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The role of sulfhydryl groups in sulfobromophthalein transport in rat liver plasma membrane vesicles

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Sulfobromophthalein (BSP) electrogenic transport activity in a plasma membrane vesicle preparation from rat liver is shown to depend on free sulfhydryl groups. These are organized in two classes, one of which does not react with the sulfhydryl group reagent 5,5′-dithiobis(2-nitrobenzoate). The two classes appear to be involved in BSP transport independently. However, reactivity of one class can be shown to be affected by alkylation of the other. Hence, it is concluded that both classes are located on the same carrier system, which previous research has established to be the integral sinusoidal membrane protein bilitranslocase.

Introduction

Bilitranslocase is a membrane protein isolated in our laboratory from rat liver plasma membrane [1,2]. Its functional role in the transport of organic anions by the liver has been inferred on the basis of different lines of evidence [3–6]. Particularly significant was the observation that bilitranslocase could reconstitute in vitro BSP transport by liposomes [4]. The isolated protein was found to be somewhat unstable in that it could lose its reconstitutive activity upon aging. Similarly, but not necessarily in parallel, the BSP binding ability of the protein was lost. In our experience (unpublished data), both effects of aging could be prevented by adding 2-ME to the preparation.

All these observations suggest that sulfhydryl groups may be constitutive of the protein.

In this work, the possible functional role of sulfhydryl groups has been investigated by testing the effect of specific group reagents on the rate of electrogenic BSP uptake by plasma membrane vesicles [5]. This approach allowed us to identify two classes of sulfhydryl groups with different reactivity. We show and discuss a case of influence of one class of sulfhydryl group over the other.

Materials and Methods

Stock solutions of sulfhydryl group reagents were the following: 10 mM cupric sulfate (Carlo Erba, Milano, Italy) in 100 mM sodium-potassium tartrate (pH 7.5); 10 mM pHMB (Sigma, St. Louis, MO, U.S.A.) in 1 mM NaOH, and then adjusted at pH 7.4; 10 mM DTNB (Sigma) in 100 mM Hepes (pH 7.4); 10 and 30 mM NEM (Sigma) in 10 mM Hepes (pH 7.4). All other reagents were the same as those used in previous work [5,6]. Vesicles were prepared, stored and utilized as described in Ref. 6.

Pre-incubation for inhibition experiments was started by adding aliquots of 0.025 ml vesicles (0.442 ± 0.0685 mg protein) to 0.005 ml reagents, at concentrations, temperatures and for times indicated in the legends to the figures. Reactivation experiments were performed by adding 0.5–1 μl 40% (v/v) 2-ME (BDH, Poole, U.K.) diluted with 100 mM potassium phosphate buffer (pH 8.0) to the pre-incubation mixture, where indicated in the figures. Phosphate buffer per se has no effect on the activity measured.

BSP transport activity was measured in all cases at room temperature by the spectrophotometric technique described in Ref. 5, as applied in Ref. 6. The wavelength pair was 580–514.4 nm. 0.025 ml vesicles (0.368 ± 0.0571 mg protein), withdrawn from the pre-incubation tubes, were added to 1.975 ml medium, whose composition was 100 mM potassium phosphate buffer (pH 8.0) and 0.010 mM BSP. The valinomycin-induced uptake phase was started by adding 5 μg of the ionophore dissolved in 5 μl methanol [6]. Protein determination was performed by the Bio-Rad protein assay, taking γ-globulin (standard I) as the standard.

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

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Results

In view of the fact that the electrogenic transport of BSP 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 [5], all the data presented in this paper are expressed as percentage change of this parameter. Fig. 1 shows the concentration-dependence of inhibition of BSP transport by four sulfhydryl group reagents. Cupric sulfate, pHMB and NEM are strong inhibitors, whereas DTNB inhibition levels off at about 50% of the maximal transport rate.

Fig. 2 shows the time-course of inhibitions. Cupric sulfate reacts so rapidly, that, to follow the kinetics of the process, pre-incubations had to be performed at 0°C. pHMB and NEM reaction rates may be followed at 37°C. Clearly, DTNB reaction is slower than that of pHMB. In the same figure, the effect on transport inhibition of 2-ME was investigated in order to ascertain that these reagents reacted with sulfhydryl groups. 2-ME reverses inhibitions by all reagents except that by NEM, which forms a stable thioether bond with sulfhydryls. The rates of reactions and of their reversal are approximately the same with a given reagent. From these data, it can be postulated that BSP transport in vesicles depends on free sulfhydryl groups.

