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Phosphate Transport in Mitochondria and Submitochondrial Particles: The Influence of Thiol Oxidation

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Abstract

Diamide, a thiol oxidizing agent, partially inhibited P_i uptake by rat liver mitochondria.* The inhibition was temperature dependent; at 20°C, the optimal temperature for maximum inhibitory effect, diamide also reduced the minimal amount of mersalyl required for the inhibition of P_i transport. Under the same conditions no inhibitory effect on P_i efflux was observed. The amount of mitochondrial thiol groups titrated by the amounts of diamide needed for the inhibition of P_i uptake was on the order of 5 nmole/mg protein. Unlike liver mitochondria, the P_i transport system of heart mitochondria was insensitive to diamide. On the contrary, accumulation of P_i into submitochondrial heart vesicles, previously loaded with $MnCl_2$, was inhibited by diamide. These results outline the different positional character of membrane thiol groups of mitochondria from various sources, and provide further evidence of an asymmetric orientation of the P_i transport system in mitochondrial membranes.

* Abbreviations: P_i = inorganic phosphate; DTNB = 5,5′-dithiobis-2-nitrobenzoic acid; NEM = *N*-ethylmaleimide; diamide = (diazenedicarboxylic acid bisdimethylamide); DTE = dithioerythritol; FCCP = *p*-trifluoromethoxyphenylhydrazone; RLM = rat liver mitochondria; RHM = rat heart mitochondria.

Introduction

It is generally accepted that the transport of P_i across the mitochondrial membrane is a "carrier"-mediated process. The process is specifically inhibited by reagents, such as mercurials, DTNB, and NEM, which react with single thiols to form mercaptide, mixed disulfide, and adducts, respectively [1].

The relationship between the oxidation state of mitochondrial membrane thiols and P_i transport is, however, poorly understood at present. Diamide is a thiol reagent that appears suitable for the study of the influence of perturbations in the thiol–disulfide balance on P_i transport across the mitochondrial membrane [2].

This paper describes the various effects induced by diamide on P_i transport in liver and heart mitochondria as well as in heart submitochondrial particles. Different mitochondrial and submitochondrial preparations respond quite differently to diamide. Furthermore the results obtained with these preparations provide additional evidence for asymmetry in mitochondrial membranes, with respect to P_i transport.

The asymmetric orientation of the P_i transport system in mitochondria has been suggested by Rhodin and Racker [3] and, on a different basis, by some of us in a preceding study [4].

Materials and Methods

Rat liver mitochondria were isolated in 0.25 M sucrose according to Schneider [5]; rat and beef heart mitochondria were isolated as described by Pande and Blanchaer [6] and Smith [7], respectively. Beef heart submitochondrial particles were prepared according to Rhodin and Racker [3]. Protein concentration was determined by a biuret method. $^{32}P_i$ uptake in mitochondria was determined as described by Papa et al. [8]. The P_i efflux from mitochondria was followed by the swelling method, based on the generation of P_i in the mitochondrial matrix by FCCP-stimulated ATP hydrolysis as described by Klingenberg et al. [9].

For the direct measurement of P_i efflux, $^{32}P_i$ generated in the FCCP [γ^{32}P] ATP system was determined both in the supernatant and in the pellet following the procedure of Lindberg and Ernster [10].

The amount of SH groups was determined by titrating the unreacted SH groups with [^{14}C]NEM, according to Klingenberg et al. [9]. $^{32}P_i$ uptake in submitochondrial particles was determined according to Rhodin and Racker [3]. $^{32}P_i$ was counted employing a Beckman LS-100 C spectrophotometer.

Results

Liver Mitochondria

Figure 1 shows that diamide partially inhibits P_i uptake by rat liver mitochondria. Maximum inhibition was reached (about 35%) with 100–120 nmole diamide/mg protein, and was temperature dependent. The temperature optimum for highest inhibition ranged between 20 and 25°C. Below 10°C no inhibition was observed.

Figure 1 also shows that the inhibition induced by mersalyl on P_i transport was complete at much lower concentrations of the reagent and was temperature independent.

Both the complete inhibition of P_i uptake by mersalyl and the partial inhibition induced by diamide in RLM were fully abolished by the subsequent addition of DTE (Table I).

