Biochimica et Biophysica Acta, 1025 (1990) 122–126
Elsevier

BBAMEM 74885

Arginine residues are involved in the transport function of bilitranslocase

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(Received 22 November 1989)

Key words: Bilitranslocase; Electrogenic transport; Arginine residue; Bilirubin; Sulfobromophthalein; (Rat liver)

Specific guanido group reagents inhibit bilitranslocase transport activity in rat liver plasma membrane vesicles. Their reaction is shown to be affected by sulfobromophthalein, Thymol blue and bilirubin, which are translocated by bilitranslocase across the plasma membrane. It is concluded that the transport function of bilitranslocase depends on arginine residues, which are involved in the interaction with the molecules to be translocated.

Introduction

The liver plasma membrane protein bilitranslocase [1–3] is the specific carrier through which bilirubin moves from the blood into the liver cell [4,5]. This pathway is shared with its functional analogues, such as BSP [3,6] and Thymol blue [5], and a number of other organic anions such as, for instance, rifamycin-SV [5]. The only structural feature shared by all of these molecules is the ability to display one or more negative charges. Although all of them contain aromatic rings, their two-dimensional structures appear to be unrelated to one another. The negative charge accounts for the electrogenic transport of BSP and Thymol blue in rat liver plasma membrane vesicles [5].

The role of the negative charge in establishing the interaction of these organic anions with bilitranslocase complies with the existence in the protein of a high density of positive charges. Purified bilitranslocase displays, in fact, a p*I* higher than 9.0 [3], suggesting a high content in both arginine and lysine residues.

The role of arginine residues in determining bilitranslocase electrogenic transport activity in rat liver plasma membrane vesicles has been studied investigating the possible inhibitory effect of α,α'-dicarbonyl reagents, such as phenylglyoxal and methylglyoxal.

It has been found that specific blockade of arginine residues reduces bilitranslocase activity by 50%. Since both bilirubin and BSP interfere with the reaction of both reagents with the protein, it is concluded that these arginine residues are involved in the interaction of bilitranslocae with the organic anions.

Materials and Methods

Solutions of the reagents used were: 0.2 M phenylglyoxal and methylglyoxal (Sigma, St. Louis, MO, U.S.A.), freshly dissolved in ethanol/water (1 : 1, v/v); 1 M arginine (Merck AG, Darmstadt, F.R.G.) in water; 1 M 2-mercaptoethanol (BDH, Poole, U.K.), dissolved in water. Solutions of BSP (Serva, Heidelberg, F.R.G.) and Thymol blue (Riedel-De Haen, F.R.G.) were prepared in water. 1 mM bilirubin (Sigma, St. Louis, MO, U.S.A.), dissolved in 0.1 M NaOH and then diluted immediately before use. The final concentration of bilirubin was 100–500 nM in 0.5 mM NaOH. All other reagents were the same as those used in previous work [5,6].

Vesicles were prepared, stored and utilized as described in Ref. 5.

Inactivations of electrogenic BSP transport by dicarbonyl compounds were obtained by reaction of the latter with vesicles. The inactivation reactions were started by the addition of 0.1 vol. reagent to a tube containing 0.1 vol. either water or BSP or Thymol blue or bilirubin and 0.8 vol. vesicles, already equilibrated at 36 °C. It was checked that the solvent of the bilirubin solution (0.5 mM NaOH) in the comparatively highly buffered suspension, did not influence per se the results.

Abbreviation: BSP, sulfobromophthalein.

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Reactivations were obtained by adding, by means of a microsyringe, 0.027 vol. arginine either without or with one of the organic anions.

Reactions were stopped by diluting 25 μl samples, withdrawn from the pre-incubation mixture, in the transport assay medium.

Measurements of BSP electrogenic transport activity were carried out by the spectrophotometric technique described in Ref. 6 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 25 μM BSP. The valinomycin-induced uptake phase was started by adding 2 μg of the ionophore dissolved in 2 μl of methanol [5]. The wavelength pair was 580–514.4 nm. As in previous work [3–6], the activity of the carrier has been measured by the initial rate of BSP uptake induced by valinomycin, in the presence of potassium. All the data presented in this paper are expressed as percent 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 that inhibition of BSP electrogenic transport activity in liver plasma membrane vesicles depends on the concentration of either phenylglyoxal (panel A) and methylglyoxal (panel B). The two inhibition curves are virtually identical, the maximal effect being 50% inactivation, obtained by the same concentration of either reagent.