The rate of DTNB reaction with thiols was followed directly at the wavelength pair 412–514.4 nm (Fig. 3). The upper curve in the panel refers to the complete

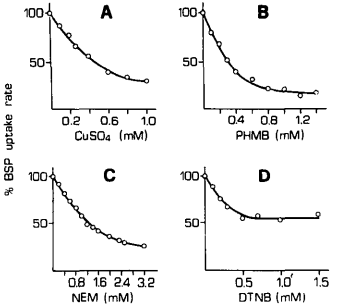

Fig. 1. BSP electrogenic transport rate inhibitions by four sulfhydryl group reagents in plasma membrane vesicles. Experimental conditions of pre-incubations: 0.442±0.068 mg protein, 4.16 mM Hepes (pH 7.4), 62.5 mM NaCl and : (panel A) curpric sulfate, 1–10 mM sodium-potassium tartrate, for 1 min at 0°C; (panel B) pHMB, 0.1 mM NaOH, for 1 min at 37°C; (panel C) NEM, 2 mM Hepes (pH 7.4), for 5 min at 37°C; (panel D) DTNB, 20 mM Hepes (pH 7.4), for 15 min at 37°C. Experimental conditions of the assay of BSP electrogenic transport rate are described under Materials and Methods.

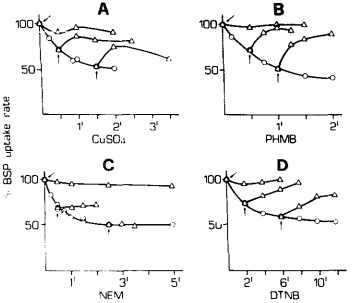

Fig. 2. Time-course of inactivation and reactivation of BSP electrogenic transport in plasma membrane vesicles. Experimental conditions: Inactivations (○) were started by adding vesicles to: (panel A) 0.5 mM cupric sulfate and 5 mM sodium-potassium tartrate, $t = 0°C$; (panel B) 0.3 mM pHMB and 0.1 mM NaOH, $t = 37°C$; (panel C) 1.2 mM NEM and 2 mM Hepes (pH 7.4), $t = 37°C$; (panel D) 2.5 mM DTNB and 20 mM Hepes (pH 7.4), $t = 37°C$. Reactivations (△) were started by the addition of 1.3% 2-ME, where indicated by the arrows. Further details as in Fig. 1.

mixture. The lower curve is the control experiment performed in the absence of the reagent. The dashed line is the difference computed for the two previous ones and corresponds to the net change in absorbance due to thiol titration. The rate of this process appears to be at least biphasic. The first phase of DTNB reaction

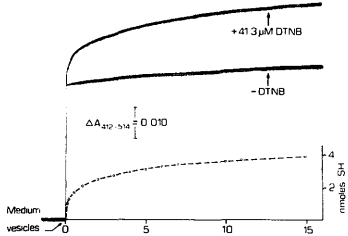

Fig. 3. Time-course of DTNB reaction with plasma membrane vesicles. Experimental conditions: The reaction was followed at the wavelength pair 412–514.4 nm at room temperature. The reaction was started by adding 0.025 ml vesicles (0.455 mg protein) to 2.0 ml medium, composed of 100 mM potassium phosphate buffer (pH 8.0) and 0.041 mM DTNB, so as to have the DTNB/protein ratio of 181.6 nmol/mg protein, which gives maximal transport activity inhibition.

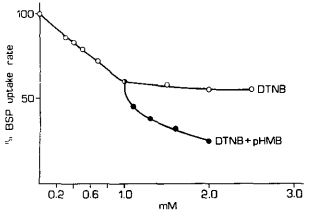

Fig. 4. Inhibition of BSP electrogenic transport rate in plasma membrane vesicles by DTNB and DTNB plus pHMB. Experimental conditions: upper curve (○) DTNB, as in Fig. 1, panel C; lower curve (●) 1 mM DTNB and 8.33 mM Hepes (pH 7.4), for 15 min. Then, addition of pHMB and 0.083 mM NaOH for 1 min. $t = 37°C$. Further details as in Fig. 1.

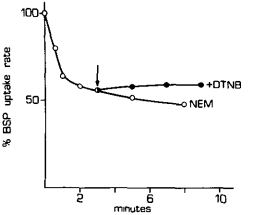

Fig. 5. Time-course of BSP electrogenic transport inactivation by NEM and NEM plus DTNB. Experimental conditions: inactivation (○) started by adding vesicles to 1.2 mM NEM and 20 mM Hepes (pH 7.4). At the time indicated by the arrow, addition of 2.5 mM DTNB and 16.6 mM Hepes (pH 7.4) to 1.2 mM NEM and 0.4 mM Hepes (pH 7.4) (●). $t = 37°C$. Further details as in Fig. 1.

is definitely too fast to account for the blockade of functional thiols, being completed within a few seconds. Rather, the rate of the second, slower phase of reaction complies with the rate of DTNB inhibition of BSP transport activity, lasting several minutes. In 15 min, 3.90 nmol SH per mg protein had reacted.

