EFFECT OF SH REAGENTS ON ATRACTYLOSIDE BINDING TO MITOCHONDRIA AND ADP TRANSLOCATION. POTENTIATION BY ADP AND ITS PREVENTION BY UNCOUPLER FCCP

P.V. VIGNAIS and P.M. VIGNAIS
Biochimie, CEN-G, BP 85 et Faculté de Médecine, 38041-Grenoble, France

Received 18 July 1972

1. Introduction

ADP at low concentrations brings about morphological [1], physical [2] and chemical [3, 4] alterations of mitochondria. One typical chemical change demonstrated upon addition of ADP consists in an unmasking of some membrane SH groups. As shown recently by Leblanc and Clauser [4], alkylation of these unmasked groups by *N*-ethylmaleimide (NEM) appears to prevent the further functioning of the ADP translocase.

On the other hand atractyloside (ATR), a competitive inhibitor of the ADP translocation [5, 6], interacts with the binding of ADP to its carrier [7–9]; conversely, the affinity of mitochondria for [^{35}S]ATR can be used for monitoring structural changes in the ADP translocator [10, 11]. Experiments* presented in this paper show that the binding of NEM concomitantly with that of a minute amount of ADP (hardly effective by itself) strikingly decreases the affinity of mitochondria for [^{35}S]ATR and inhibits the ADP translocation. This NEM-amplified effect of ADP on [^{35}S]ATR binding is given by ADP or ATP (half maximal effect at less than 2 μM) but not other nucleotides such as UDP, CDP or GDP. It is counteracted by the uncoupler FCCP which also decreases the amount of NEM alkylatable groups unmasked by small ADP additions. These results confirm and extend those independently reported by Leblanc and Clauser [4]. They not only point to a conformational change of the mitochondrial membrane, which can be trapped irreversibly by NEM, but also they demonstrate that this conformational change is initiated by the specific binding of ADP (or ATP) to its carrier.

2. Results

2.1. *Effect of NEM and ADP on [^{35}S]ATR binding*

Fig. 1 shows that rat liver mitochondria preincubated for a short period of time (2 min at 20°) with 10 μM ADP and 50 μM NEM partially loose their capacity to bind [^{35}S]ATR. However, when added separately, ADP and NEM were virtually ineffective. The concentration of ADP used in that experiment was sufficiently low so as not to interfere significantly with [^{35}S]ATR binding. Since mitochondria display a higher affinity for ATR in the presence of $MgCl_2$ than EDTA [12], $MgCl_2$ was systematically used in preliminary experiments. However, essentially similar results were obtained with an EDTA supplemented medium.

NEM can be replaced by fuscin (table 1), a quinonoid compound which reacts covalently with SH groups [13] and competes with other SH reagents such as NEM, mersalyl or *p*CMB for binding to mitochondria [14]. In our experimental conditions, mersalyl and *p*CMB were found much less effective than NEM and fuscin (table 1). The decrease in the

* Communicated at the International Conference on "Mechanisms in Bioenergetics" held in Pugnochiuso, Italy, 1–4 May 1972.
Abbrevations: ATR: atractyloside; AOPCP: adenosine 5'methylene diphosphonate; DTNB: dithio-bis-(2-nitrobenzoate); *p*CMB: *para*chloromercuribenzoate; FCCP: carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; NEM: *N*-ethylmaleimide.

North-Holland Publishing Company – Amsterdam

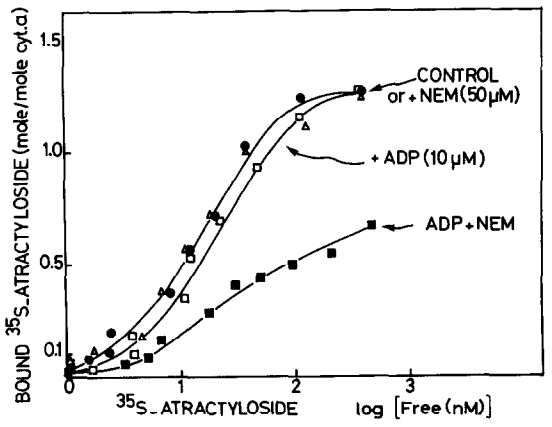

Fig. 1. Decrease of [^{35}S]ATR binding affinity to rat liver mitochondria upon simultaneous addition of NEM and ADP. Mitochondria (8.4 mg protein) were preincubated in 10 ml of 110 mM KCl, 6 mM $MgCl_2$, 10 mM Tris-sulfate, pH 7.2, with ADP or NEM or both of them for 2 min at 20°. After cooling at 2°, [^{35}S]ATR was added at concentrations ranging from zero to 0.5 μM. The incubation lasted for 45 min at 2° and was ended by centrifugation. The pellets were dissolved in formamide at 180° and the radioactivity counted by scintillation.

