THE ENERGY-DEPENDENT UNMASKING OF -SH GROUPS IN THE MITOCHONDRIAL ADP/ATP CARRIER, AND ITS PREVENTION BY NIGERICIN

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1. Introduction

Permanent SH reagents, like *N*-ethylmaleimide (NEM) [1], inhibit ADP/ATP transport, provided the mitochondria are preincubated with minute amounts of the carrier substrates, ADP or ATP [2,3]. Not only ADP/ATP transport is inhibited, but also binding of atractyloside [3], a specific non-penetrant inhibitor of the ADP/ATP carrier [4]. Probably, strategic -SH groups, which are buried within the carrier at rest, are unmasked and trapped by NEM when the carrier is functioning. More puzzling was the finding that respiration enhanced the ADP or ATP induced-unmasking of -SH groups, an effect which was abolished by uncouplers [3,5]. This suggested that the protonmotive force generated by respiration could also control the topology of the ADP/ATP carrier protein within the mitochondrial membrane.

The aim of this work was to determine which of the two components of the protonmotive force, the proton gradient (ΔpH) or the membrane potential ($\Delta\psi$), is responsible for the unmasking of -SH groups in the carrier protein within the inner mitochondrial membrane. For this purpose, nigericin and valinomycin were chosen as specific ionophores to collapse ΔpH and $\Delta\psi$, respectively. The experimental data suggest that the ΔpH component of the protonmotive force is the factor that primarily controls -SH unmasking in the ADP/ATP carrier.

Abbreviations: NEM, *N*-ethylmaleimide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone

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2. Materials and methods

Rat heart mitochondria were prepared in sucrose medium as in [6].

[^{3}H]Atractyloside was prepared as in [7]. [^{3}H]-Atractyloside binding assays were carried out either in a 'saline medium' made of 125 mM KCl buffered with 10 mM MOPS (pH 7.2) supplemented with 0.2 mM EDTA, or in a 'sucrose medium' made of 250 mM sucrose and 12 mM KCl buffered with 10 mM MOPS (pH 7.2). Incubation with [^{3}H]atractyloside was carried out for 5 min at 25°C, followed by a further 20 min standing in ice. These conditions insured full equilibration between bound and free [^{3}H]atractyloside. Mitochondria were collected by centrifugation. The pellet was digested by formamide and the radioactivity counted by scintillation.

To test the effect of NEM, the mitochondria were first preincubated for 2 min, in the saline or sucrose medium at 25°C. Then NEM was added, and incubation with NEM was continued for 5 min at 25°C, followed by addition of [^{3}H]atractyloside. When the effect of ADP plus NEM was assayed, ADP was placed directly in the medium, and left to react for 2 min prior to NEM addition. When other compounds like succinate, valinomycin, nigericin, and FCCP were tested, they were introduced in the medium prior to mitochondria, the pattern of incubation being the same as above. It is noteworthy that the valinomycin-induced swelling of heart mitochondria in the saline medium is very limited in contrast to that of liver mitochondria, and is probably not responsible for the effect of valinomycin on [^{3}H]atractyloside binding.

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3. Results

3.1. *Effect of the respiratory activity of heart mitochondria on the inhibition of atractyloside binding by NEM*

Data in fig.1 illustrate the effect of respiration and addition of ADP on the atractyloside binding capacity of rat heart mitochondria in saline and sucrose medium. Respiring mitochondria oxidizing succinate were supplemented with oligomycin to increase the value of the protonmotive force. Abolition of respiration was obtained by addition of cyanide and antimycin. There was no effect of oligomycin, cyanide and antimycin on atractyloside binding. The concentration of ADP used in atractyloside binding assays was sufficiently low (routinely 3 μM) not to interfere with atractyloside binding, since atractyloside is a competitive inhibitor of ADP/ATP transport [4]. In agreement with [3], atractyloside binding was more inhibited by ADP plus NEM than by NEM alone. The inhibitory effect of NEM alone, or NEM plus ADP, was markedly higher with respiring mitochondria than with non-respiring mitochondria. Inhibition was also higher when the respiring mitochondria were incubated in a saline medium rather than in a sucrose medium. Whereas the binding capacity of heart mitochondria for atractyloside was decreased by NEM (control value of high affinity binding sites, 1 nmol/mg protein), their binding affinity (control K_d-value, 20–30 nM) was virtually not altered. The NEM effect can therefore be considered an all or none process, in agreement with the covalent nature of the alkylation reaction mediated by NEM.