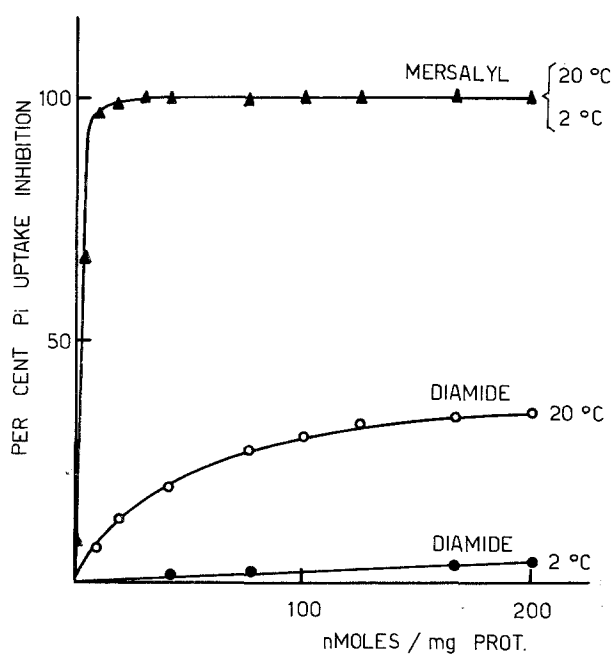

Figure 1. Percent inhibition of P_i transport by rat liver mitochondria as a function of diamide and mersalyl concentrations at different temperatures. Rat liver mitochondria (5 mg protein/ml) were preincubated 4 min in 125 mM KCl, 20 mM Tris-HCl (ph 7.00), 3 μg/ml rotenone, 0.5 μg/ml antimycin A, and 15 μg/ml oligomycin. $^{32}P_i$, 1 μmole/ml (110,000 cpm), was then added and after 45 sec, mitochondria were rapidly centrifuged. $^{32}P_i$ was counted by a Beckman LS-100 G spectrophotometer. When present diamide was added before mitochondria, and mersalyl 3 min after mitochondria.

TABLE I. Reversible inhibition of diamide and mersalyl on P_i transport by RLM [a]

Conditions	nmole P_i/mg protein		IRC	
	−DTE	+DTE	−DTE	+DTE
None	9.3	9.4	3	5.8
Diamide	6.0	9.7	1	3.4
Mersalyl	1.9	9.5	1	2.6

[a] Experimental conditions as in Fig. 1. When added, mersalyl was 40 nmole/mg protein and diamide 120 nmole/mg protein. DTE, 250 nmole/mg protein, was added with $^{32}P_i$. Temperature was 20°C. Respiratory control index (IRC) was determined under the same conditions in a medium containing 125 mM KCl, 20 mM Tris-HCl (pH 7.4), 2 mM $MgCL_2$, 10 mM succinate, and 3 μg/ml rotenone. Added ADP was 200 nmole. Correction for the sucrose-permeable space was not made.

Parallel experiments carried out in the absence of respiratory chain and oxidative phosphorylation inhibitors permitted the recognition of the state of the mitochondria following restoration of P_i transport by DTE. After incubation with DTE, both mersalyl- and diamide-treated mitochondria were still coupled, thus indicating a complete recovery of the native state of mitochondria (Table I).

Figure 2 shows that in the presence of 100 nmole diamide/mg protein the amount of mersalyl needed for 75% inhibition of P_i uptake was significantly lower. Furthermore, in line with the observation that diamide is inactive below 10°C (Fig. 1) no synergism between diamide and mersalyl was observed at 0°C.

In contrast with its inhibitory effect on the influx of P_i into RLM, diamide did not affect the efflux of P_i. Diamide behavior was, in this case also, different from that of mersalyl (Fig. 3). That the effect demonstrated in Fig. 3 is dependent on phosphate impermeability in the case of mersalyl, as well as on phosphate permeability in the case of diamide, is confirmed by the results reported in Table II.

These results clearly show that, unlike mersalyl, diamide did not induce an accumulation of $^{32}P_i$ in the pellet, though FCCP-stimulated ATPase activity was not substantially modified by either thiol reagent.

The thiol titration results reported in Fig. 4 show that at 20°C diamide, even at concentrations much higher than that needed to inhibit P_i transport, did not affect more than 10 nmole of mitochondrial thiols. The amount of mitochondrial thiols oxidized by diamide therefore corresponds to about 20% of the thiols titratable with radioactive NEM (see also Gautheron [11]).