Table 1
Effect of ADP and SH reagents on the binding of [^{35}S]ATR.

Preincubation medium SH reagent	ADP	[^{35}S]ATR bound (mole/mole cyt. *a*)	Inhibition (%)
None	–	1.05	
None	+	1.00	5
NEM	–	1.07	0
NEM	+	0.36	66
Fuscin	–	1.05	0
Fuscin	+	0.20	76
Mersalyl	–	1.07	0
Mersalyl	+	1.01	4
*p*CMB*	–	0.51	51
*p*CMB*	+	0.35	67

* Swelling of mitochondria.

Rat liver mitochondria (8.5 mg protein) were preincubated for 2 min at 20° in 10 ml of 110 mM KCl, 6 mM $MgCl_2$, 10 mM Tris-sulfate, pH 7.2, with 50 μM NEM, fuscin, mersalyl or *p*CMB and 10 μM ADP, as indicated. After the preincubation period, the tubes were cooled, 1.65 nmole of [^{35}S]ATR in 0.3 ml water added and incubation carried out for 45 min at 2°. The incubation was ended by centrifugation.

Table 2
Inhibition of [^{35}S]Atractyloside binding to mitochondria by preincubation with ADP + NEM; specificity of ADP and ATP, counteracting effect of ATR and FCCP.

No.	Additions in preincubation medium	Bound [^{35}S]Atractyloside (nmoles/cytochrome *a*)
1	Nil	0.86
2	NEM	0.78
3	ADP	0.77
4	NEM + ADP	0.31
5	ATR + NEM + ADP	0.60*
6	ATP	0.83
7	NEM + ATP	0.35
8	UDP, CDP or GDP	0.83 – 0.89
9	NEM + UDP, CDP or GDP	0.71 – 0.78
10	FCCP (+ Oligomycin)**	0.86
11	FCCP + NEM (+ Oligomycin)	0.70
12	FCCP + ADP (+ Oligomycin)	0.81
13	FCCP + NEM + ADP (+ Oligomycin)	0.62

* Corrected for dilution by the unlabelled ATR.
** Oligomycin by itself has no significant effect on [^{35}S]ATR binding.

Mitochondria (6.6 mg protein) in 5 ml of 110 mM KCl, 0.1 mM EDTA, 10 mM Tris-sulfate, pH 7.2, were first preincubated for 2 min at 20° with 100 μM NEM, 10 μM ADP, 10 μM ATP, 20 μM UDP, CDP or GDP, 0.2 μM ATR, 2 μM FCCP, 10 μg oligomycin as indicated. After the preincubation period, the tubes were cooled and 1.65 nmole of [^{35}S]ATR in 0.3 ml water was added to each tube. The incubation lasted for 45 min at 2° and was ended by centrifugation.

amount of bound [^{35}S]ATR observed after addition of 50 μM *p*CMB alone may be due to the rapid mitochondrial swelling induced by *p*CMB. In contrast, no significant swelling occurred upon addition of NEM, mersalyl or fuscin.

Using 100 μM NEM, the half maximal inhibition of [^{35}S]ATR binding is observed at about 2 μM, a value which is similar to the K_m^{ADP} found for the ADP translocation [15, 16]. This effect of ADP is shared by ATP and by the methylene analogue of ADP (AOPCP), but not by UDP, CDP, GDP (table 2, lines 8, 9). In other words, only the nucleotides which can be translocated in mitochondria, i.e. ADP, ATP

Table 3
Variation in the amount of NEM reactive groups in mitochondria upon addition of ADP and FCCP.

Additions	Bound [^{14}C]NEM (nmoles/mg protein)
Nil	34
ADP	37
FCCP	27
ADP + FCCP	28

Mitochondria (8.8 mg protein) were incubated for 2 min at 20° in 5 ml of 110 mM KCl, 0.1 mM EDTA and 10 mM Tris-sulfate, pH 7.2, 100 μM [^{14}C]NEM and, as indicated, 10 μM ADP or (and) 2 μM FCCP. The incubation was ended by rapid centrifugation.

[16, 17] and AOPCP [16], are also able, *when preincubated with NEM,* to inhibit [^{35}S]ATR binding.