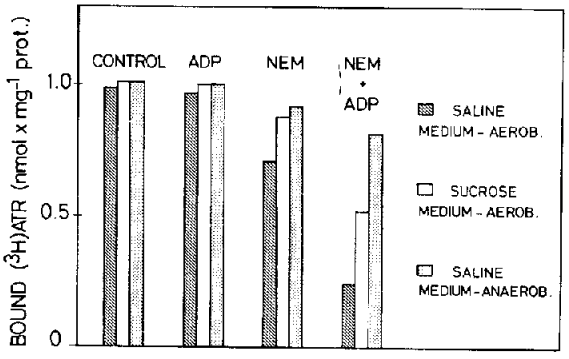

Fig.1. Effect of respiration and medium composition on inhibition of [^{3}H]atractyloside binding by NEM, or NEM + ADP in heart mitochondria. Rat heart mitochondria (1 mg) were preincubated at 25°C for 2 min in 2.5 ml saline or sucrose medium, in the presence of 5 mM succinate and oligomycin (1 μg/mg protein). Incubation of respiring mitochondria was carried out under constant aeration. In the case of non-respiring mitochondria, the medium was supplemented with 1 mM KCN and antimycin (1 μg/mg protein). After 2 min preincubation, NEM was added (150 μM final conc.) and left to react at 25°C for 5 min. Then, [^{3}H]atractyloside was added (2 μM final conc.). After another 5 min incubation at 25°C, samples were placed at 0°C for 20 min, and centrifuged to sediment mitochondria. The bound ^{3}H radioactivity in the pellet was counted by scintillation.

Since internal adenine nucleotides released outside mitochondria by spontaneous leakage in the course of the incubation could mimic externally added ADP by enhancing the NEM inhibitory effect, the amount of released adenine nucleotides was determined. With rat heart mitochondria incubated under the experimental conditions of fig.1 without further additions, 0.3–0.6 nmol adenine nucleotides/mg protein (60–70% being AMP) were released after 7 min at 25°C, which corresponded to 0.1–0.2 μM adenine nucleotides in external medium. ADP at ~1–2 μM was capable of inducing the half-maximal response to NEM. It is therefore likely that the protonmotive force developed by respiration plays a decisive role in the unmasking of -SH groups in the ADP/ATP carrier protein, and that alkylation of these groups by NEM leads to inhibition of atractyloside binding. One cannot, however, eliminate the possibility that the traces of ADP or ATP released from mitochondria to the external medium could not act in synergy to enhance the effect of the protonmotive force.

3.2. *Effect of nigericin and valinomycin on inhibition of atractyloside binding by NEM, or NEM plus ADP, in respiring mitochondria*

To determine which of the two components ($\Delta\psi$ and ΔpH) of the protonmotive force generated by respiration was responsible for enhancing the inhibitory effect of NEM on atractyloside binding, mitochondria oxidizing succinate were preincubated with valinomycin or nigericin to collapse $\Delta\psi$ and ΔpH, respectively. The appropriate concentrations of valinomycin and nigericin required for abolition of $\Delta\psi$ and ΔpH were determined by fluorimetry with dipropylthiodicarbocyanine [8] and double wavelength spectrophotometry with fluoresceine [9]. Nigericin at 200–500 ng/mg protein was required for full inhibition of ΔpH. On the other hand, as low as 10 ng valinomycin/mg protein was sufficient to abolish $\Delta\psi$.

As shown in table 1, nigericin was able to partially

Table 1
Modulation of the NEM-dependent inhibition of [^{3}H]atractyloside binding by nigericin, valinomycin and FCCP in respiring heart mitochondria

No.	Additions	Bound [^{3}H]-atractyloside (nmol/mg protein)
1	None	0.96
2	ADP	0.94
3	Nigericin, FCCP, or Nigericin + valinomycin	0.95–1.02
4	Valinomycin	0.58
5	NEM	0.70
6	NEM + nigericin	0.88
7	NEM + valinomycin	0.26
8	NEM + nigericin + valinomycin	0.92
9	NEM + FCCP	0.90
10	NEM + ADP	0.34
11	NEM + ADP + nigericin	0.64
12	NEM + ADP + valinomycin	0.21
13	NEM + ADP + nigericin + valinomycin	0.81
14	NEM + ADP + FCCP	0.92

