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Phosphate Carrier of Liver Mitochondria: The Reaction of Its SH Groups with Mersalyl, 5,5′-Dithio-bis-nitrobenzoate, and *N*-Ethylmaleimide and the Modulation of Reactivity by the Energy State of the Mitochondria

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Abstract

The inhibitory effect of three SH reagents, mersalyl, 5,5′-dithio-bis-nitrobenzoate, and *N*-ethylmaleimide, on P_i transport in rat liver mitochondria was investigated under a variety of conditions. Mersalyl binds at room temperature with both high ($K_d < 10\ \mu M$) and low affinity to mitochondria. Inhibition of P_i transport by mersalyl goes in parallel with titration of the high-affinity sites, inhibition being complete when 3.5–4.5 nmol/mg protein is bound to the mitochondria. At concentrations of mersalyl equal to or higher than 10 μM, inhibition of P_i transport occurs in less than 10 sec. At concentrations of mersalyl lower than 10 μM, the rate of reaction with the P_i carrier is considerably decreased. At a concentration of 100 μM, 5,5′-dithio-bis-nitrobenzoate fully inhibits P_i transport in about 1 min at room temperature. Nearly total inhibition is attained when as little as 40–50 pmol/mg is bound to mitochondria. Upon incubation longer than 1 min, additional SH groups, not belonging to the P_i carrier, begin to react. The uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone decreases the rate of reaction of mersalyl, 5,5′-dithio-bis-nitrobenzoate, and *N*-ethylmaleimide with the P_i carrier. Preincubation with P_i has a similar effect. We propose that both carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and P_i act by increasing the acidity of the mitochondrial matrix. Protonation of the P_i carrier at the matrix side would change the accessibility of its SH groups at the outer surface of the inner membrane. This might correspond to a membrane-Bohr effect, possibly related to the opening of a gating pore in the P_i carrier.

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Introduction

Organic mercurials, such as *p*-hydroxymercuribenzoate and mersalyl (sodium salt of *O*-[(3-hydroxymercuri-2-methoxypropyl)carbamyl] phenoxy acetic acid), are powerful inhibitors of the mitochondrial P_i carrier ([1–4]; see also [5–6] for review). The action of the mercurials is rapid [7] and they "freeze" the P_i content of the mitochondria at the moment of their addition. This rapid action made it possible to stop P_i transport at any given moment and to measure the rate of transport by the "inhibitor-stop" principle [8–10]. Recently, however, we found that whereas mersalyl blocked P_i efflux immediately and completely from mitochondria treated by valinomycin, it inhibited P_i efflux only after some delay if the mitochondria were uncoupled [11]. This finding was considered to be important for two reasons:

1. Because current methodology of the measurement of P_i transport is based on the assumption of an immediate quenching of transport by mercurials [10].
2. Because it pointed to the possibility that the reactivity of the P_i carrier may be altered by proton-conducting uncouplers like carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP); this might be indicative of the relationship which is known to exist between proton and P_i transport in mitochondria [7, 12–14].

In the present paper we describe the FCCP- and P_i-induced change of reactivity of the SH groups of the mitochondrial P_i carrier with three SH reagents, mersalyl, 5-5′ dithio-bis-nitrobenzoate (DTNB), and *N*-ethylmaleimide (NEM). Because no described data were available on the quantitative aspects of mersalyl and DTNB action, these are included in the present report.

Materials

[^{14}C]-mersalyl and [^{14}C]DTNB were synthesized by the Centre d'Etudes Nucléaires de Saclay, Gif-sur-Yvette. The former was purified before use by thin-layer chromatography on F-1440 cellulose plate (20 × 20 cm) of Schleicher and Schull by using a solvent system made of ethanol–water–NH_4OH (80:20:10). The labeled compound was localized by autoradiography; its chemical purity was checked by ultraviolet spectrophotometry (λ_{max} = 288 nm, λ_{min} = 266 nm). The [^{14}C]DTNB was used without further purification.

Methods

Rat liver mitochondria were isolated by conventional differential centrifugation in 0.25 M sucrose and 5 mM Tris-HCl at pH 7.4. The protein content was determined immediately by a rapid biuret method and the suspension was diluted to contain 50 mg protein/ml.

