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Phosphate Transport in Rat Liver Mitochondria: Location of Sulfhydryl Groups Essential for Transport Activities[1]

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Abstract

The membrane orientation and symmetry of protein thiol group(s) necessary for transport of P_i in rat liver mitochondria have been assessed by comparing inhibition of transport in intact mitochondria to that in inverted vesicles of purified inner membrane. The permeability characteristics of a variety of inhibitors have been determined under specified conditions. The sensitivities of the uptake pathways in mitochondria and in inverted vesicles appear thus far to be identical. By comparing results with permeant and nonpermeant inhibitors, or sequential treatment with different inhibitors, arguments can be made in favor of a single reorienting site of thiol sensitivity.

Key Words: Membranes; transport; inorganic phosphate; mitochondria; Thiol groups; orientation.

Introduction

The physical characteristics of the transport system(s) responsible for the uptake and release of P_i from mitochondria are still largely unknown. Klingenberg *et al.* (1974) proposed a rotating carrier model in which a single critical SH group could be exposed at either the matrix or the cytosolic surface, but not at the same time. Fonyo *et al.* (1975) suggested that inhibition of transport might require the modification of two SH groups per transport protein, but presumably both on the same side of the membrane, as the phenomena he described could be observed with a single, nonpermeant

[1]DABS, *p*-(diazonium)-benzenesulfonic acid; IMV, inner membrane vesicles; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; GSH, reduced glutathione; TMPD, *N*, *N*, *N'N'*-tetramethyl-*p*-phenylenediamine; EGTA, ethylene glycol-bis (β-aminoethyl ether); *p*-CMB, *p*-chloromercuribenzoate; NEM, *N*-ethylmaleimide; FCCP, *p*-trifluoromethoxycarbonyl cyanide phenylhydrazone.

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reagent, mersalyl. Studies on P_i uptake in inverted vesicles of inner membrane (Wehrle *et al.*, 1978) have established that a matrix-facing SH group is essential for P_i transport from the matrix to the cytosolic surface. If this transport is via the same protein system responsible for uptake in intact mitochondria, then either a fixed, symmetrical arrangement or a mobile arrangement providing possible exposure of a single SH group at either surface is suggested. In intact mitochondria it is impossible to compare the effects of binding inhibitor at the cytosolic surface alone with the effects of treating only the matrix surface. However, this comparison can be made for the uptake process in IMV, provided the permeability characteristics of the system are unequivocally established.

In order to study the location of the SH group(s) of the P_i transport system it is necessary to have both reagents reversible by free thiol competition and reagents which react essentially irreversibly, which do not migrate when the membrane is disrupted by sonication or detergent. In addition the ability of each reagent to cross the inner membrane under the conditions used must be experimentally determined. Data in the literature regarding permeability of the membrane to these reagents is notoriously contradictory. In the present study a variety of potential labels have been tested, including the classical P_i transport inhibitors *p*-CMB, mersalyl, and NEM, as well as certain other reagents which may react with free SH groups. Ethacrynic acid has been reported to inhibit P_i uptake in mitochondria under certain conditions, and to have little effect on efflux (Goldschmidt *et al.*, 1976). The SH oxidizing agent sodium tetrathionate is highly charged and expected to be nonpenetrant. DABS is a reagent of rather broad specificity which has been shown to inhibit P_i transport in mitochondria under certain conditions (Dawson, 1974). The experiments described below were designed to determine the characteristics of potential labels for the two surfaces of the phosphate transporter and to establish the distribution of the SH groups involved in mitochondrial phosphate transport.

Experimental

Materials

Bovine serum albumin (Fraction V, essentially fatty acid free) and yeast glutathione reductase (Type III) were obtained from Sigma Chemical Company. Sodium Edecrin (ethacrynic acid sodium MSD) was obtained from Merck, Sharp, and Dohme, reconstituted with water, and used directly as the mannitol-containing solution. [^{32}P]Orthophosphoric acid was obtained from New England Nuclear and was incubated at 100°C for 3 hr in 1 N HCl before titration, dilution, and use. Sodium tetrathionate ($Na_2S_4O_6 \cdot 2\ H_2O$)

was synthesized and crystallized as described by Gilman *et al.* (1946). DABS was synthesized as described by Dilley *et al.* (1972) and used as the P_i-containing solution. Other reagents were the highest grades commercially available and were used as provided.