Same conditions as in fig.1; saline medium, respiring mitochondria. ADP (3 μM), nigericin (500 ng/mg protein), valinomycin (30 ng/mg protein), and FCCP (1 μM) were present in the medium prior to the addition of mitochondria

counteract inhibition of atractyloside binding by NEM, or NEM plus ADP. A maximal effect was obtained at 100–500 ng nigericin/mg protein. It is noteworthy that P_i at ⩾1 mM behaved like nigericin on NEM inhibition. In contrast to nigericin, valinomycin enhanced the inhibition of atractyloside binding caused by NEM, or NEM plus ADP. A maximal effect of valinomycin was obtained at as low as 5 ng/mg protein and was steady till 50 ng/mg protein. These data showed that the concentrations of nigericin and valinomycin which were able to affect the inhibition of atractyloside binding caused by NEM, or NEM plus ADP, were of the same order as those required to collapse ΔpH and $\Delta\psi$, respectively. Nigericin and valinomycin added together (which is equivalent to addition of an uncoupler) or the uncoupler FCCP could reverse the inhibition of [^{3}H]atractyloside binding by NEM better than nigericin alone.

The opposite effects of nigericin and valinomycin could be related to the fact that the ΔpH and $\Delta\psi$ are interconvertible [10]; i.e., valinomycin collapses $\Delta\psi$ and increases ΔpH. However, it is noteworthy that, in the absence of NEM, atractyloside binding is not modified by nigericin, but is severely diminished by valinomycin. This effect of valinomycin per se, in the absence of NEM, may be related to a modification of the charge of the membrane surface. There was no significant change in the above mentioned effects when MOPS buffer was replaced by MES or Hepes buffers. It must be stressed that, in spite of quantitative differences, the responses of heart mitochondria and liver mitochondria to inhibition by NEM with or without ADP, and the sensitivity to nigericin and valinomycin are basically similar in direction.

4. Discussion

These data make it clear that the protonmotive force developed by mitochondrial respiration results in a marked increase in the NEM reactivity of the ADP/ATP carrier protein. This may be due to a facilitated access of -SH groups of the carrier to NEM, or more likely to the unmasking of -SH groups in the carrier. The unmasked -SH groups are in an hydrophobic environment, probably in contact with the lipid core of the membrane, since they are reactive towards penetrant -SH reagents like NEM, but not towards non-penetrant reagents like mersalyl [3]. The alkylation of the unmasked -SH groups by NEM leads to a conformation of the ADP/ATP carrier which is unable to react with and to bind atractyloside. Assuming that the substrate site and the atractyloside site correspond to the same molecular entity, it was postulated [11] that, upon addition of ADP to mitochondria, the carrier site turns to the inside and that its motion is associated with some conformational changes, including -SH unmasking; alkylation of the unmasked -SH groups would freeze the 'internal' conformation of the carrier and prevent atractyloside binding. In keeping with this idea, one has to further assume that the development of a protonmotive force would also favour the 'internal' conformation of the carrier. There are however recent data which do not fit entirely with the postulate that the atractyloside and ADP/ATP binding sites are strictly identical [12].

The decreased sensitivity of the ADP/ATP carrier to NEM upon addition of uncouplers recalls the uncoupler-induced decrease in sensitivity of the P_i carrier to -SH reagents like mersalyl, NEM and 5,5′-dithio-bis-nitrobenzoate [13]. In both cases, it appears that the conformational state of the carrier, and therefore its chemical reactivity depends not only on substrate binding, but also on the membrane

environment.

The component of the protonmotive force that primarily controls the unmasking of -SH groups in the ADP/ATP carrier appears to be the proton gradient, since nigericin, an ionophore which collapses the proton gradient, can reverse the inhibitory effect of NEM on atractyloside binding; one may imagine that nigericin favors the return to the original conformation of the carrier where -SH groups are buried in the protein and not accessible to NEM. P_i has the same effect as nigericin. It may be recalled that preincubation of mitochondria with P_i also decreases the rate of inhibitin of the P_i carrier with -SH reagents [13]. This was interpreted by an increased acidity of the mitochondrial matrix, which could modify the conformation of the P_i carrier and mask reactive -SH groups. A similar interpretation can be formulated for the ADP/ATP carrier.

It is well admitted that ADP/ATP transport is a primarily electrogenic process, i.e., the transport of ADP^{3-} against ATP^{4-} is not charge-compensated by cotransport of H^+ [14–16]. The electrogenic nature of ADP/ATP transport and the conformational changes, including -SH unmasking, that appear to be dependent on the magnitude of the generated proton gradient, are probably non-related processes.

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