The amount of mitochondrial thiol groups oxidized with a concentration of diamide equal to that needed to inhibit P_i transport (i.e.,

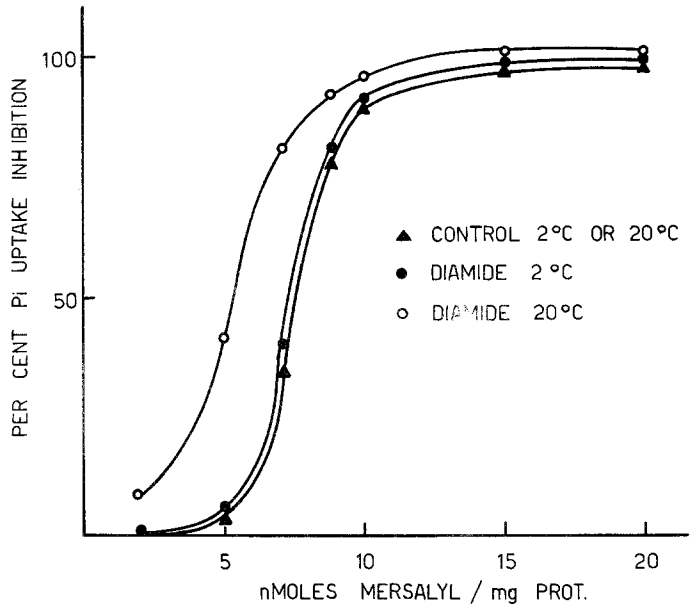

Figure 2. Percent inhibition of P_i transport in RLM by different concentrations of mersalyl in the presence and absence of diamide. Experimental conditions as in Fig. 1, except for the time of preincubation, which was 3 min. Mersalyl was added 2 min after mitochondria. When present, diamide was 100 nmole/mg protein.

~60 nmole) can be calculated to the order of 5 nmole/mg protein. Similarly, the amount of thiols oxidized by the lowest amount of mersalyl (about 9 nmole) capable of completely inhibiting P_i transport can be estimated at around 1.5 nmole/mg protein. These figures should be considered as only indicative, since they are at the lower sensitivity limit of

TABLE II. Action of diamide and mersalyl on the efflux of $^{32}P_i$ formed inside the mitochondria from [^{32}P]ATP[a]

	$^{32}P_i$ cpm/mg protein		
	Supernatant	Pellet	Total
Control	3139	117	3256
+ diamide	2604	100	2704
+ mersalyl	1153	2092	3245

[a] Rat liver mitochondria (2.5 mg/ml) were incubated 7 min at 20°C in the medium described in Fig. 3 containing 1.2 μmole/ml [γ^{32}P]ATP (35,000 cpm). When present diamide was 400 nmole/mg protein and mersalyl 40 nmole/mg protein.

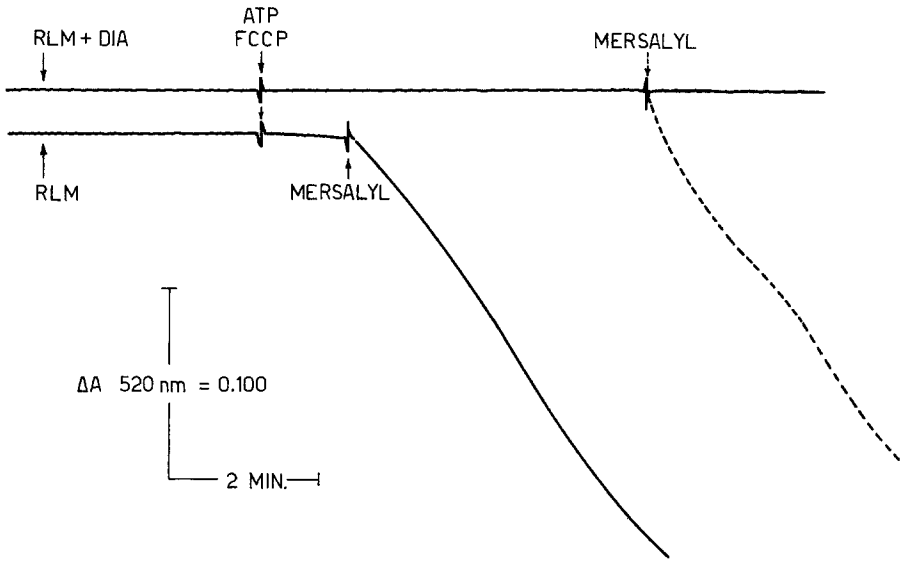

Figure 3. Influence of diamide and mersalyl on P_i efflux as recorded by the "FCCP–ATP system." Rat liver mitochondria (2.5 mg protein/ml) were added to a medium containing 250 mM sucrose, 20 mM Tris-HCl (pH 7.4), 1 mM ATP, 0.8 μM FCCP; final volume: 3 ml. When present, diamide was 400 nmole/mg protein. Mersalyl added was 40 nmole/mg protein. The changes in absorbance were recorded in an Aminco spectrophotometer at 520 nm with a scale extension of $A = 0.5$ cm^{-1}. Temperature was 20°C.