Methods

Rat liver mitochondria were prepared by the high-yield method of Bustamante *et al.* (1977) in H medium: 220 mM D-mannitol, 70 mM sucrose, 0.5 mg/ml bovine serum albumin, 2.0 mM Hepes, K^+, pH 7.4. IMV were prepared by sonication of water-washed mitoplasts as described by Wehrle *et al.* (1978). Mitochondria were always used within 1–2 hr of preparation. IMV ($\leq$1 ml, 50 mg/ml in H medium) were stored in liquid N_2 before use, but were never refrozen after thawing.

In all cases where sulfhydryl-group reagents were used, mitochondria or IMV (either at 2 mg protein/ml in H medium) were incubated with reagent at the level indicated in the tables and figures at 0°C for 5 min (except DABS, 10 min). Samples were centrifuged (20,000 *g*, 20 min for mitochondria; 200,000 *g*, 30 min for IMV), and resuspended in inhibitor-free H medium before use.

P_i transport was assayed in mitochondria by recording swelling in NH_4P_i, measuring decrease in absorbance at 540 nm spectrophotometrically. P_i transport in IMV was assayed by uptake of $^{32}P_i$. IMV, control or treated, were added to assay medium: H medium plus 1.5 mg/ml additional bovine serum albumin, 2.5 mM KP_i containing 0.25 μCi/ml $^{32}P_i$, 10 mM ascorbate, 0.75 mM TMPD. Control experiments also contained either 0.4 mM KCN or 1 μM FCCP, either of which has been shown to block completely carrier-mediated P_i uptake by IMV, which is completely energy dependent (Wehrle *et al.*, 1978). After 5 min at 25°C *p*-CMB (100 nmol/mg of protein in 5 ml of cold H medium) was added to stop transport. Samples were centrifuged (200,000 *g*, 30 min), supernatants drained, and sediments rinsed, dissolved in 88% formic acid, and counted by liquid scintillation. Glutathione content of control and treated mitochondria was determined as described by Wehrle *et al.* (1978), using the glutathione reductase colorimetric assay of Tietze (1969). Substrate oxidation was assayed polarographically using a Clark electrode (Yellow Springs Instruments).

Results

Characterization of Potential Labels

Several combinations of permeant and nonpermeant, reversible and irreversible labels were required to examine the orientation of SH groups

critical to the activity of the major P_i transport system in mitochondria. A variety of reagents, some previously examined (Wehrle *et al.*, 1978) and some previously untested, were incubated under defined uniform conditions with intact mitochondria and with inverted vesicles of purified inner membrane (IMV). All reagents were tested for ability to cross the membrane during the defined incubation period and ability to react covalently with the transporter. In every case mitochondria or IMV were exposed to potential labels, then washed free of unreacted material before assay.

Two criteria were used to assess the degree to which the various SH-group reagents (Fig. 1) crossed the inner membrane of intact mitochondria during the incubation period: reduction in levels of matrix glutathione and inhibition of respiration with β-hydroxybutyrate (relative to inhibition in inverted inner membrane). These methods have been used previously to determine reagent penetration under specified conditions (Gaudemer and Latruffe, 1975 and Wehrle *et al.*, 1978). Inhibition of phosphate transport in