Fig. 2 shows the progression curves of inactivation of BSP transport by both 20 mM phenylglyoxal (panel A) and 20 mM methylglyoxal (panel B) and subsequent reactivation by arginine. It is known, in fact, that the reaction of dicarbonyl derivatives with arginine is reversible either at alkaline pH or by removing the excess reagent [7]. Here, shifting of the equilibrium has been obtained by adding free arginine. It is shown that the

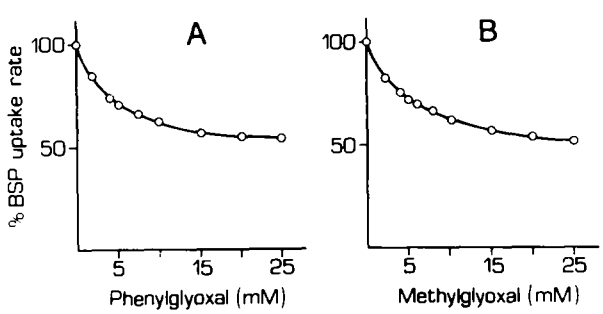

Fig. 1. Effect of phenylglyoxal (panel A) and methylglyoxal (panel B) on BSP electrogenic transport activity in rat liver plasma membrane vesicles. Experimental conditions of pre-incubation: 40 mM Hepes (pH 7.4), 60 mM NaCl, 8.7 mg protein/ ml, 15 min, t = 36 °C.

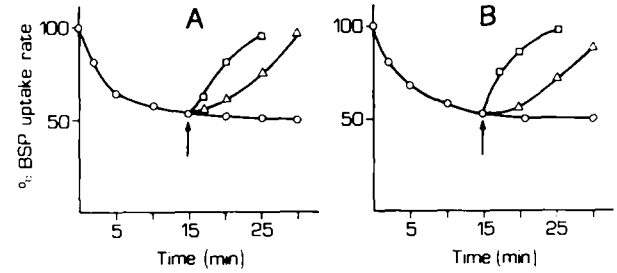

Fig. 2. Time-course of inactivation by phenylglyoxal (panel A) and methylglyoxal (panel B) of electrogenic BSP transport in rat liver plasma membrane vesicles and of reactivation by arginine. Experimental conditions of the pre-incubation: 20 mM phenylglyoxal or methylglyoxal (○); at 15 min (arrow), addition of 20 mM (△) or 40 mM (□) arginine; t = 36 °C; further details as in Fig. 1.

rate of recovery depends on arginine concentration. The fact that full recovery from both phenylglyoxal and methylglyoxal inhibition can be achieved with arginine indicates that in both cases the loss of activity is to be ascribed only to arginine derivatization. These data say nothing about the possible, irreversible deamination of primary amines, which is known to be a side-reaction when dicarbonyls react with polypeptides [7]. Had it occurred, no effect on transport activity results therefrom. Phenylglyoxal has been reported to react with sulfhydryl groups [8]. Addition of 2-mercaptoethanol failed however to reverse inhibition by 20 mM phenylglyoxal (not shown), indicating that no reversible derivatization of sulfhydryl groups has occurred.

Inactivation and reactivation obtained with either reagents display similar, if not identical, kinetic patterns. Intriguing is the fact that none of the reagents can inhibit transport activity by more than 50%. Although both reagents react with the guanido group of arginine residues by the same mechanism [9], their reactivity and accessibility are expected to be affected by the methyl- or phenyl-substitution on their dicarbonyl backbone, an effect not seen in these experiments.

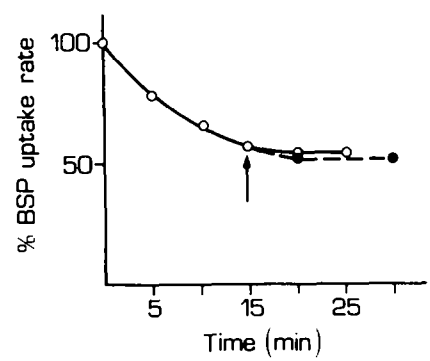

Fig. 3. Effect of methylglyoxal on maximal inactivation by phenylglyoxal of BSP electrogenic transport in rat liver plasma membrane vesicles. Experimental conditions of the pre-incubation: 20 mM phenylglyoxal (○); at 15 min (arrow), addition of 20 mM methylglyoxal (●); t = 36 °C; further details as in Fig. 1.

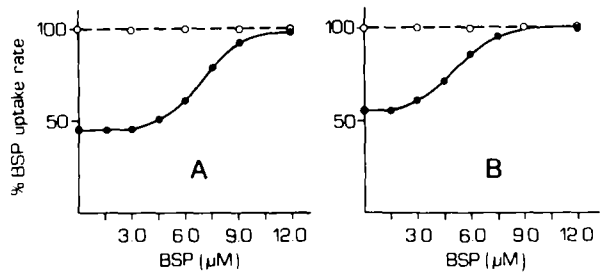

Fig. 4. Effect of BSP on inactivation by phenylglyoxal (panel A) and methylglyoxal (panel B) of electrogenic BSP transport in rat liver plasma membrane vesicles. Experimental conditions of pre-incubations: no dicarbonyl reagent (○) or 20 mM phenylglyoxal (●) (panel A) and 20 mM methylglyoxal (panel B) (●) for 15 min in the presence of BSP; $t = 36\,^{\circ}C$; further details as in Fig. 1.