On the basis of the data so far presented, it may be suggested that there exists a class of sulfhydryl groups involved in transport which is not accessible to DTNB. In Fig. 4 we show that DTNB-resistant transport activity can be further inhibited by increasing concentrations of pHMB. Thus, two classes of thiols are involved in transport. The first is accessible to DTNB, whereas the second one is not.

Do better inhibitors, such as NEM or pHMB, react at random with thiols of both classes, or is their reaction ordered by affinities specific to each class? Fig. 5 shows that 50% inhibition of transport obtained with NEM leaves this function no more susceptible to DTNB attack. This indicates that NEM also distinguishes the two classes of thiols, reacting preferentially with those which are accessible to DTNB rather than with those that are not. Note also that the weak progress of inhibition by NEM is blocked by DTNB, which, during the reaction, provides a free thiol group (2-nitro-5-thiobenzoate) available for binding the excess of NEM still free in solution. A similar experiment (not shown) was performed after 50% inhibition by pHMB. Also in this case, DTNB fails to inhibit further BSP uptake rate. However, the higher reactivity of mercuri-derivatives of protein thiols with 2-nitro-5-thiobenzoate, contaminating the DTNB stock solution, makes this result less significant.

We have attempted to discover possible relationships existing between the two classes of thiols identified. We have therefore designed experiments of inactivation and reactivation in order to see whether the expected rates of either one or both processes were affected by thiols in either a free or a bound state.

The rates of inactivation found in combined inhibitions shown in Fig. 6 to 8 do not appear to be substantially affected, as if no prior reaction to DTNB-accessible thiols could determine changes in the affinity of DTNB-inaccessible thiols for the second reagent.

In Fig. 6, reactivation by 2-ME of DTNB and pHMB combined inhibition is shown to occur first at a high rate and then at a lower one. The first phase is as fast as the recovery from pHMB inhibition already shown in

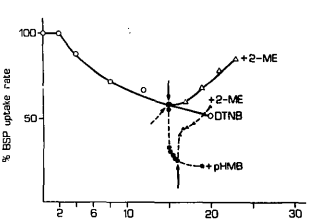

Fig. 6. Time-course of inactivation of BSP electrogenic transport in plasma membrane vesicles by DTNB and DTNB plus pHMB and of reactivation by 2-ME. Experimental conditions: upper inactivation curve (○) 1 mM DTNB, 10 mM Hepes (pH 7.4); lower inactivation curve (●) at the time indicated by the arrow, addition of 1 mM pHMB and 0.083 mM NaOH to 1 mM DTNB and 10 mM Hepes (pH 7.4); upper reactivation curve (△) at the time indicated by the arrow, addition of 1.3% 2-ME to 1 mM DTNB and 10 mM Hepes (pH 7.4), lower reactivation curve (▲) at the time indicated by the arrow, addition of 1.3% 2-ME to 1 mM DTNB, 10 mM Hepes (pH 7.4), 1 mM pHMB, 0.083 mM NaOH. $t = 37°C$. Further details as in Fig. 1.

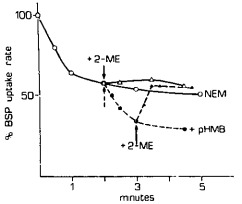

Fig. 7. Time-course of inactivation of BSP electrogenic transport in plasma membrane vesicles by NEM and NEM plus pHMB and of reactivation by 2-ME. Experimental conditions: upper inactivation curve (○) 1.2 mM NEM and 2 mM Hepes (pH 7.4), lower inactivation curve (●), at the time indicated by the arrow, addition of 0.25 mM pHMB and 0.1 mM NaOH to 1.2 mM NEM and 1 mM Hepes (pH 7.4); upper reactivation curve (△) at the time indicated by the arrow, addition of 0.65% 2-ME to 1.2 mM NEM and 2 mM Hepes (pH 7.4); lower reactivation curve (▲) at the time indicated by the arrow, addition of 0.65% 2-ME to 1.2 mM NEM, 1 mM Hepes (pH 7.4), 0.25 mM pHMB and 0.1 mM NaOH. $t = 37°$ C. Further details as in Fig. 1.