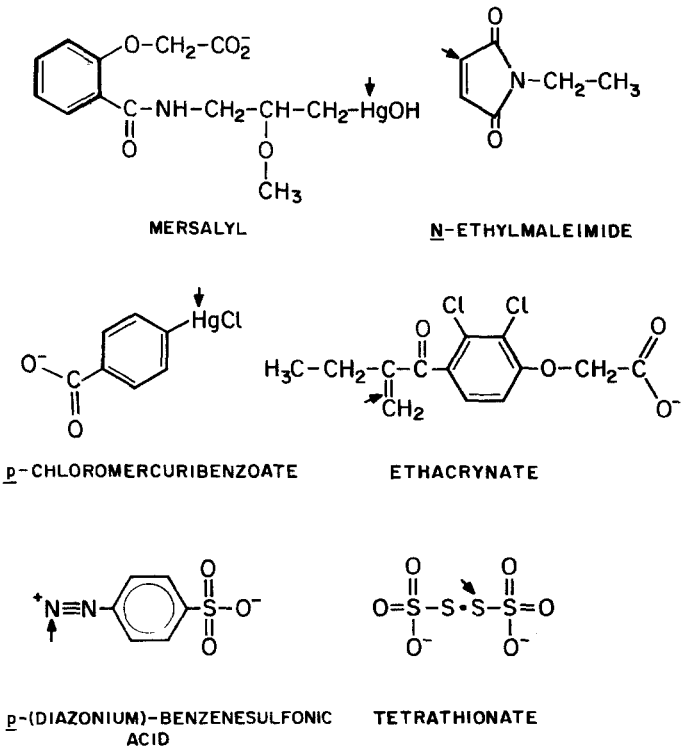

Fig. 1. Structures of sulfhydryl reagents used in this study. Arrows denote point of reaction with sulfhydryl groups.

Table I. Permeability Characteristics of Inhibitors[a]

Pretreatment	GSH content in mitochondria (nmol/mg)	Respiration (% of control)		Swelling in NH_4P_i in mitochondria (OA/0.5 min)
		Mitochondria	IMV	
Control	5.8	100	100	0.32
*p*CMB (25 nmol/mg)	4.6	96	28	0
Mersalyl (25 nmol/mg)	4.5	100	15	0.05
N-ethylmaleimide (50 nmol/mg)	0.5	15	10	0
Ethacrynate (50 nmol/mg)	3.0	39	28	0.37
Tetrathionate (50 nmol/mg)	7.0	85	25	0.32
DABS (40 nmol/mg)	5.2	107	26	0.05

[a]Mitochondria (or IMV) were treated with SH reagents by incubation in H medium (2 mg protein/ml) for 5 min at 0°C with reagents as indicated, then sedimented and resuspended in inhibitor-free H medium. For GSH determination samples were extracted with trichloroacetic acid/HCl and assayed as described in Methods. Mean of three experiments, duplicate assays, error ± 1 nmol/mg in all cases. The respiration assay (pH 7.4, 25°C) contained H medium, 10 mM β-hydroxybutyrate, 75 μM NAD^+ (IMV assays only), 2.5 mM KP_i, 1 μM FCCP, 0.5 mg/ml IMV, or mitochondrial protein. Control rates of respiration were 71 and 40 ng-atoms oxygen per min per mg for mitochondria and IMV respectively. Values represent the averages of four experiments. The swelling assay for P_i uptake by intact mitochondria contained, at 25°C, 120 mM ammonium phosphate, 0.5 mM EGTA, and 0.04 μM rotenone, pH 7.2. Control or treated mitochondria (1.5 mg/ml) were added with rapid mixing. The decrease in absorbance due to light scattering at 540 nm for the first 0.5 min is indicated.

intact mitochondria was assayed by prevention of swelling of mitochondria in isotonic NH_4P_i. Table I summarizes briefly the results of these studies. In the standard incubation neither ethacrynate (permeant) nor sodium tetrathionate (impermeant) inhibited P_i uptake. Even at much higher concentrations (not shown) on inhibiton was found. Inhibition of P_i transport by ethacrynate has been reported to occur only under special conditions, including the presence of Mg^{+2} (Goldschmidt *et al.*, 1976). The other compounds inhibited P_i uptake essentially completely, whether permeant (NEM) or nonpermeant (*p*-CMB, mersalyl, DABS).

Which Reagents Inhibit P_i Uptake in IMV?