[^{14}C]-Mersalyl Binding

A 5-mg sample or in some instances 1 mg of mitochondrial protein was incubated at room temperature in 0.25 M sucrose, 2 mM Tris-HCl, 0.5 mM EDTA, and 2 μM rotenone, pH 7.4, in 1 ml final volume for 1 min. [^{14}C]-mersalyl was rapidly added, and after 1 min further incubation the suspension was centrifuged in the cold in the Sorvall SS1 centrifuge for 2 min at 20,000 *g*. The supernatant was decanted, and the pellet rinsed with 0.15 M KCl, drained, and dissolved in 1 ml formamide. The radioactivity of the whole pellet and of an aliquot of the supernatant was counted by liquid scintillation counting. The radioactivity of the pellet was corrected for the [^{14}C]-mersalyl in the extramitochondrial space. This latter was determined in separate experiments by the distribution of [^{14}C]-sucrose. The result of this determination (2.6 μl/mg protein) was in agreement with those of Harris and Van Dam [15].

[^{14}C]DTNB Binding

A 5-, 10,- or 20-mg sample of mitochondrial protein was incubated at room temperature in 0.25 M sucrose, 20 mM Tris-HCl, 0.5 mM Na-EDTA, and 2 μM rotenone at pH 7.4. After a 1-min preincubation period, [^{14}C]-DTNB was rapidly added and the unreacted DTNB was inactivated by addition of 2.6 mM 2-mercaptoethanol at given times. For control, DTNB and mercaptoethanol were added to the incubation medium before addition of the mitochondria. After addition of the mercaptoethanol, the suspension was chilled in ice, and an aliquot (3 ml) was immediately transferred on top of 7 ml of 0.5 sucrose and 20 mM Tris-HCl pH 7.4 in 10 × 1.2 cm centrifuge tubes. The mitochondria were centrifuged at 20,000 *g* for 6 min through this sucrose layer; this washing procedure was found necessary because only a very small fraction of the [^{14}C]DTNB reacted with the mitochondria. The pellet was rinsed with 0.15 M KCl, drained, dissolved in formamide, and counted by liquid scintillation counting.

P_i Transport

Inorganic phosphate (P_i) uptake in 100 mM ammonium phosphate (pH 7.4) and 0.5 mM EDTA was followed at room temperature by three methods.

a. Continuous Recording of Swelling ("Conventional" Technique). Mitochondria containing 2.5 mg protein were preincubated in 0.25 M sucrose, 20 mM tris-HCl, 0.5 mM EDTA, and 2 μM rotenone at pH 7.4 in a final volume of 1.5 ml. Further additions were made as described with the individual experiments. In the experiments with mersalyl the reaction was terminated by rapid injection of 0.5 ml of the suspension into the cell of the spectrophotometer at the time indicated. In the experiments with either DTNB or NEM the reaction was terminated by the addition of 2-mercaptoethanol and dithiothreitol respectively, and 0.5 ml was added rapidly to the spectrophotometer cell at a standard time (3 min). The spectrophotometric cell contained 2.5 ml of the ammonium phosphate solution. Recording was started immediately in the LERES spectrophotometer equipped with a SEFRAM recorder at 546 nm. The rate of P_i transport and its inhibition was calculated from the decrease of absorbance [16].

b. Increase in the P_i Content of Centrifuged Mitochondria. The preincubation was carried out as described in the preceding paragraph. After the preincubation period, a 0.5-ml aliquot of the suspension was rapidly injected into a centrifuge tube containing 2.5 ml of ammonium phosphate solution: after 5, 10, or 15 sec the addition of a large excess of mersalyl terminated the transport. The mitochondria were immediately centrifuged at 20,000 *g* for 90 sec in the Sorvall SSl centrifuge in the cold, and the tubes rinsed with 0.15 M KCl to remove any adherening ammonium phosphate solution, drained, and extracted with 1.5 ml of 1 N perchloric acid. Inorganic phosphate was determined in an aliquot of the extract by the method of

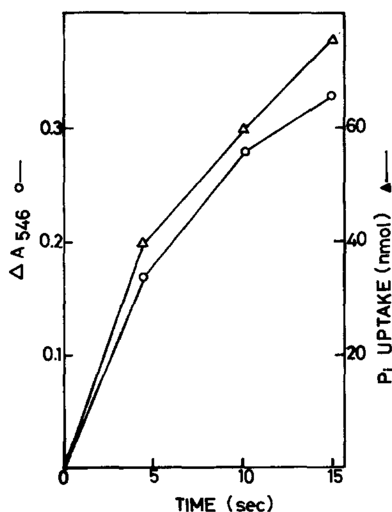

Fig. 1. The "stopped swelling" and the simultaneously measured P_i transport of mitochondria. Mitochondria (3.3 mg protein/ml) were preincubated as described in Methods. The preincubated suspension (0.5 ml) was injected into 2.5 ml of 100 mM ammonium phosphate–0.5 mM EDTA at pH 7.4. At the times indicated (0, 5, 10, and 15 sec) 300 μM mersalyl was added to stop P_i transport. The absorbance of one set of tubes was read immediately at 546 nm (o). Another set of tubes was immediately centrifuged for P_i determination as described under Methods (Δ).