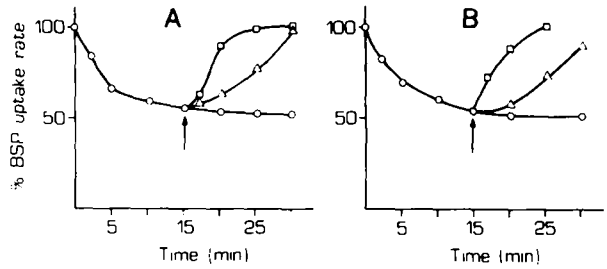

Fig. 6. Time-course of inactivation by phenylglyoxal (panel A) and methylglyoxal (panel B) of electrogenic BSP transport in rat liver plasma membrane vesicles and of reactivation by arginine with BSP. Experimental conditions of pre-incubation: 20 mM phenylglyoxal (○) (panel A) or 20 mM methylglyoxal (○) (panel B); at 15 min (arrow), addition of 20 mM arginine without (△) and with 10 μM BSP (□); $t = 36\,^{\circ}C$; further details as in Fig. 1.

Fig. 3 shows that inhibition of BSP transport activity by 20 mM phenylglyoxal leaves the carrier refractory to further inhibition by 20 mM methylglyoxal. The same picture results from prior reaction with methylglyoxal, followed by addition of phenylglyoxal (not shown). These data show that both reagents may have access and derivatize the arginyl residue(s) important for the transport function.

From the data collected so far it can be concluded that BSP electrogenic transport activity in plasma membrane vesicles depends on arginyl residues. The mechanism(s) whereby they accomplish their role in the transport activity of bilitranslocase might be: (a) interaction with the anionic moiety of membrane phospholipids; (b) interaction with the anionic moiety of the organic anions to be translocated. To check the second hypothesis, inactivation experiments were performed in the presence of anions translocated by bilitranslocase.

Fig. 4 shows the effect of increasing concentrations of BSP on the level of inactivation of transport activity reached after 15 min of reaction with phenylglyoxal (panel A) and methylglyoxal (panel B). In both cases BSP behaves as a protective agent. As shown in Fig. 5, this effect against phenylglyoxal-induced derivatization can be reproduced by either Thymol blue, a phthalein like BSP (panel A), and unconjugated bilirubin (panel B). Noticeable is the low concentration of bilirubin which succeeds in protecting BSP transport activity.

Fig. 6 shows that the rate of reactivation from both phenylglyoxal (panel A) and methylglyoxal (panel B) can be accelerated if arginine is added along with BSP. In particular, 20 mM arginine plus BSP allows reactivation to proceed at a rate similar to that obtained with 40 mM arginine (compare with Fig. 2). As shown in Fig. 7, both Thymol blue (30 μM) and bilirubin (30 nM) promote the acceleration of arginine-induced reactivation from phenylglyoxal inhibition as well.

Discussion

The approach of chemical modification of a carrier protein in situ, while allowing valuable conclusions concerning the role of specific amino acid residues in the transport function, suffers from the obvious limitation that a quantitative measure of the number of residues involved cannot be obtained in view of the complexity

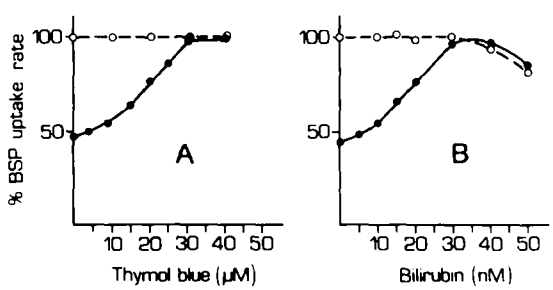

Fig. 5. Effect of Thymol blue (panel A) and bilirubin (panel B) on inactivation by phenylglyoxal of electrogenic BSP transport in rat liver plasma membrane vesicles. Experimental conditions of pre-incubations: no dicarbonyl reagent (○) or 20 mM phenylglyoxal (●) for 15 min in the presence of Thymol blue (panel A) or bilirubin (panel B); $t = 35\,^{\circ}C$; further details as in Fig. 1.