Uptake of P_i by IMV requires coupled respiration (Wehrle *et al.*, 1978). To avoid sulfhydryl-sensitive dehydrogenases, ascorbate (plus TMPD) was used as substrate. If IMV are treated with sulfhydryl reagents under conditions identical to those used for intact mitochondria, the inhibition pattern is strikingly similar (Table II). Mersalyl, *p*-CMB, and NEM all inhibit phosphate uptake by IMV essentially completely. DABS also inhibits uptake of P_i by IMV, but ethacrynate and tetrathionate fail to inhibit. As was the case with intact mitochondria, under the conditions used these latter two

Table II. Effect of Sulfhydryl-Group Reagents on Phosphate Uptake by IMV[a]

Pretreatment	P_i uptake (nmol/mg)
Experiment 1	
Control	26
Mersalyl (50 nmol/mg)	4
p-CMB (50 nmol/mg)	0
N-ethylmaleimide (100 nmol/mg)	0
Experiment 2	
Control	29
DABS (40 nmol/mg)	0
Ethacrynate (50 nmol/mg)	25
Tetrathionate (50 nmol/mg)	31

[a]Inner membrane vesicles were treated with sulfhydryl reagents as described in Table I. Phosphate uptake was assayed exactly as described in Methods. The assay (5 min, 25°C) contained in H medium, IMV (0.5 mg/ml), 10 mM ascrobate, 0.15 mM TMPD, 2.5 mM KP_i (0.25 μCi/ml), and 1.5 mg/ml additional bovine serum albumin. Controls contained, in addition, 0.14 mM KCN. Values are averages of quadruplicates; range of error in all cases, ±1 nmol P_i/mg.

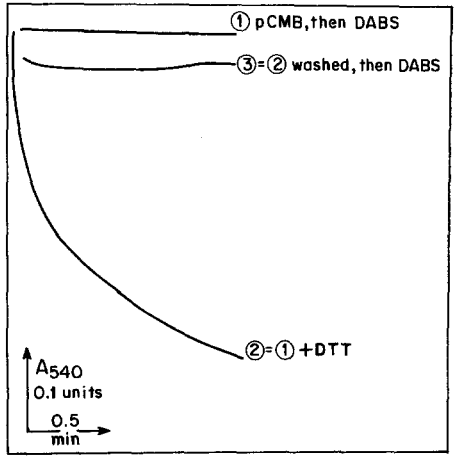

Fig. 2. Protection by *p*-CMB against DABS inhibition. P_i uptake by intact mitochondria was measured by swelling in ammonium phosphate as described in Table I. In (1) mitochondria were incubated with *p*-CMB (15 nmol/mg) for 1 min at 0°C, then DABS (20 nmol/mg) was added for a further 5 min at 0°C. In (2) *p*-CMB + DABS-treated mitochondria were washed with excess DTT in H medium, then in H medium alone. In (3) the washed mitochondria were re-exposed to DABS (20 nmol/mg) for 5 min at 0°C. Mitochondria before any treatment produced a trace identical to (2).

reagents fail to inhibit significantly even at levels as high as 200 nmol/mg of protein (data not shown).

Is DABS Reacting with an SH Group?

Of the reagents which inhibited transport under the conditions used, only DABS could be shown not to cross the membrane (Table I) and formed stable chemical bonds not subject to thiol exchange. However, DABS is not specifically an SH reagent and it was necessary to determine whether DABS was inhibiting P_i transport through the same SH group as the classical inhibitors, or by an entirely separate interaction. Figure 2 illustrates that treatment of mitochondria or IMV with *p*-CMB, which is relatively SH-specific, protects their transport activities against DABS inhibition. Mitochondria were first treated with levels of *p*-CMB sufficient to fully inhibit transport activity, then DABS was added and incubated as usual (Fig. 2, trace 1). After excess DTT was added to remove bound *p*-CMB and any unreacted inhibitors, transport activity was fully restored (Fig. 2, trace 2). After the "protection" sequence, transport activity could be inhibited normally by DABS (Fig. 3, trace 3). The same results were obtained for uptake in IMV (not shown). Inhibition by DABS itself is not reversed with DTT (Dawson, 1974).

