EFFECT OF SULPHYDRYL-BLOCKING REAGENTS ON MITOCHONDRIAL ANION-EXCHANGE REACTIONS INVOLVING PHOSPHATE

A.J. MEIJER, G.S.P. GROOT and J.M. TAGER
Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, Plantage Muidergracht 12, Amsterdam-C, The Netherlands

Received 14 March 1970

1. Introduction

Chappell and coworkers [1-4] have proposed that the transport of anions across the mitochondrial membrane is brought about by specific carrier systems that mediate exchange-diffusion processes. Two of these carrier systems, or translocators, mediate an exchange between phosphate and hydroxyl and an exchange between phosphate and dicarboxylate, respectively.

Fonyo [5] and Tyler [6] have shown that certain sulphydryl-binding reagents specifically interfere with the transport of phosphate across the mitochondrial membrane. The problem arises of whether these inhibitors not only specifically block the exchange diffusion between phosphate and hydroxyl, but also that between dicarboxylate and phosphate. We have recently presented evidence for inhibition of both transport systems by the sulphydryl-binding reagent mersalyl [7]. According to Johnson and Chappell [8], however, these sulphydryl-blocking reagents inhibit only the phosphate-hydroxyl exchange.

We have now found that there is a difference in sensitivity of the phosphate-hydroxyl and phosphate-dicarboxylate exchange reactions, depending on the sulphydryl-blocking reagent used. Mersalyl [6] and *p*-hydroxymercuribenzoate [5] inhibit both of these anion-exchange reactions (see ref. [7]). On the other hand, *N*-ethylmaleimide inhibits the phosphate-hydroxyl exchange only. These results provide additional evidence for the postulate [1-4] that the phosphate-hydroxyl and the phosphate-dicarboxylate exchange reactions are mediated by distinct translocating systems (see also refs. [7,9]).

2. Materials and methods

Rat-liver mitochondria were prepared according to Myers and Slater [10]. Oxygen uptake was determined at 25° with a Clark-type electrode. ^{32}P radioactivity was determined in a gas-flow counter. Phosphate was determined according to the method of Wahler and Wollenberger [11]. The oligomycin-sensitive P_i - ATP exchange reaction was measured as described by Groot [12], using rotenone instead of KCN as a respiratory inhibitor. The reaction conditions for P_i-P_i exchange are indicated in the legends to the figures.

3. Results

In order to study the nature of the inhibition of the transport of phosphate by sulphydryl-blocking reagents, the effect of the inhibitors on the K_m for phosphate in activating the respiration of succinate in the presence of ADP (and rotenone) was determined. Fig. 1 shows a Lineweaver-Burk plot of the phosphate-induced stimulation of respiration in the absence and presence of *p*-hydroxymercuribenzoate. It can be seen that the inhibition is competitive with respect to the phosphate concentration. Similar plots were obtained using mersalyl or *N*-ethylmaleimide. Two observations are noteworthy. Firstly the straight lines in the Lineweaver-Burk plots were obtained by subtracting the "resting" respiration ob-

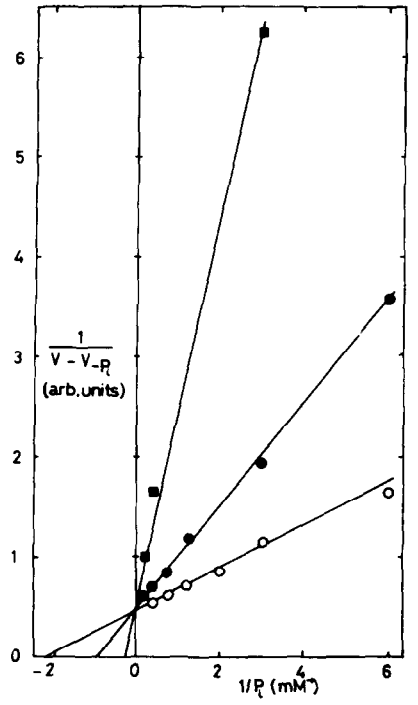

Fig. 1. Inhibition of phosphate-stimulated respiration by *p*-hydroxymercuribenzoate. The reaction medium (1.5 ml) contained 120 μmoles sucrose, 35 μmoles tris-HCl (pH 7.4), 75 μmoles KCl, 15 μmoles succinate, 15 μmoles $MgCl_2$, 1 μg rotenone, 0.25 μmole ADP, 30 μmoles glucose, 5 units hexokinase, 1.5 mg rat-liver mitochondrial protein and (where present) *p*-hydroxymercuribenzoate in the concentrations indicated. After 2 min preincubation increasing amounts of phosphate were added. ○——○, Control; ●——●, 4 μM *p*-hydroxymercuribenzoate; ■——■, 12 μM *p*-hydroxymercuribenzoate (in order to accomodate these points on the graph, the scale on the abscissa was reduced 20 times). V-V_{-P_i} represents the rate of respiration in the presence of phosphate *minus* that in its absence (in arbitrary units).

