BBA 45833

COMPARATIVE STUDIES OF THE ADP-ATP AND THE P_i-ATP EXCHANGE REACTIONS RELATED TO OXIDATIVE PHOSPHORYLATION IN RAT-LIVER MITOCHONDRIA

G. S. P. GROOT

Laboratory of Biochemistry, B. C. P. Jansen Institute, University of Amsterdam, Amsterdam (The Netherlands)*

(Received April 11th, 1969)

SUMMARY

1. The ADP–ATP and P_i–ATP exchange reactions have been studied in rat-liver mitochondria under conditions in which both exchanges are completely sensitive to oligomycin. Endogenous respiration was inhibited by cyanide.

2. Phosphate stimulates the ADP–ATP exchange.

3. ADP inhibits the P_i–ATP exchange and, above 0.5 mM, the ADP–ATP exchange. At ADP concentrations lower than 0.5 mM, the rate of the ADP–ATP exchange is lower than that of the P_i–ATP exchange. At higher concentrations, the rates are equal. Under these conditions, the rate of both exchange reactions is probably limited by the rate of entry of ATP *via* the adenine nucleotide translocator.

4. Both exchange reactions are inhibited by succinate, β-hydroxybutyrate or malonate. Inhibition by malonate is competitive with ATP. Thus, inhibition by substrate is not due to reduction of the respiratory chain but to competition between the substrate anion and ATP for entry into the mitochondrion.

INTRODUCTION

Mitochondria catalyse ADP–ATP[1–6] and P_i–ATP[7–12] exchange reactions that, under carefully defined[5,13] conditions, are inhibited completely by uncouplers and inhibitors of respiratory-chain phosphorylation. There is, therefore, reason to believe that these reactions reflect a part of the phosphorylation mechanism.

One of the arguments for the existence of a high-energy phosphate compound was the finding that the ADP–ATP exchange is considerably faster than the P_i–ATP exchange[3]. However, KULKA AND COOPER[14] found that the rate of the oligomycin-sensitive ADP-ATP exchange is equal to that of the P_i–ATP exchange. They concluded that the possibility of a concerted mechanism for ATP formation could not be ruled out.

Since the discovery of a translocation system for adenine nucleotides[15,16], it has become clear that this system also is involved in the exchange reactions. Atractyloside, an inhibitor of the translocation of externally added nucleotides, inhibits both exchange reactions[6,17]. Translocation systems for substrate anions have also been

* Postal address: Plantage Muidergracht 12, Amsterdam, The Netherlands.

described[18–20]. There appears to be a mutual effect of substrates on their rate of metabolism[19,20] and VELDSEMA-CURRIE AND SLATER[21] have interpreted the known effects of substrates on the uncoupler-induced ATPase on the basis of competition for transport through the mitochondrial inner membrane between ATP, uncoupler and these substrates. Similar inhibitory effects of substrates have been described for the ADP–ATP[3] and P_i–ATP exchange reactions[10].

It seemed, therefore, worthwhile to study in more detail the requirements of the oligomycin-sensitive ADP–ATP and P_i–ATP exchange reactions under the appropriate conditions, taking into account the known properties of the adenine nucleotide translocator and the mutual effect of anions on their transport across the inner membrane of the mitochondria.

METHODS AND MATERIALS

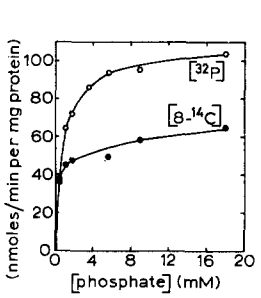
The ADP–ATP exchange reaction was measured in a medium containing 25 mM Tris–HCl buffer (pH 7.4), 25 mM phosphate buffer (pH 7.4), 5 mM EDTA, 100 mM sucrose, 1 mM KCN, 6 mM ATP and 1 mM [8-^{14}C]ADP ($1.5 \cdot 10^5$–$2.5 \cdot 10^5$ disint./min) in a total volume of 1 ml. The reaction was started by the addition of rat-liver mitochondria, prepared according to MYERS AND SLATER[22]. When oligomycin was used, the mitochondria were preincubated with the inhibitor for 2 min in the complete reaction mixture, except for the radioactive ADP, which was added to start the reaction. The reaction was stopped by the addition of 0.1 ml 35% $HClO_4$ and was cooled to 0°. After neutralization with KOH, the protein and the $KClO_4$ were removed by centrifugation and an aliquot of the supernatant was applied to a Dowex-1 (formate) column. The nucleotides were separated and counted as described earlier[13]. The P_i–ATP exchange reaction was measured in the same medium except that ADP was omitted and $^{32}P_i$ was used ($1.5 \cdot 10^6$–$2.0 \cdot 10^6$ counts/min). The reaction was stopped by the addition of 1.0 ml 10% trichloroacetic acid. The ^{32}P incorporation into ATP was determined by extraction of the P_i with molybdate and isobutanol–benzene (1:1) according to NIELSEN AND LEHNINGER[23] and by counting the aqueous layer in a Nuclear Chicago gas-flow counter. An aliquot was used to determine the P_i concentration by the method of Fiske and SubbaRow, as modified by SUMNER[24]. Protein was determined by the biuret method as described by CLELAND AND SLATER[25].