Although it is still possible that *p*-CMB may be hindering sterically the access of DABS to some other residue near the critical thiol, the simplest interpretation of the phenomenon of *p*-CMB protection is that in this case DABS has reacted with the thiol group.

Can the IMV Uptake Mechanism be Inhibited from the Cytosolic Surface?

From the results described above it was concluded that DABS alone could be used to answer this question. Mitochondria were treated with DABS under exactly the conditions for which permeability and inhibition properties had been determined. To prevent any carry-over of unreacted DABS into the IMV preparation, excess dithiothreitol was added to the mitochondria at the end of the treatment period, after which the mitochondria were washed as usual. IMV were prepared from the treated mitochondria by the usual procedure and assayed for P_i uptake capacity. The results are shown in Table III. P_i uptake in IMV was found to be inhibited when the IMV were prepared from DABS-treated mitochondria.

Table III also describes another characteristic of P_i uptake in IMV. A reversible, impermeant reagent (*p*-CMB), added first, prevents irreversible inhibition of the carrier not only by another impermeant reagent (e.g., DABS, Fig. 3), but also by a permeant reagent, in this case NEM. This had been found to be the case for intact mitochondria (Coty and Pedersen, 1974).

Table III. Orientation of Inhibitor Sites in IMV[a]

Pretreatment	P_i uptake (nmol/mg)
Experiment 1	
IMV	20
IMV* (from DABS mitochondria)	1
IMV + DABS	0
Experiment 2	
IMV	17
IMV** (*p*-CMB, then NEM)	2
IMV,** then DTT wash	16
IMV,** DTT wash, then NEM	1

[a]Experiment 1: IMV were prepared as described in Methods, either from control mitochondria (IMV) or from mitochondria treated with DABS as in Table I followed by DTT (0.5 mM), then washed twice with H medium. IMV* were prepared from these mitochondria by the standard method. Normal IMV were also treated with DABS followed by DTT and washing. These are designated "IMV + DABS." Experiment 2: IMV were treated with *p*-CMB for 1 min, followed by NEM for 2 min (IMV**). IMV** were washed with excess DTT, then re-exposed to NEM. Inhibitors were used as described in Table II. P_i uptake in all cases was assayed as described in Table II except that 1 μM FCCP was added to controls in place of KCN. Values are the averages of duplicates, range of error, ± 1 nmol P_i/mg.

Discussion

Inhibition of the activity of a membrane-bound transport protein varies with reaction condition in at least two ways, beyond simple changes in chemical reactivity. First, the ability of the inhibitor to penetrate into or across the membrane may be a function of reaction conditions (Gaudemer and Latruffe, 1975). Second, the accessibility of membrane protein thiols may vary due to changes in protein conformation (le Quoc *et al.*, 1977). In the present work, therefore, permeability studies have been carried out for each inhibitor, and under precisely the conditions of inhibitor treatment used in the transport assays. By this method it has been possible to determine that of the inhibitors expected to form nonexchangeable addition products with SH groups (NEM, DABS, ethacrynate) only the first two inhibit P_i transport under the conditions used, and of these only DABS is nonpenetrant. NEM crosses the membrane quite freely, reacting extensively with both membrane-bound matrix-face thiol (β-hydroxybutyrate dehydrogenase) and soluble matrix thiol (glutathione) (Table I). The inability of the thiol oxidizing agent tetrathionate to inhibit the phosphate transporter was not unexpected. Another thiol oxidizing agent, diamide, has also been reported to inhibit P_i transport only slightly (Zaccarato *et al.*, 1977). Mersalyl and *p*-CMB inhibit and are nonpenetrating under the conditions used. These reagents have the advantage that their inhibition can be reversed by added free thiol, but this capacity to exchange thiol sites makes them inappropriate