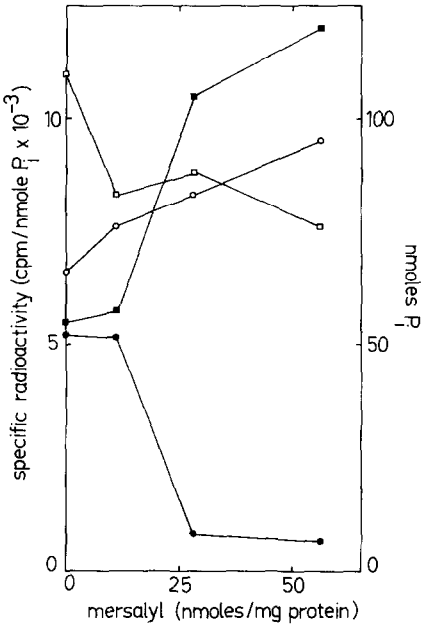

Fig. 2. Inhibition of phosphate-phosphate exchange by mersalyl. The reaction medium (1 ml) contained 25 μmoles sucrose, 50 μmoles tris-HCl (pH 7.5), 15 μmoles KCl, 5 μmoles $MgCl_2$, 2 μmoles EDTA, 3 μg rotenone, 5.3 mg mitochondrial protein and (where present) mersalyl in the concentrations indicated. After 1 min, ^{32}P-labelled phosphate (carrier-free) was added. One min later the mitochondria were separated from the suspending medium by centrifugation in an Eppendorf micro-centrifuge for 1 min at full speed. The mitochondrial pellet and the supernatant were then immediately acidified with $HClO_4$. ●——●, Specific radioactivity of intramitochondrial phosphate; ■——■, specific radioactivity of extramitochondrial phosphate; ○——○, intramitochondrial phosphate (chemically determined); □——□, extramitochondrial phosphate (chemically determined)

served in the absence of phosphate from that found in its presence (cf. ref. [13]). Secondly, in order to calculate true K_i values for the inhibitors one has to consider the fact that a sigmoidal curve is obtained when the percent inhibition is plotted as a function of inhibitor concentration; it was found that no inhibition of phosphate transport could be seen until the inhibitor concentration was increased beyond a certain level (cf. ref. [6]).

In fig. 2 the effect of mersalyl on the exchange-diffusion between extra- and intramitochondrial phosphate was studied. In this experiment the entry of ^{32}P-labelled phosphate in exchange for endogenous phosphate (and other anions) was measured. This exchange was extensively inhibited by mersalyl when the concentration of mersalyl was increased above about 10 nmoles/mg protein. Analogous results were obtained when mitochondria were preloaded with $^{32}P_i$ and the exit of the latter was induced by the addition of unlabelled phosphate. In this case, mersalyl almost completely prevented the discharge of the radioactivity from the mitochondria (not shown).

In fig. 3 the effect of *N*-ethylmaleimide and of mersalyl on the exchange between intra- and extramitochondrial phosphate is shown. With no further

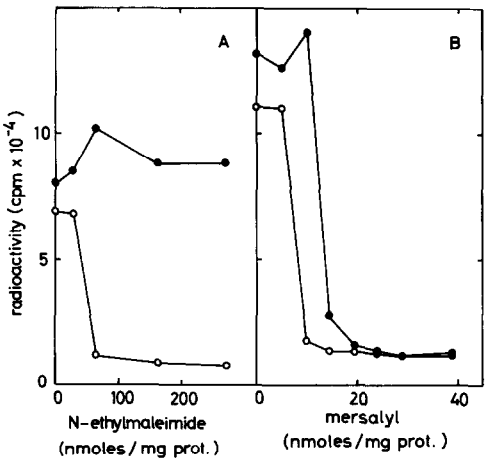

Fig. 3. Inhibition of phosphate-phosphate exchange by *N*-ethylmaleimide and mersalyl. Reaction conditions as described in fig. 2, except that oligomycin (10 μg) was also included. Exchange was initiated by addition of 0.1 mM ^{32}P-labelled phosphate. Mitochondrial protein: 3.7 mg (fig. 3A), 5.2 mg (fig. 3B). ●—●Control; ○—○ 5 mM butylmalonate present. The radioactivity given in the figure represents that in the mitochondrial pellet. *Abbreviation*: cpm, counts per minute; prot., protein.

addition, there was no inhibition by *N*-ethylmaleimide of the phosphate-phosphate exchange. However, in the presence of butylmalonate, which inhibits the phosphate-dicarboxylate exchange [3, 7-9], *N*-ethylmaleimide at a concentration of about 60 nmoles/mg protein inhibited the phosphate-phosphate exchange almost completely (fig. 3A). Although mersalyl in the absence of butylmalonate inhibited the phosphate-phosphate exchange almost completely, addition of butylmalonate brought about a decrease in the amount of mersalyl required for maximal inhibition (fig. 3B). Entirely analogous results were obtained when the oligomycin-sensitive P_i - ATP exchange was used as a measure of the exchange-diffusion between intra- and extramitochondrial phosphate (not shown).