[8-^{14}C]ADP was purchased form Calbiochem and purified before use on a Dowex-1 (formate) column[13]. Carrier-free $H_3{}^{32}PO_4$ was obtained from Philips-Duphar. It was boiled with 0.25 M HCl for 1 h and neutralized with 1 M Tris before use. ADP and ATP were obtained from Boehringer und Soehne. Oligomycin was kindly provided by the Upjohn Co. and atractyloside by Dr. V. Sprio. Antimycin was obtained from Sigma Chemical Co. and rotenone from Penick and Co. All other chemicals were from the British Drug Houses.

RESULTS

In order to exclude a possible contribution of ATP synthesis during measurement of the exchange reaction, endogenous respiration was blocked by 1 mM KCN. Rotenone, antimycin and arsenite were found to inhibit both the P_i–ATP exchange (*cf.* ref. 11) and the ADP–ATP exchange to the same extent as KCN.

WADKINS AND LEHNINGER[3] found with intact rat-liver mitochondria, in contrast to digitonin particles, that the ADP–ATP exchange reaction is stimulated by phosphate. GUILLORY AND SLATER[5] pointed out, however, that this stimulation could be accounted for by net synthesis of ATP coupled with the oxidation of endogenous substrate. It was therefore, necessary to re-investigate the effect, of phosphate under conditions in which endogenous respiration is inhibited. Fig. 1 shows that also under these conditions the ADP–ATP exchange is stimulated by phosphate. Half-maximal velocity was reached at about the same concentration that gave half-maximal velocity of the P_i–ATP exchange. Fig. 2 shows that the P_i–ATP exchange reaction is markedly



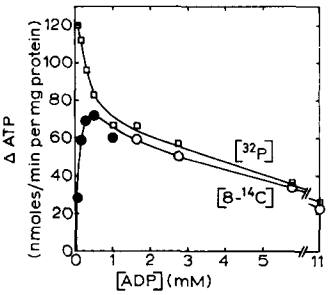


Fig. 1. The effect of the phosphate concentration on the P_i–ATP and ADP–ATP exchange reactions. The reaction mixture as described in METHODS AND MATERIALS (except for the phosphate concencentration) contained 1.1 mg mitochondrial protein. Reaction for 5 min at 25°.

Fig. 2. The effect of the ADP concentration on the P_i–ATP and ADP–ATP exchange reactions. The reaction mixture as described in METHODS AND MATERIALS (except for the ADP concentration) contained 1.1 mg mitochondrial protein. In the incubations indicated by the filled circles, 0.2 mg mitochondrial protein was used. Reaction for 5 min at 25°.

inhibited by added ADP. At concentrations above 0.5 mM, it also inhibits the ADP–ATP exchange. At concentrations of ADP lower than 0.5 mM the ADP–ATP exchange reaction is slower than the P_i–ATP reaction. At higher concentrations the rates are equal.

In order to determine whether the inhibitory effect of ADP on the P_i–ATP exchange reaction is related to the known inhibitory effect of ADP on the transport of ATP, we compared ADP with atractyloside, a potent inhibitor of the translocator. In Fig. 3 the effect of oligomycin on mitochondria is shown with or without ADP or

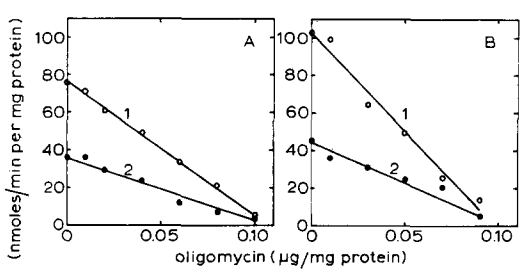


Fig. 3. The effect of oligomycin on the P_i–ATP exchange reaction in the absence and the presence of ADP or atractyloside. The reaction mixture as described in METHODS AND MATERIALS contained 1.0 mg mitochondrial protein. Expt. A: Curve 1, control; Curve 2, 2.4 mM ADP added. Expt. B: Curve 1, control; Curve 2, 2 μM atractyloside added. Reaction for 5 (Expt. A) or 7 min (Expt. B) at 25°.

atractyloside in concentrations that inhibit the P_i–ATP exchange about 50%. As can be seen from Fig. 3, in both the presence and absence of the inhibitors, a linear inhibition curve was obtained. In this respect ADP and atractyloside have the same effect.