TABLE III. P_i transport in beef heart mitochondria and submitochondrial particles in the presence of diamide and mersalyl and DTE restoration of inhibited P_i uptake

	nmole P_i/mg protein		
		Particles[b]	
Conditions	Mitochondria[a]	−DTE	+DTE
None	14.5	1.37	1.36
Mersalyl	5.1	0.06	1.0
Diamide	14	0.7	1.2

[a] *Beef heart mitochondria:* 2.5 mg/ml was incubated to determine P_i uptake as described in Fig. 1. Temperature was 20°C. Added mersalyl was 40 nmole/mg protein and diamide was 500 nmole/mg protein. Correction for the sucrose-permeable space was not made.

[b] *Submitochondrial particles:* 1 mg protein/ml was incubated for 5 min at 20°C in 125 mM KCl, 20 mM Tris-HCl pH 8.1. $^{32}P_i$, 1 μmole/ml (380,000 cpm), was then added, and after 2 min the reaction was stopped by filtering 1.5 ml suspension through a 0.45-μm Millipore filter. The filters were washed with 10 ml of 150 mM KCl, dried, and counted in a Beckman LS-100 C spectrophotometer [3]. Diamide (500 nmole/mg protein) was added before submitochondrial particles and mersalyl (40 nmole/mg protein) 2 min before addition of $^{32}P_i$. DTE, 3 mM, was added with $^{32}P_i$.

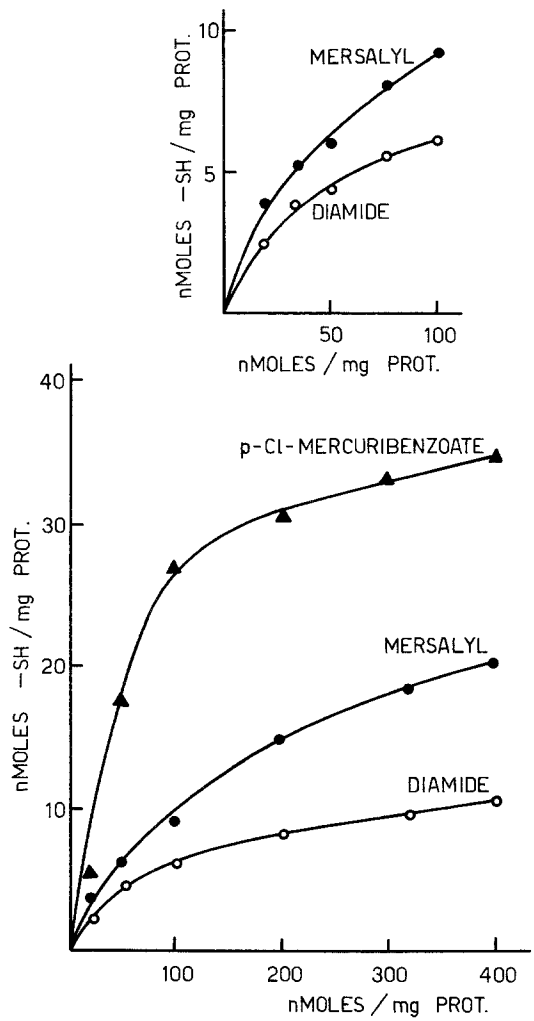

Figure 4. Titration of mitochondrial thiol groups of RLM by sulfydryl reagents. Assay medium as described in Fig. 1. The amount of untitrated SH groups was determined in the mitochondria sedimented after incubation, by titrating with [^{14}C]NEM. The pellet was resuspended in 1% deoxycholate, 150 nmole/mg protein [^{14}C]NEM (30,000 cpm) was added, and, after 40 min, the proteins were precipitated by 0.2 M TCA. The precipitate was washed twice with TCA, and then dissolved in 0.5 ml HCOOH for scintillation counting. Temperature was 20°C.

the titration method and also because a certain variability was observed in the response of different mitochondrial preparations.