To check whether the effect of the preincubated ADP was mediated through its binding to its own carrier, use was made of ATR, as a specific inhibitor of the adenine nucleotide translocation. Unlabelled ATR was added to mitochondria, immediately followed by ADP and NEM; the ATR concentration (0.2 μM) was sufficient to inhibit the binding of the preincubated ADP (10 μM) to more than 80%. Preincubation with ATR prevented the inhibition by NEM + ADP of the binding of [^{35}S]ATR (compare lines 4 and 5, table 2).

The proton conductor FCCP did not interfere by itself with [^{35}S]ATR binding, in agreement with a previous report [10] but antagonized the inhibitory effect of NEM + ADP on [^{35}S]ATR binding (table 2, lines 10–13). In this experiment oligomycin had been added together with FCCP to avoid ADP or ATP dephosphorylation; contrary to FCCP, oligomycin does not significantly interfere with NEM + ADP.

By using [^{14}C]NEM, we have tested whether the antagonism between FCCP on the one hand and NEM + ADP on the other could reflect a FCCP dependent alteration of the binding capacity for NEM. Whereas the amount of bound [^{14}C]NEM is slightly increased upon addition of ADP, in agreement with Zimmer [3] and with Leblanc and Clauser [4], it is decreased by FCCP either in the presence or in the absence of ADP (table 3). Using DTNB as a SH reagent, Gautheron et al. [18] have also observed a decrease of SH groups in mitochondria upon addition of uncouplers. These data indicate that the masking or unmasking of some NEM

Table 4
Inhibition of the [^{14}C]ADP translocation by NEM + ADP; antagonistic effect of FCCP.

No.	Additions in preincubation medium	[^{14}C]ADP translocation (nmoles/min/mg prot.)
1	Nil	6.9
2	NEM	6.8
3	ADP	6.9
4	NEM + ADP	2.2
5	ATR	4.1
6	ATR + NEM + ADP	3.2
7	ATP	6.5
8	ATP + NEM	2.9
9	UDP, CDP or GDP	6.6 – 6.8
10	NEM + UDP, CDP or GDP	5.8 – 5.9
11	FCCP (+ Oligomycin)	6.5
12	FCCP + NEM (+ Oligomycin)	6.7
13	FCCP + ADP (+ Oligomycin)	6.9
14	FCCP + NEM + ADP (+ Oligomycin)	5.6

Rat liver mitochondria (6.6 mg protein) were preincubated in 5 ml of 110 mM KCl, mM EDTA and 10 mM Tris-sulfate, pH 7.2, for 2 min at 20° with 100 μM NEM, 10 μM ADP, 10 μM ATP, 20 μM UDP, CDP or GDP, 0.2 μM ATR, 2 μM FCCP, 10 μg oligomycin as indicated. After cooling at 2°, [^{14}C]ADP in 200 μl was added to a final conc. of 300 μM. The incubation lasted for 30 sec at 2° and was stopped by addition of 4 μM gummiferin [19] followed by rapid centrifugation. The amount of [^{14}C]ADP incorporated in the matrix space was calculated from the amount of [^{14}C]ADP present in the pellet [16] after correction for the [^{14}C]ADP in the sucrose space.

reactive groups in mitochondria upon addition of FCCP or ADP, respectively, reflects a conformational change of membrane areas which include the [^{35}S]ATR binding sites. Other experiments under way indicate that the respiratory inhibitor, antimycin A, similarly to FCCP counteracts the ADP + NEM effect and also decreases the amount of bound [^{14}C]NEM.

2.2. *Effect of NEM and ADP on the adenine nucleotide translocation*

That the synergistic inhibitory effect of NEM and ADP on the [^{35}S]ATR binding affinity is related to ADP translocation is corroborated by the further observation, in agreement with Leblanc and Clauser [4], that preincubation of mitochondria with NEM + ADP results in a striking decrease of the rate of ADP translocation (table 4). To ensure that the effect due

to the preincubated ADP was mediated by the binding of ADP to its carrier, we took advantage of the competitive inhibition of the ADP translocation by ATR, ADP being used in the preincubation medium at a concentration (10 μM ADP) much lower than in the incubation medium (300 μM [^{14}C]ADP). The concentration of ATR, 0.2 μM, was chosen to inhibit nearly completely the binding of ADP at 10 μM (preincubation period), but only partially, about 40%, (line 5, table 4) at 300 μM (incubation period). Under these conditions, ATR added to mitochondria just before ADP and NEM in the preincubation period significantly prevented the inhibitory effect of ADP + NEM on the [^{14}C]ADP translocation (compare lines 6 and 4, table 4).