TABLE I

THE EFFECTS OF ANIONS ON THE ADP–ATP EXCHANGE REACTION AND ON THE P_i–ATP EXCHANGE REACTION

The reaction mixture, as described in METHODS AND MATERIALS (except that KCN was omitted) contained 0.7 mg mitochondrial protein. Reaction for 9 min at 25°. Results are given in nmoles/min per mg protein.

Additions	$\Delta[8\text{-}^{14}C]ATP$	$\Delta[^{32}P]ATP$
None	107	126
KCN (1 mM)	50	76
KCN + succinate (20 mM)	38	64
KCN + β-hydroxybutyrate (20 mM)	36	54
KCN + succinate + β-hydroxybutyrate	30	47
Rotenone (5 μg)	49	77
Rotenone + malonate (20 mM)	35	62

The influence of anions on the ADP–ATP and the P_i–ATP exchange reaction is shown in Table I. In agreement with WADKINS AND LEHNINGER[3,10], we find an inhibition of the exchange reactions by succinate and β-hydroxybutyrate. However, the additions of malonate in the presence of rotenone gives the same inhibition. It appears, then, that the inhibitory effect of succinate or of β-hydroxybutyrate is not due to reduction of the respiratory chain. This conclusion is also supported by the results of other experiments showing that the rates of the exchange reactions are equal, whether KCN, with which the respiratory chain is completely reduced, or arsenite, with which it is completely oxidized, is added. The inhibition by malonate was found to be competitive with respect to ATP. For the ADP–ATP and the P_i–ATP exchange, a K_i of respectively 8 and 13 mM was found, using a fixed malonate concentration of 10 mM (*cf.* ref. 21) and variable concentrations of ATP.

DISCUSSION

In agreement with GUILLORY AND SLATER[5], the amount of labeled ATP formed by oxidation of endogenous substrate can form an appreciable part of the total amount of radioactive ATP measured in exchange experiments (see Table I.) This is especially true with liver mitochondria which have a much higher endogenous respiration than heart mitochondria. This is probably the explanation for the difference between the results of GUILLORY AND SLATER[5] who used liver mitochondria and BYGRAVE AND LEHNINGER[6] who used heart mitochondria, with respect to the contribution of endogenous respiration. The effect of varying the concentration of the reactants on the two

exchange reactions can be clearly investigated only if the endogenous respiration is inhibited.

There are two possible explanations for the inhibition of the P_i–ATP exchange by ADP. The first is that an increased ADP concentration decreases the amount of $X \sim P$ which is a possible intermediate in the P_i–ATP exchange reaction. This explanation is, however, not very likely since increasing concentrations of added ADP do not increase the amount of intramitochondrial ADP[16,26]. Moreover, this cannot explain the inhibition of the ADP–ATP exchange reaction. A second and more likely explanation is that the entry of ATP is the rate-limiting step in the exchange reactions. It is known that ADP inhibits this reaction[16]. If the entry of ATP is the rate-limiting step, the fact that the velocities of the ADP–ATP and P_i–ATP exchanges are equal is understandable, since both are limited by the same process. At very low ADP concentrations, however, the entry of ADP is probably the rate-limiting step for the ADP–ATP exchange reaction. The low K_m of ADP for this reaction is in agreement with the low K_m of ADP for the translocator[16] and for the phosphorylation of added ADP[27]. Since the entry of ADP is not necessary for the P_i–ATP exchange, the discrepancy in the rates of the two reactions at low ADP concentrations is understandable. If ADP is necessary for the P_i–ATP exchange reaction, sufficient amounts are probably present in the mitochondria.

A further indication that the translocator is the rate-limiting step in the exchange reactions is the very high activation energy of 29 kcal (ref. 28), which agrees very well with that reported for the adenine nucleotide translocator[16], and is much higher than the activation energy reported for the phosphorylation of intramitochondrial ADP [28,29]. Moreover, the maximal rate of the P_i–ATP exchange reaction approximates the rate of ATP uptake as reported by KLINGENBERG AND PFAFF[16]. The stimulatory action of P_i on the ATP transport[16] might explain the stimulation of the ADP–ATP exchange reaction by P_i.

The inhibition by various anions, whether or not they are metabolized, can be explained on the basis of the findings of VAN DAM AND TSOU[19] and QUAGLIARIELLO AND PALMIERI[20] and are analogous to the effects of these anions on the uncoupler-induced ATPase as described by VELDSEMA-CURRIE AND SLATER[21].

As a consequence of the findings reported in this paper, it is clear that the arguments brought forward in favour of a concerted mechanism[14] lose much of their cogency.

The most striking conclusion is the close relationship between the adenine nucleotide translocator as the rate-limiting step in the exchange reactions and the phosphorylation enzymes. Even when the entry of ATP is inhibited by atractyloside or ADP, the whole phosphorylation system seems to be necessary for the P_i–ATP exchange, since addition of a small amount of oligomycin is inhibitory. This close relationship between the two systems, of which the nature remains obscure, is also evident in other experiments[16,30].