Wahler and Wollenberger [17]. In the control tubes mersalyl was added before the mitochondria. The course of P_i uptake is shown in Fig. 1.

c. "Stopped Swelling." In the conventional recording of mitochondrial swelling, the initial, most rapid phase of P_i transport was not measured. This difficulty was overcome by stopping the transport of P_i by mersalyl (as described in *b* above) and reading the absorbance of the suspension at 546 nm. This reading was usually stable for at least 2 min. The zero time control was a sample into which mersalyl was added before the mitochondria, and the rate of swelling was calculated from the difference in the absorbance values. In Fig. 1, the increase in P_i content and the change of absorbance measured by the stopped swelling are compared. Both processes are parallel.

Results

Binding of Mersalyl to Mitochondria and Its Inhibitory Action on P_i Transport

The binding of mersalyl to mitochondria was measured under two slightly different conditions. In order to get the approximate affinity and dissociation constant for mersalyl, 1 mg of mitochondrial protein/ml had to be used. On the other hand, the relationship between mersalyl binding and inhibition of P_i transport was investigated with 5 mg protein/ml. Figure 2 (inset) shows that at least two different types of mersalyl binding sites can be distinguished: one with a K_d value in the range of 3–5 μM and one or more to which mersalyl is bound with much lower affinity. The number of high-affinity sites was between 3.5 and 4.5 nmol/mg protein. The relationship between P_i transport and bound mersalyl is shown in Fig. 2. Inorganic phosphate transport was little affected when less than 0.5 nmol mersalyl/mg protein was bound. The inhibition of transport became practically complete on binding of 2.5–3.0 nmol/mg protein. It is implicit in these results that the SH groups which are essential for P_i transport coincide with the mersalyl high-affinity sites. It should, however, be emphasized that there is no evidence that all the high-affinity SH groups should belong to the P_i carrier.

A similar relationship between P_i transport and mersalyl binding was found when an excess of mersalyl was first applied and variable levels of dithiothreitol were added to partially remove the bound mersalyl (results not shown).

The rate of reaction of mersalyl with the P_i carrier was obtained by measuring the inhibition of P_i transport (Fig. 3). At concentrations lower than 10 μM, mersalyl reacts with the P_i carrier with a measurable rate; for example, full inhibition of transport by 8 μM mersalyl was reached after 30

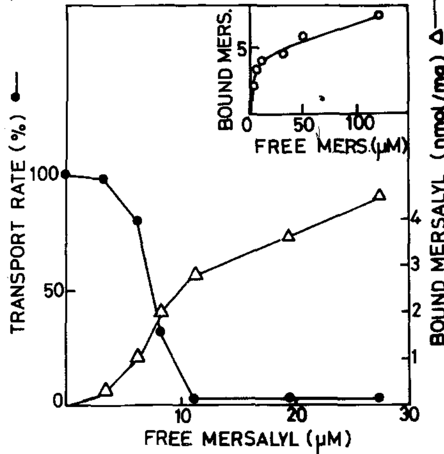

Fig. 2. Relationships of the P_i transport to the concentration of free mersalyl and the mersalyl binding to mitochondria. Mitochondria (5 mg protein) were incubated as described in Methods with increasing concentrations of [^{14}C]mersalyl for 1 min. In one set of tubes P_i transport was measured by the continuous recording of absorbance in 100 mM ammonium phosphate–0.5 mM EDTA at pH 7.4 (●). The calculation was based on the change between 5 and 15 sec. In another set of tubes, bound [^{14}C]mersalyl was measured in the pellet after centrifugation of mitochondria (Δ). The inset gives the relationship between free mersalyl concentration and mersalyl bound to mitochondria for a large range of mersalyl concentrations; in the latter case 1 mg protein/ml was incubated with [^{14}C]mersalyl.

sec. With concentrations of mersalyl lower than 6 μM, P_i transport was no longer inhibited even after a 2-min period of contact with the inhibitor. At concentrations of mersalyl higher than 10 μM, full inhibition was attained in less than 10 sec and the initial kinetics of inhibition could not be resolved.