Heart Mitochondria and Submitochondrial Particles

Unlike RLM, mitochondria isolated from beef or rat heart were completely insensitive to diamide. In fact, concentrations of diamide up to 0.5 μmole/mg protein failed to affect P_i transport (Table III). This lack of sensitivity to diamide may be related to the possibility that heart mitochondria thiol groups involved in P_i transport are not accessible to diamide.

As found by Rhodin and Racker, the P_i transport system is active in bovine heart submitochondrial particles [3]. This transport leads to P_i accumulation within the vesicles, provided a proper P_i-trapping system (i.e., $MnCl_2$) is present inside. Transport is inhibited by mersalyl and *N*-benzylmaleimide, but is unaffected by *N*-ethylmaleimide. Using the same particles, we have confirmed the inhibitory effect of mersalyl on P_i accumulation within the vesicles, and observed that diamide is capable of inhibiting P_i influx to a certain extent. The subsequent addition of DTE abolished both mersalyl and diamide inhibition (Table III). If we assume that the sonic particles used are inside out, and take into account the fact that P_i transport in intact heart mitochondria is insensitive to diamide (Table III), these results provide further evidence of an asymmetric orientation of the P_i transport system of the mitochondrial inner membrane.

Discussion

Diamide brings about a partial but constant inhibition of P_i influx into RLM. This effect indicates that only a portion of the rat liver mitochondria thiols involved in P_i transport is susceptible to the oxidative action of diamide. The methodology followed in the present paper allows an evaluation of the global transmembrane phosphate transport, sensitive to organic mercurials, and does not discriminate between P_i–OH exchange, sensitive to NEM [12], and P_i–dicarboxylate exchange, inhibited by substrate analogues such as *n*-butyl malonate [13]. Consequently no conclusion about the possible specificity of diamide toward either carrier system is allowed.

The complete lack of sensitivity to diamide observed in heart mitochondria may be related either to the poor reactivity of the thiol groups of these mitochondria or to the unavailability to the reagent of those involved in phosphate transport. Therefore, diamide, unlike monothiol reagents, in

a sense, is able to discriminate between different types of mitochondria (heart and liver in this case) on the basis of the positional and steric character of their membrane thiol groups.

The significant synergistic effect of diamide and mersalyl (Fig. 2) in inhibiting P_i transport that was observed in RLM may be interpreted by assuming that the oxidation of some thiol groups by diamide facilitates the approach of mersalyl to the critical thiols on which P_i transport depends. This may be possible if massive structural modifications in the outer surface of inner mitochondrial membrane are induced by the attachment of diamide molecules. Otherwise, a simple summation of the diamide and mersalyl effects on inhibition of the P_i carrier, or of the thiol population involved in P_i transport, should be assumed. The synergism between diamide and mersalyl could also be interpreted in terms of binding to a nonspecific site which has a high affinity for mersalyl and which, therefore, in the absence of diamide, reacts with mersalyl preferentially, that is, before mersalyl acts on the phosphate carrier. The synergism between diamide and mersalyl was observed only at 20°C, and therefore is not in contrast with our previous finding of lack of synergism at 0°C, which is further confirmed in this report [4].

The observation that diamide, unlike mersalyl, does not affect P_i efflux from RLM (see Fig. 4) may be related to poor penetrability of diamide across the mitochondrial membrane. If this is true, diamide could affect the binding of P_i to the "translocator" in correspondence to the outer surface but not the inner surface of the inner mitochondrial membrane. The objection that added ATP could prevent diamide binding to mitochondria and the consequent inhibition of P_i efflux, can be ruled out by the observation that 1 mM ATP did not modify the inhibition of P_i uptake by diamide under the conditions described for Fig. 1 (results not reported).

The finding that intact heart mitochondria are insensitive to diamide, in contrast to the relative sensitivity observed in heart submitochondrial particles (Table III), lends further support to our former assumption that the mitochondrial membrane is structurally asymmetrical, in the sense of a vectorial function and arrangement of the thiol groups involved in P_i translocation. The present results are also in full agreement with the conclusions of Rhodin and Racker which indicate that the whole system of mitochondrial P_i transport is oriented in an asymmetric fashion [3].

Finally, the finding that diamide action on both P_i transport and SH oxidation is temperature dependent is worth stressing. Temperature dependence could imply the existence of an activation barrier and therefore of complex structural transitions in the membrane components that should precede diamide action, which requires the proper vicinity of two thiols [14].

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