Fig. 2, whereas the second one is as slow as DTNB inhibition reversal, indicating that, at least for a while, reacted thiols are reduced by 2-ME at a rate comparable to that of their direct reaction. It should be added (not shown in the figure) that, when the inhibition is

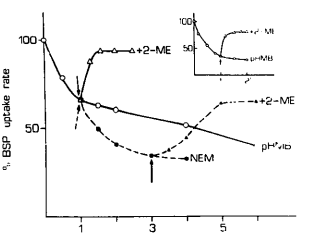

Fig. 8. Time-course of inactivation of BSP electrogenic transport in plasma membrane vesicles by pHMB and pHMB plus NEM and of reactivation by 2-ME. Experimental conditions: upper inactivation curve (○) 0.25 mM pHMB and 0.1 mM NaOH; lower inactivation curve (●) at the time indicated by the arrow, addition of 3 mM NEM and 1 mM Hepes (pH 7.4) to 0.25 mM pHMB and 0.1 mM NaOH; upper reactivation curve (△) at the time indicated by the arrow, addition of 0.65% 2-ME to 0.25 mM pHMB and 0.1 mM NaOH; lower reactivation curve at the time indicated by the arrow, addition of 0.65% 2-ME to 0.25 mM pHMB, 0.1 mM NaOH, 3 mM NEM and 1 mM Hepes (pH 7.4); the arrow, addition of 0.65% 2-ME (△). $t = 37°$ C. Further details as in Fig. 1.

virtually complete, full recoveries may not be achieved, a phenomenon well known in the literature [7].

Here, the last reacting class has been the first to be released by 2-ME. We do not know, however, whether or not the rate of recovery of the DTNB-accessible thiols depends on prior reduction of the DTNB-inaccessible thiols. These possible situations can be investigated by promoting 2-ME reversal of reaction with pHMB, while either one or the other class of thiols is irreversibly bound to NEM. Fig. 7 shows that blockade of DTNB-accessible thiols with NEM does not hinder accessibility of pHMB to DTNB-inaccessible thiols (compare with Fig. 5). Moreover, 2-ME allows fast and total recovery of transport accounted for by this class of thiols. In Fig. 8 we show that partial inhibition of transport with pHMB leaves again thiols accessible to NEM. 2-ME added after NEM has reacted allows a slow recovery of activity, which is to be ascribed to the release of pHMB-reacted thiols. The rate of this process is heavily affected by derivatization with NEM of thiols of the DTNB-inaccessible class. On the contrary, if thiols of this class are still reduced (see upper curve of reactivation) or reducible (see inset), recovery is much faster. It should be noted that, in both experiments shown in this figure, at the time of 2-ME addition, BSP uptake rate is inhibited by pHMB to the same extent as by the combination of pHMB and NEM, so that the conditions of reactivation are strictly equivalent. It can therefore be concluded that the DTNB-inaccessible class of thiols, when bound to NEM, affects the rate of reactivation of the DTNB-accessible class of thiols.

Discussion

Data presented in this paper point to a central role of thiol groups in BSP transport by plasma membrane vesicles. The importance of sulfhydryl groups in transport systems seems to have attracted more and more attention in recent literature and a number of isolated carrier proteins have been shown to include such a functional group in regions of their molecule directly connected with their function [8–10].

The facts that different thiol group reagents are good inhibitors of the function in question and that restoration can be obtained with 2-ME support our conclusion.

The fact that, in contrast to the other reagents, DTNB far from inhibits completely the activity measured suggests that we are dealing with two different transport systems, both located in the plasma membrane, one accessible to this thiol reagent and the other virtually inaccessible. In line with this interpretation would also be the observation that DTNB-sensitive groups are also blocked by the other reagents preferentially or, anyway, at a higher rate.

A phenomenologically equivalent situation would be the existence in suspension of two population of vesicles

with opposite orientation. In the latter case, accessibility of thiol groups would be dependent on the side of the membrane exposed to the medium

Data shown in Fig. 8 lead us to conclude, however, that irreversible blockade of one class of sites (DTNB-inaccessible) has a profound influence on the rate of functional recovery brought about by 2-ME of the activity measured. Such an observation would greatly favor the idea of a perturbation caused by the ligand and passed over to a different region of the same system, rather than interaction between two independent transport systems. Even more difficult to accept would be an effect propagated from one vesicle to another with different orientation.

On the basis of these considerations, we are inclined to conclude that we are measuring the activity of a single transport system. In line with this interpretation also are previous data demonstrating the specificity of the measure of valinomycin-induced BSP uptake in plasma membrane preparations. This process had been shown to be performed by a single carrier, called bilitranslocase, which is responsible for a specific function of the sinusoidal domain of the hepatocyte plasma membrane [5] and is inhibited competitively by both rifamycin-SV and bilirubin [6].

If this interpretation holds true, it remains to be understood why, while availability of both classes of thiols provides maximal BSP transport activity, blockade of one of them still permits this process to occur, although at a reduced rate.

Visualization of the spatial arrangement of these functionally relevant sulfhydryl groups requires further investigation of bilitranslocase at the structural level. These studies are currently under way in our laboratory.

Acknowledgments

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