Inhibition of P_i Transport by DTNB and DTNB Binding to Mitochondria

DTNB inhibited the P_i carrier rather slowly: the time needed for inhibition depended on the concentration of the reagent (Fig. 4). Full

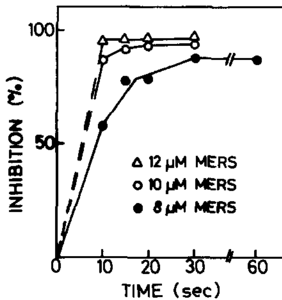

Fig. 3. The rate of the reaction of the P_i transport system with mersalyl. Mitochondria (1.67 mg protein/ml) were preincubated as described under Methods (conventional technique). After 60-sec preincubation mersalyl (MERS) was added at the concentration indicated. At the times indicated on the abscissa, a 0.5-ml aliquot of the suspension was rapidly injected into 2.5 ml of 100 mM ammonium phosphate–0.5 mM EDTA and recording of absorbance was started immediately.

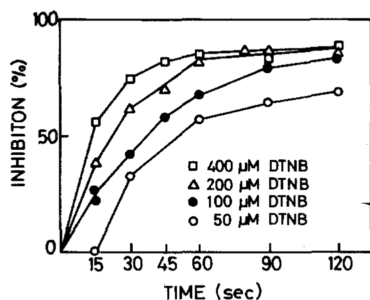

Fig. 4. The rate of reaction of the P_i transport system with DTNB. Mitochondria (1.67 mg protein/ml) were preincubated as described under Methods. After 60-sec preincubation, DTNB was added at the concentrations indicated. At the times indicated on the abscissa, 2.7 mM 2-mercaptoethanol was added to terminate the reaction. At 180 sec a 0.5-ml aliquot was injected into 2.5 ml of 100 mM ammonium phosphate–0.5 mM EDTA, the other experimental details being identical with those described in Fig. 3.

inhibition required a 1-min preincubation with 200-400 μM DTNB. The rate of inactivation was decreased by changing the external pH from 7.0 to 7.5.

The amount of [^{14}C]DTNB bound to the mitochondria increased in a parallel manner to the inhibition of P_i transport (Fig. 5). However, when the maximum inhibition of P_i transport was attained, the amount of bound [^{14}C]DTNB was only 45 pmol/mg protein. [^{14}C]DTNB binding to mitochondria proceeded in time after the inhibition of P_i transport has reached its maximum.

Effect of FCCP on the Rate of Reaction of Mersalyl, DTNB, and NEM with the P_i Carrier

We reported recently [11] that whereas mersalyl blocked immediately and completely P_i efflux from mitochondria hydrolyzing ATP in the presence of valinomycin, the same reagent inhibited P_i exit only after a 0.5-min delay period under uncoupled conditions. These data are corroborated by the experiment illustrated in Fig. 6A, in which the effect of mersalyl was tested on P_i uptake in the absence or presence of FCCP. Clearly, FCCP counteracted the inhibitory effect of mersalyl on P_i transport. Using 10-sec preincubation periods with different concentrations of mersalyl, a comparable

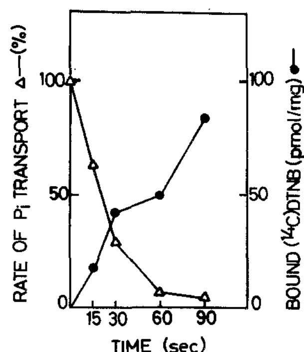

Fig. 5. The relationship between P_i transport and the binding of DTNB to mitochondria. Mitochondria (6.67 mg protein/ml) were preincubated as described under Methods. After 60 sec preincubation, 100 μM [^{14}C]DTNB was added. At the times indicated on the abscissa, 2.7 mM 2-mercaptoethanol was added to terminate the reaction. In one set of tubes, a 0.125-ml aliquot (0.83 mg protein) was injected into 2.9 ml of 100 mM ammonium phosphate–0.5 mM EDTA and recording of the absorbance was started immediately. In another set, 3 ml of the suspension (20 mg protein) was layered on the top of a sucrose layer and centrifuged as described under Methods. Full inhibition corresponds to the binding of 45 pmol of [^{14}C]DTNB/mg protein.