We have also studied the effect of other sulphydryl-blocking reagents. *p*-Hydroxymercuribenzoate behaves like mersalyl. The effect of 5,5′-dithio-*bis*-(2-nitrobenzoate) [14] is intermediate between those of *N*-ethylmaleimide and mersalyl (or *p*-hydroxymercuribenzoate).

4. Discussion

Table 1 summarizes the effect of different inhibitors on the phosphate-hydroxyl and phosphate-dicarboxylate exchange-diffusion reactions. All the sulphydryl-blocking reagents inhibit the phosphate-hydroxyl translocator [5-9, 13, 14]. Butylmalonate has no effect on this translocating system [7-9], but does inhibit the phosphate-dicarboxylate translocator [3, 7-9].

Table 1 provides a satisfactory explanation of the apparent discrepancy between the results obtained by Johnson and Chappell [8] and by Meijer et al. [7,9] on the effect of sulphydryl-blocking reagents on the exchange between extra- and intramitochondrial phosphate. This exchange can occur via the phosphate-hydroxyl and the phosphate-dicarboxylate translocating systems. Both systems are inhibited by mersalyl [7,9]

In contrast, *N*-ethylmaleimide inhibits only the phosphate-hydroxyl translocating system, so that the phosphate-phosphate exchange-diffusion can still occur via the phosphate-dicarboxylate translocation system (cf. ref. [8]). In this connection it should be pointed out that the inhibition by 5,5′-dithio-*bis*-(2-nitrobenzoate) of the 2,4-dinitrophenol-induced ATPase and the lack of inhibition of the oligomycin-sensitive P_i - ATP exchange reaction reported by Miyahara [15] can easily be explained by the differential effect of the inhibitor on the two translocating systems responsible for phosphate transport. Since only the phosphate-hydroxyl exchange is operative when the ATPase is studied, 5,5′-dithio-*bis*-(2-nitrobenzoate) will inhibit. On the other hand, the P_i - ATP exchange reaction involves an exchange of intra- and extramitochondrial phosphate, that can take place via both translocating systems.

Finally our results show that the sulphydryl groups involved in the two translocating systems differ in their sensitivity to different sulphydryl-blocking reagents. Whether these two translocating systems represent distinct entities on the mitochondrial membrane or whether they are different functional groups on the same molecule remains to be established.

Table 1
Effect of inhibitors on the phosphate-hydroxyl and phosphate-dicarboxylate translocators.

Inhibitors	Effect on phosphate transport via	
	phosphate-hydroxyl translocator	phosphate-dicarboxylate translocator
Mersalyl	+	+
p-Hydroxymercuribenzoate	+	+
N-Ethylmaleimide	+	–
5,5'-Dithio-*bis*-(2-nitro benzoate)	+	±*
Butylmalonate	–	+

* Inhibition only at high concentrations (60% at 250 nmoles/mg protein).
+ = inhibiton, – = no effect.

Acknowledgements

This study was supported in part by grants from the Life Insurance Medical Research Fund and The Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from The Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

References

[1] J.B.Chappell and A.R.Crofts, in: Regulation of Metabolic Processes in Mitochondria, eds. J.M.Tager, S.Papa, E.Quagliariello and E.C.Slater, BBA Library, Vol. 7 (Elsevier, Amsterdam, 1966) p. 293.
[2] J.B. Chappell and K.N. Haarhoff, in: Biochemistry of Mitochondria, eds. E.C.Slater, Z.Kaniuga and L.Wojtczak (Academic Press and Polish Scientific Publishers, London and Warsaw, 1967) p. 75.
[3] B.H.Robinson and J.B. Chappell, Biochem. Biophys. Res. Commun. 28 (1967) 249.
[4] J.B.Chappell, P.J.F. Henderson, J.D. McGivan and B.H. Robinson, in: The Interaction of Drugs and Subcellular Components in Animal Cells ed. P.N. Campbell (Churchill, London, 1968) p. 71.
[5] A.Fonyo, Biochem. Biophys. Res. Commun. 32 (1968) 624.
[6] D.D. Tyler, Biochem. J. 111 (1969) 665.
[7] A.J. Meijer and J.M. Tager, Biochim. Biophys. Acta 189 (1969) 136.
[8] R.N.Johnson and J.B. Chappell, Biochem. J. 116 (1970) 37P.
[9] A.J.Meijer, N.E. Lofrumento, S. Papa, J.M. Tager and E. Quagliariello, Biochem. J. 116 (1970) 35P.
[10] D.K. Myers and E.C. Slater, Biochem. J. 67 (1957) 558.
[11] B.E. Wahler and A. Wollenberger, Biochem. Z. 329 (1958) 508.
[12] G.S.P. Groot, Biochim. Biophys. Acta 180 (1969) 439.
[13] A. Fonyo and S.P. Bessman, Biochem. Med. 2 (1968) 145.
[14] N. Haugaard, N.H. Lee, R. Kostrzewa, R.S. Horn and E.S. Haugaard, Biochim. Biophys. Acta 172 (1969) 198.
[15] M.Miyahara, Arch. Biochem. Biophys. 134 (1969) 590.