nicotinic acid are quite interesting for a number of reasons. Its protective effect is exerted through a very low K_d value (10.8 nM, only 5-fold higher than that of bilirubin), raising the issue that bilitranslocase could be the door for the entry of this vitamin precursor into the liver. Moreover, these data confirm earlier observations concerning the inhibitory effect of this anion on BSP transport activity and its competitive binding on purified bilitranslocase [16].

The fact that neither BSP nor rifamycin SV provided a protective effect comes as an acute note of peculiarity of the PMSF target in bilitranslocase, the more so since BSP translocation is indeed affected, though only partially.

This entire phenomenonology could indicate that the site of sulfonylation is within a segment of the protein devoted to the exclusive binding of bilirubin (or its strictest analogue, nicotinic acid). None the less, sulfonylation of this segment affects the function of BSP translocation, possibly as a consequence of a conformational adjustment or steric hindrance by the adduct.

Thus, by the use of PMSF, we have found that bilitranslocase contains a portion of its sequence that can operate a fine discrimination among its substrates. In particular, the discrimination is operated among natural, physiological compounds and xenobiotic molecules.

These features make quite possible that this site occupies a strategical position in the protein, perhaps on an ectodomain at the sinusoidal surface of the cell membrane. At this level, free bilirubin could find a specific, high-affinity binding site, representing the first step of the translocation process into the liver cell.

If indeed the segment in question represents the portion of the carrier conferring the high affinity for its physiological substrate, it is reasonably expected that a modification at this level would drastically reduce the ability of the carrier to operate the uptake of unconjugated bilirubin under physiological conditions. As a matter of fact, the impairment of bilirubin uptake might occur even in the presence of mild reduction of the carrier activity with respect to other substrates, commonly used in the experimentation. This consideration calls therefore for a word of caution when comparing results obtained in different experimental models, measuring the rates of transport of either bilirubin or phthaleins. This becomes of paramount importance when PMSF is included in the isolation media during subfractionation of the tissue, to forestall proteolysis.

As to the identity of the sulfonylation site, this might be, in the first place, a serine. Other sites, however, cannot be disregarded a priori; for instance, threonine and tyrosine could lend their hydroxyl and histidine its imidazole [17].

The fact that 2-ME did not cause reactivation stands as a proof that none of the functionally relevant thiol groups of bilitranslocase has participated in the reaction.

In this regard, it might turn useful to examine the effects of sulfonylation, as opposed to those of thiol- and arginine-modification [12,13], on bilitranslocase transport function. For simplicity, only the DTNB-

Table 1
Bilitranslocase modification by phenylglyoxal, DTNB and PMSF

Effects	Phenylglyoxal	DTNB	PMSF
Extent of inactivation	50%	50%	30%
Protective ligands	all [a]	all [a]	some
Induced reactivation	yes	yes	yes
Acceleration of reactivation by ligands	yes	yes	no

[a] Nicotinic acid not tested.

sensitive [12] class of thiols is surveyed. The facts to be examined are summarized in Table 1.

Thiol- and arginine-modification has common features and additional evidence [18] has been provided that the DTNB-sensitive cysteine and the arginine are: (a) close to each other and (b) belonging to a common functional domain of the carrier. By contrast, sulfonylation is a different event, that causes no more than 30% loss of activity, is prevented only by the natural substrates and induced reactivation cannot be speeded up by the presence of ligands. All of these considerations focus on the fact that the site of sulfonylation has functional properties quite distinct from those of the site of either cysteine- or arginine-specific modification. This segment plays a critical role in the binding of bilirubin, or nicotinic acid; its chemical modification causes only limited disorder to the transport process of BSP into the vesicle.

It cannot be forgotten that other transport proteins have been found to occur in the rat liver plasma membrane fraction, which employ BSP as a substrate (see [19] for a review). Of course, PMSF could cause their covalent modification, possibly with inhibition of their BSP transport function. If the rate of BSP entry into vesicles, as collected by our method, were the measure of a sum of of BSP transporters, then the overall rate of PMSF inhibition would be the sum of a number of individual modification reactions. Were this the case, the rate of PMSF inactivation would not appear as a simple, pseudo-first order process, unless all transporters reacted with the reagent at the same rate. Also the protection data indicate that a single binding site for either bilirubin or nicotinic acid is located with these experiments. This is proven by the fact that plotting the data of Fig. 4 according to the Scrutton and Utter equation results in a simple, straight line. A more complex pattern should arise if either bilirubin or nicotinic acid exerted protection upon a variety of binding sites with their own binding affinities. The effect of PMSF on the rate of BSP transport in vesicles is ascribed to the modification of bilitranslocase on the basis of the established relationship between the electrogenic BSP uptake by vesicles and the purified protein [5]. Further, direct proof of bilitranslocase covalent modification by this reagent is currently under test in our laboratory.

A last word should be spent about the widespread use of PMSF in buffer solutions, as an inhibitor of protease activity. This work shows that this reagent has a wide spectrum of activity, because it appears that reactive serines are not confined to the categories of serine-proteases or esterases. Thus, its employment should pass an experimental test of inoffensiveness.

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