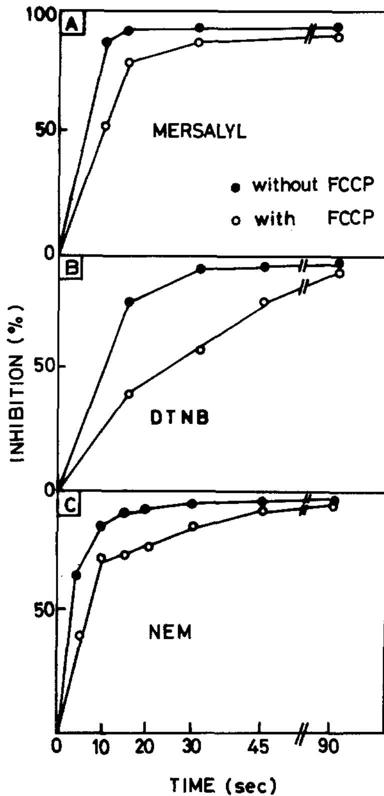

Fig. 6. The effect of preincubation with FCCP on the rate of the reaction of the P_i transport system with mersalyl, DTNB, and NEM. Mitochondria (1.67 mg protein/ml) were preincubated as described under Methods. (A) FCCP (1.3 μM) was added at 30 sec where indicated (o). 13.3 μM mersalyl was added at 60 sec and after the time period indicated on the abscissa, 0.5 ml aliquot of the suspension was injected into the ammonium phosphate solution as described in Fig. 3. (B) FCCP (1.3 μM) was added at either 30 sec (before DTNB addition, o) or after the reaction with DTNB was terminated by 2-mercaptoethanol (•). All other experimental details are identical with those of Fig. 4. (C) FCCP (1.3 μM) was added at either 30 sec (before NEM addition, o) or after the reaction with NEM was terminated by dithiothreitol (•). At 60 sec 200 μM NEM was added. After the reaction time indicated on the abscissa, 1 mM dithiothreitol was added to stop the reaction with NEM. At 180 sec 0.5 ml of the suspension was injected into 2.5 ml of 100 mM ammonium phosphate–0.5 M EDTA at pH 7.40 and recording of the absorbance was started immediately.

inhibition of transport required a higher concentration of mersalyl when FCCP was present (Fig. 7).

These results point to the possibility that in the presence of the uncoupler, it is a change in the reactivity of the SH groups of the P_i carrier which results in slower inhibition. There is, however, an alternative explanation. According to this, FCCP could unmask some SH groups in mitochondria which are not related to P_i transport and which could bind mersalyl. This

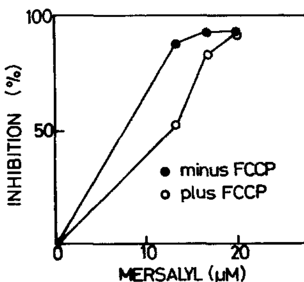

Fig. 7. The effect of preincubation with FCCP on the reaction of the P_i transport system with different concentrations of mersalyl. Same conditions as in Fig. 6A. The preincubation with mersalyl lasted for 10 sec.

would reduce the available concentration of free mersalyl and consequently slow down the rate of inhibition of the P_i carrier.

To distinguish between these two possibilities, we decided to use two other SH-group reagents, DTNB and NEM, which have to be added in a large molar excess over the reacting SH groups to inhibit P_i transport. Data in Fig. 6B and 6C show that preincubation of the mitochondria with FCCP decreased the rate of inactivation of P_i transport by DTNB and NEM. The conventional technique of continuous recording of swelling in ammonium phosphate solution was routinely used in the above experiments. Similar results were obtained by direct determination of P_i uptake (Table I) or by the stopped-swelling technique (results not shown). NEM is a penetrant reagent, and DTNB and mersalyl are nonpenetrant. Whatever the penetrant or nonpenetrant nature of the SH reagents used, uncoupling delays their inhibitory effect.

Effect of P_i Addition on the Rate of Reaction of the P_i Carrier with Mersalyl and DTNB

FCCP, a proton-conducting uncoupler, abolishes both components of the total proton motive force, ΔpH and $\Delta\psi$. As a secondary effect, it also causes the efflux of P_i from the mitochondrial matrix [18, 19]. FCCP could have affected the reactivity of the P_i carrier toward various SH-group reagents by changing $\Delta\psi$, ΔpH, or both, or by depleting intramitochondrial P_i.