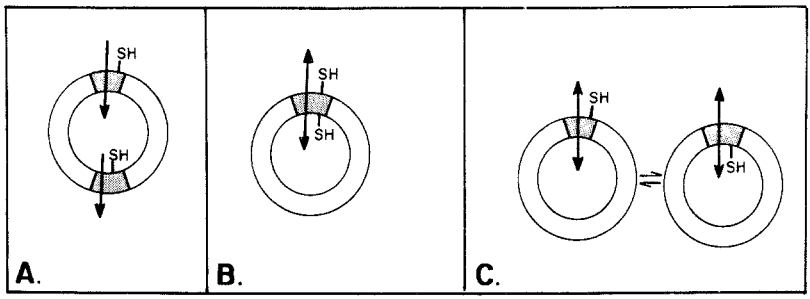

Fig. 3. Models for orientation of P_i transport thiol(s). (A) Independent asymmetric carriers for influx and efflux; (B) symmetric, fixed channel; (C) mobile carrier with a single thiol.

for use as protein orientation markers when the membrane is to be disrupted by detergents or sonication.

Several models for the P_i transporter SH groups which had been suggested in the past are illustrated in Fig. 3. Figure 3A illustrates characteristics which had been unequivocally established prior to the current work. It was known that mitochondrial uptake could be inhibited by reagents which react at the cytosolic surface (e.g., Dawson, 1974). Uptake in inverted vesicles could be inhibited by nonpenetrant reagents at the matrix surface (Wehrle *et al.*, 1978). No assumption is made about identity of the outward with the inward carrier. Two observations support the concept of separate carriers for influx and efflux. First, Azzone *et al.* (1976) observed that inhibition of influx by certain reagents resulted in enhancement of P_i efflux, suggesting independent, competing carriers. Second, uptake in both mitochondria and IMV can be driven by coupled respiration, even though both the membrane potential and the pH gradient have been shown to be reversed in IMV (Wehrle *et al.*, 1978). This implies different mechanisms for energy coupling in the two phenomena.

It is not, however, impossible that the same protein is responsible for both processes. Each of the reagents tested had an effect on transport in IMV identical to its effect (or lack of effect) in intact mitochondria. With the use of other reagents it may become possible to detect differences between the two systems. However, to date the results are not inconsistent with the sites being identical. In this case only a fixed symmetrical arrangement (Fig. 3B) or an alternating site mechanism (Fig. 3C) is possible. A fixed, symmetric model is more difficult to reconcile with the observation of Coty and Pedersen (1974) that inhibition by a reversible nonpermeant reagent protects against inhibition by a freely permeant reagent. Of course it is impossible to rule out the possibility of an indirect, allosteric reduction in reactivity of a matrix SH

caused by mercaptan formation at the cytosolic SH group. However, Fig. 3C is certainly the most consistent with the Coty and Pedersen result. Klingenberg *et al.* (1974) have interpreted the results of experiments with several types of maleimide under a variety of conditions as supporting a model similar to Fig. 3C, in which the P_i binding site and the critical SH group may be exposed at either one surface or the other, but not both simultaneously.

In the present study it has been possible using DABS to demonstrate directly that the carrier responsible for P_i uptake by IMV can be inhibited from either the matrix (Table II) or the cytosolic surface (Table III). In addition it has been shown (Table III) that *p*-CMB also protects against NEM in IMV (Table III). From this it must be concluded that Fig. 3C represents the most likely configuration for the matrix-to-cytosol transport process. The existence of a matrix-facing position for an SH group in mitochondrial uptake cannot be directly established as there is currently no way to return inverted inner membrane to its original orientation. However, if the processes occur via a common carrier, then Fig. 3C represents the best phenomenological model for both processes.

It should be stressed that Fig. 3C is precisely a phenomenological illustration. The rotation of the entire protein component is not a necessary part of the "alternating site" model. Any conformational change which would allow access to the SH group from one aqueous phase while restricting access from the other would fulfill the requirements of the data available so far. This could conceivably involve relatively small changes in protein structure in some type of channel through the molecule.

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