ACKNOWLEDGEMENTS

The author wishes to thank Professors E. C. SLATER and S. G. VAN DEN BERGH for their continuous interest and advice and Miss J. H. DE KONING for excellent technical assistance. This work was supported in part by the Life Insurance Medical Research Fund.

REFERENCES

1 C. COOPER AND A. L. LEHNINGER, *J. Biol. Chem.*, 224 (1957) 561.
2 C. L. WADKINS, *J. Biol. Chem.*, 236 (1961) 221.
3 C. L. WADKINS AND A. L. LEHNINGER, *J. Biol. Chem.*, 238 (1963) 2555.
4 F. HUIJING AND E. C. SLATER, *J. Biochem. Tokyo*, 49 (1961) 493.
5 R. J. GUILLORY AND E. C. SLATER, *Biochim. Biophys. Acta*, 105 (1965) 221.
6 F. L. BYGRAVE AND A. L. LEHNINGER, *J. Biol. Chem.*, 241 (1966) 3894.
7 P. D. BOYER, A. B. FALCONE AND W. H. HARRISON, *Nature*, 174 (1954) 401.
8 M. A. SWANSON, *Biochim. Biophys. Acta*, 20 (1956) 85.
9 W. C. HÜLSMANN, *Over het Mechanisme van de Ademhalingsketenphosphorylering*, M. D. Thesis, Poortpers, Amsterdam, 1958.
10 C. L. WADKINS AND A. L. LEHNINGER, *J. Biol. Chem.*, 234 (1959) 681.
11 P. D. BOYER, L. L. BIEBER, R. A. MITCHELL AND G. SZALBOLCSI, *J. Biol. Chem.*, 241 (1966) 5384.
12 P. D. BOYER, in D. R. SANADI, *Current Topics in Bioenergetics*, Vol. 2, Academic Press, New York, 1967, p. 99.
13 G. S. P. GROOT AND S. G. VAN DEN BERGH, *Biochim. Biophys. Acta*, 153 (1968) 22.
14 R. G. KULKA AND C. COOPER, *J. Biol. Chem.*, 237 (1962) 936.
15 M. KLINGENBERG, E. PFAFF AND A. KRÖGER, in B. CHANCE, Q. H. GIBSON, R. H. EISENHARDT AND K. K. LONBERG-HOLM, *Rapid Mixing and Sampling Techniques Applied to the Study of Biochemical Reactions*, Academic Press, New York, 1964, p. 333.
16 M. KLINGENBERG AND E. PFAFF, in T. W. GOODWIN, *Metabolic Roles of Citrate*, Academic Press, New York, 1968, p. 105.
17 P. V. VIGNAIS, P. M. VIGNAIS AND E. STANISLAS, *Biochim. Biophys. Acta*, 60 (1962) 284.
18 J. B. CHAPPELL AND A. R. CROFTS, in J. M. TAGER, S. PAPA, E. QUAGLIARIELLO AND E. C. SLATER, *Regulation of Metabolic Processes in Mitochondria*, BBA Library, Vol. 7, Elsevier, Amsterdam, 1966, p. 293.
19 K. VAN DAM AND C. S. TSOU, *Biochim. Biophys. Acta*, 162 (1968) 301.
20 E. QUAGLIARIELLO AND F. PALMIERI, *European J. Biochem.*, 4 (1968) 20.
21 R. D. VELDSEMA-CURRIE AND E. C. SLATER, *Biochim. Biophys. Acta*, 162 (1968) 310.
22 D. K. MYERS AND E. C. SLATER, *Biochem. J.*, 67 (1957) 558.
23 S. O. NIELSEN AND A. L. LEHNINGER, *J. Biol. Chem.*, 215 (1955) 555.
24 J. B. SUMNER, *Science*, 100 (1944) 413.
25 K. W. CLELAND AND E. C. SLATER, *Biochem. J.*, 53 (1953) 547.
26 H. H. WINKLER, F. L. BYGRAVE AND A. L. LEHNINGER, *J. Biol. Chem.*, 243 (1968) 20.
27 F. L. BYGRAVE AND A. L. LEHNINGER, *Proc. Natl. Acad. Sci. U.S.*, 57 (1967) 1409.
28 A. KEMP, JR., G. S. P. GROOT AND H. J. REITSMA, *Biochim. Biophys. Acta*, 180 (1969) 28.
29 H. W. HELDT AND M. KLINGENBERG, *European J. Biochem.*, 4 (1968) 1.
30 W. D. J. GRAAFMANS, R. CHARLES AND J. M. TAGER, *Biochim. Biophys. Acta*, 153 (1968) 916.