Treatment of mitochondria by P_i results in the transport of acidic equivalents into the mitochondria [7, 13, 14] and in an increase of the matrix P_i content [8]. As shown in Fig. 8, preincubation of mitochondria with 6 mM P_i decreased the rate of reaction of the P_i carrier with mersalyl. Using 10-sec reaction time with changing mersalyl concentrations revealed that more mersalyl was needed for a given inhibition of P_i transport in the presence than in the absence of P_i. Inactivation of the P_i carrier by DTNB and NEM was similarly affected by P_i (data not shown).

Table I. Effect of DTNB on P_i Transport without and with FCCP as Assayed by Direct Determination of Phosphate Uptake by Mitochondria[a]

Time for DTNB action	FCCP added	P_i uptake, nmol · mg^{-1} · 10 sec^{-1}	Percent inhibition
0	—	48.9	0
15	—	15.3	68.7
30	—	1.4	97.1
15	+	23.0	53.0
30	+	17.1	65.0

[a]Experimental conditions are described under Methods. FCCP was used at the final concentration of 1.3 μM.

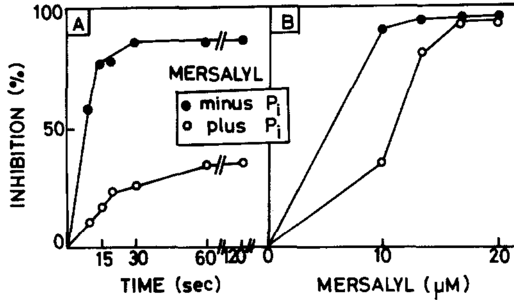

Fig. 8. The effect of preincubation with P_i on the reaction of the P_i transport system with mersalyl. Experimental conditions are similar to those described in Fig. 6A. Tris-phosphate (6 mM, pH 7.4) was added where indicated (○) at 30 sec, before addition of mersalyl. (A) Mersalyl (8 μM) was added at 60 sec, and after the time period indicated on the abscissa a 0.5-ml aliquot of the mitochondrial suspension was injected into the ammonium phosphate solution. (B) Mersalyl was added at the concentrations indicated on the abscissa, and a 0.5-ml aliquot of the mitochondrial suspension was injected into ammonium phosphate at 10 sec.

Discussion

Relationship between Binding of SH Groups and Inhibition of P_i Transport

Comparison of the data on mersalyl and DTNB binding when transport was inhibited reveals a difference between the two reagents. Binding of 3.5 to 4.5 nmol mersalyl/mg protein was required to inhibit P_i transport. Only part of the bound mersalyl probably corresponds to the SH groups of the P_i carrier, since several mitochondrial proteins bind mersalyl [20]. On the other hand, only 45 pmol DTNB/mg protein was bound when P_i transport was totally inhibited. This amount of bound DTNB is of about the same order of magnitude as the number of SH groups reported by Coty and Pedersen [9, 21] and Hadvary and Kadenbach [22], i.e., about 60 pmol/mg protein in liver mitochondria. The DTNB binding may indicate the amount of SH groups of the P_i carrier. On the other hand, it is possible that DTNB binding underestimates the amount of SH groups. It has been reported that if disulfide reagents react with vicinal dithiols the primary product formed from the disulfide and one of the SH groups is easily cleaved by the other SH group in the vicinal position. The result is displacement of the originally bound reagent and secondary formation of an intramolecular S–S bridge [23]. This possibility cannot be excluded at present in our experiments. Evidence has been presented that the P_i carrier contains two SH groups per active unit [22, 24]. These two groups may be in close juxtaposition. We found earlier that the inhibition of the P_i carrier by DTNB is easily reversed by the dithiol dithiothreitol but little reversal occurs by the monothiol 2-mercaptoethanol (Fonyo and Vignais, unpublished). This difference in reactivation could mean

that dithiols are present in the P_i transport system. Further work is needed to clarify this point.