A similar inhibition of the [^{14}C]ADP translocation is observed when ADP is replaced in the preincubation medium by ATP, the half maximum effect being obtained at less than 2 μM for the two nucleotides; in contrast UDP, CDP, GDP at 20 μM are virtually ineffective (table 4, line 9, 10). As for [^{35}S]ATR binding, the inhibition of the [^{14}C]ADP translocation by NEM + ADP is counteracted by FCCP (added in the presence of oligomycin to prevent ADP or ATP dephosphorylation) (table 4, lines 11–14).

3. Discussion

The data reported in this paper strongly suggest that NEM-reactive groups, possibly SH groups, which are buried in the mitochondrial membrane in the absence of external ADP, are unmasked upon addition of small concentrations of ADP (or ATP). This effect is likely related to a propagated change of conformation of the inner mitochondrial membrane initiated by the binding of minute amounts of ADP or ATP to the ADP carrier. Such a conformational change which is probably reversible in the absence of NEM would be trapped irreversibly by the binding of NEM to unmasked NEM-reactive groups. This hypothesis is in line with the fact that FCCP, which markedly decreases the amount of NEM-reactive groups in mitochondria, even in the presence of ADP, counteracts the inhibitory effect of NEM + ADP both on the translocation of ADP or ATP and the binding of [^{35}S]ATR.

Several lines of evidence indicate that during the preincubation period ADP binds to the ADP carrier: i) its effect is prevented by ATR, ii) the half maximal inhibition of [^{35}S]ATR binding or of [^{14}C]ADP translocation is given by a concentration of ADP (2 μM) which is similar to the K_m^{ADP} for the ADP translocation, iii) the effect of ADP is shared by ATP and AOPCP, i.e. by nucleotides which are liable of translocation but not by UDP, CDP or GDP, which are ineffective in translocation.

Acknowledgements

The authors gratefully acknowledge the excellent technical assistance of Mr. J. Doussière and Mrs. J. Chabert. This investigation was supported by research grants from the C.N.R.S. (E.R.A. no. 36), from the "Fondation pour la Recherche Médicale" and the D.G.R.S.T.

References

[1] N.E. Weber and P.V. Blair, Biochem. Biophys. Res. Commun. 41 (1970) 821.
[2] C.D. Stoner and H.D. Sirak, Federation Proc. 29 (1970) 734.
[3] G. Zimmer, FEBS Letters 9 (1970) 274.
[4] P. Leblanc and H. Clauser, FEBS Letters 23 (1972) 107.
[5] A. Bruni, S. Luciani and C. Bortignon, Biochim. Biophys. Acta 97 (1965) 434.
[6] P.V. Vignais, E.D. Duée, P.M. Vignais and J. Huet, Biochim. Biophys. Acta 118 (1966) 465.
[7] H.H. Winkler and A.L. Lehninger, J. Biol. Chem. 243 (1968) 20.
[8] M. Klingenberg, in: Essays in Biochemistry, Vol. 6, eds. P.N. Campbell and F. Dickens (Acad. Press, London, 1970) p. 119.
[9] P.V. Vignais, E.D. Duée, M.G. Colomb, A. Reboul, A. Cheruy, O. Bârzu and P.M. Vignais, Bull. Soc. Chim. Biol. 52 (1970) 471.
[10] P.V. Vignais, P.M. Vignais and M.G. Colomb, FEBS Letters 8 (1970) 328.
[11] P.V. Vignais and P.M. Vignais, FEBS Letters 13 (1971) 28.
[12] P.V. Vignais, P.M. Vignais, M.G. Colomb, J. Chabert and J. Doussière, in: Energy Transduction in Respiration and Photosynthesis, eds. E. Quagliarello, S. Papa and C.S. Rossi (Adriatica Editrice, Bari, Italy, 1971) p. 675.
[13] D.H.R. Barton and J.B. Hendrickson, J. Chem. Soc. (1956) 1028.
[14] J. Meyer, P.M. Vignais, G. Brandolin and P.V. Vignais, 8th FEBS Meeting, Amsterdam, August 1972, Abst. no. 169.

[15] E. Pfaff, H.W. Heldt and M. Klingenberg, European J. Biochem. 10 (1969) 484.
[16] E.D. Duée and P.V. Vignais, J. Biol. Chem. 244 (1969) 3920.
[17] E. Pfaff and M. Klingenberg, European J. Biochem. 6 (1968) 66.
[18] D.C. Gautheron, N. Sabadie-Pialoux, D. Goldschmidt, R. Morelis, B. Foucher and Y. Gaudemer, in: Energy Transduction in Respiration and Photosynthesis, eds. E. Quagliarello, S. Papa, C.S. Rossi (Adriatica Editrice, Bari, Italy, 1971) p. 807.
[19] P.V. Vignais, P.M. Vignais and G. Defaye, FEBS Letters 17 (1971) 281.