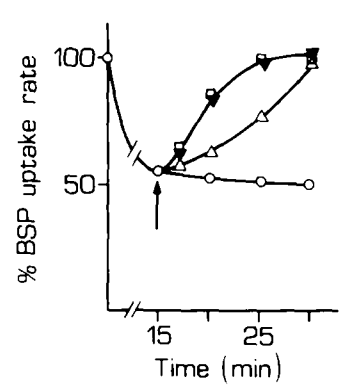

Fig. 7. Inactivation by phenylglyoxal of electrogenic BSP transport in rat liver plasma membrane vesicles and time-course of reactivation by arginine with Thymol blue or bilirubin. Experimental conditions of pre-incubation: 20 mM phenylglyoxal (○); at 15 min (arrow), addition of 20 mM arginine without (△) and with either 30 μM Thymol blue (□) or 30 nM bilirubin (▼); $t = 36\,^{\circ}C$; further details as in Fig. 1.

of the system under study. Such a drawback is, on the other hand, compensated by the invaluable advantage to follow the effect of protein modification on its function in the natural milieu.

The fact that the inhibition of BSP transport activity in plasma membrane vesicles by dicarbonyl reagents can be reversed by arginine shows that the effect of the reagents is to impair specifically this function, which has been shown to be performed by bilitranslocase [3,6]. Although dicarbonyl reagents are used mostly for the reversible modification of arginine residues in proteins, an irreversible side-reaction of deamination can occur with available α-amino groups as well as ε-amino groups of lysine [7]. The fact that arginine allows full recovery from transport activity inhibition indicates that inactivation has been produced only by the reaction occurred with arginine residues. The possibility that phenylglyoxal could inhibit BSP transport activity by reacting reversibly with sulfhydryl groups is ruled out by the lack of reactivation with 2-mercaptoethanol.

Data from protection experiments indicate that inhibition of BSP transport is due to modification of arginine residues which are involved in the interaction of bilitranslocase with the molecules to be translocated. Of course, nothing can be said about the nature of any interaction, particularly whether it is direct or mediated by arginine interaction with other amino acid residues. The positive charge on the guanidinium group could work as a recognition site for the anion. This seems to be a general rule for a number of enzymes which deal with negatively charged substrates [10]. Moreover, the charge on the guanidinium group would set it in a planar structure stabilized by resonance [10], which could accommodate the planar, quinoid structure of phthaleins, which, in turn, is selectively recognized by bilitranslocase [5]. A pure electrostatic interaction, though, is not likely to occur in a carrier system, since this would challenge the anions dissociation from the translocator along with the latter's very reason to exist. A high density of positive charges could, however, favour arginine residues reactivity with dicarbonyls by lowering their pK_a values. It has been shown, in fact, that dicarbonyl reagents react only with the unprotonated form or arginines, at neutral-alkaline pH [9]. It can be speculated then that, just for having been modified, the relevant arginine residues should be only negligibly charged. The weak binding of the anions with low charged guanidinium groups could be a step in the transport process.

The effect of organic anions on the rate of reactivation by arginine has been searched because the same anions were shown to accelerate the reactivation by 2-mercaptoethanol from inhibition of BSP transport activity in plasma membrane vesicles by the specific sulfhydryl group reagent 5,5′-dithiobis(2-nitrobenzoate) [11]. Here, the same anions, at similar concentrations, behave again as positive effectors in the reactivation reaction. The molecular mechanism by which this effect is produced cannot be predicted from these data. The sole conclusion to draw is that the interaction of bilitranslocase with the anions promotes the dissociation of the dicarbonyls from the carrier. It may be assumed that anions association with the carrier reduces the positive electric field around the reactive arginine residue(s), thus increasing its pK_a value [9] and hence promoting the regeneration of a charged arginine residue. This event could also explain the mechanism of protection.

The reason for the partial inhibition obtained by saturative concentrations of reagents is far from being seized. A resembling phenomenon has already been described, using 5,5′-dithiobis(2-nitrobenzoate) as an inhibitor of BSP transport in vesicles [11,12]. Reactivity of protein sulfhydryl groups toward this reagent is, however, known to follow no general rule, with both extreme situations possible.

In this research, two dicarbonyl reagents have been employed on the assumption that they could discriminate among arginyl residues with different reactivities/accessibilities toward the reagents. Although both reagents react with the guanido group by the same mechanism, i.e., an amine-carbonyl addition [9], their reactivity is affected also by the nature of their substitution on one of the carbonyls, but this appears to have no relevance in the reaction with bilitranslocase arginine residues.

The partial inhibition of bilitranslocase transport function by dicarbonyls could mean that a critical step in the transport process requires either arginyl residues which are inaccessible to the reagents used or the simultaneous involvement or other amino acid residues. The role of two classes of sulfhydryl groups in contributing to the transport function of bilitranslocase has already been described [11,12]. A cooperation of arginine and cysteine residues in bilitranslocase function, though not demonstrated in this paper, could be envisaged.

Acknowledgements

Supported by grants for Ministero della Pubblica Istruzione and Fondo per lo Studio e la Ricerca Scientifica delle Malattie del Fegato. Thanks are due to Mr. B. Gazzin for graphical work.

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