Reaction of the P_i Carrier with Penetrant and Nonpenetrant SH Reagents

Two types of SH reagents, penetrant (NEM) and nonpenetrant (mersalyl and DTNB), were used in this study. The penetrant reagent NEM can penetrate the inner mitochondrial membrane and react with the SH groups within the matrix as well as at the inner surface of the membrane. Mersalyl and DTNB, which do not penetrate the inner mitochondrial membrane, react exclusively with SH groups accessible from the outer surface of the membrane ([25, 26]; see further [5] for a review). It was proposed that the selective action of the penetrant and nonpenetrant reagents on P_i transport could reveal the orientation of the SH groups of the P_i carrier in the membrane and also changes in orientation when the carrier was functioning [16, 27, 28, 29, 30]. In the first of these studies, the inhibitory action of nonpenetrant (ASPM) and penetrant (NEM) maleimide derivatives was analyzed [16, 27]. Based on the finding that ionophores as well as P_i itself changed strongly the accessibility of the SH groups to ASPM whereas the reaction with NEM was less affected, it was concluded that during a P_i transport cycle the SH groups undergo reorientation from one side of the membrane to the other [16]. These observations were, however, contradicted by other data [31] showing that the action of NEM on P_i transport was strongly diminished by P_i and by the ionophores of the nigericin class. On the other hand, the action of a nonpenetrant SH reagent, *p*-diazobenzene sulfonate, was changed by various effectors in the opposite sense with respect to the action of ASPM, another nonpenetrant SH reagent [28]. These observations cast some doubt on the postulate of a transmembrane reorientation of some SH groups in the P_i carrier during a P_i transport cycle [16].

In the present work, we found that FCCP antagonized similarly the inhibition of P_i transport by penetrant and nonpenetrant SH reagents. Briand et al. [32] found no effect of FCCP preincubation on the inhibition by NEM; however, in their experiment the incubation with 100 μM NEM was much longer (90 sec) than in our experiments (cf. Fig. 6); therefore the effect of FCCP could have been overlooked. The failure to find an effect of P_i on the inhibition given by mersalyl in the experiments reported by Guérin [33, 34] may have a similar basis; the concentration of mercurial employed was so high that no effect of P_i could be detected (cf. Fig. 8).

Inhibition of P_i transport was measured in some earlier reports by monitoring swelling of the mitochondria in the presence of ATP, an uncoupler and inhibitor of P_i transport. Swelling follows indeed closely the P_i retention within the matrix [11]. By using this method we found, contrary to the suggestion of Reynafarje and Lehninger [35], that there was no extra exit

pathway for P_i, linked to ATP uptake. However, in this method the rate of swelling depends on both the rate of ATP hydrolysis and the inhibition of P_i exit. The decrease in the rate of swelling therefore does not automatically mean a decrease of the inhibitory action of P_i transport inhibitors. We found that in this system the rate of ATP hydrolysis itself was seriously changed even by the sequence of addition of the uncoupler and the transport inhibitor (unpublished observations).

Is There a Common Factor in the Action of FCCP and P_i on the P_i Carrier?

As shown in this paper, P_i decreases the rate of reaction of the P_i carrier with mersalyl, DTNB, and NEM. It was reported earlier that P_i had a similar effect when ASPM and NEM were used as inhibitors of P_i transport [16, 31, 32]. Because P_i acted in the same sense as did FCCP, this opened the question whether there was a common step in their action.

It was suggested in [16] that it is the increase in intramitochondrial P_i content that decreases the reaction of the SH groups of the P_i carrier with NEM and ASPM. FCCP and other uncouplers, however, are known to deplete the matrix of P_i; yet they act similarly to added P_i. Both FCCP and P_i abolish the ΔpH across the mitochondrial membrane and under usual conditions lower the intramitochondrial pH value [7, 13]. We propose that it is this internal acidification and protonation of the P_i carrier at the matrix surface that is responsible for the decrease in reactivity of its SH groups.

The effect of protonation at the matrix side should be clearly distinguished from the effect of acidification at the external surface. The latter increases SH group reactivity with DTNB and with mersalyl while it decreases the reactivity with NEM (see also [5] and [16]). The acidification at the matrix side may trigger a "membrane-Bohr effect" resulting in a change of the geometry of the polypeptide chain(s) of the P_i carrier system. The result would be that the SH groups are withdrawn into deeper "pouches" of the protein where they are less accessible to the SH group reagents. It is thus not necessary to postulate a "cycling" of the SH groups of the P_i carrier with each transport cycle as was proposed in [16]. The effect of protonation at one part of the P_i carrier on the accessibility of SH group(s) at a distant part of the carrier may be fundamental in the functioning of the carrier itself; for example, it may be related to the opening of a gating pore in the P_i carrier.

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