EFFECT OF SH-REAGENTS ON THE MITOCHONDRIAL ATPase AND INDUCTION OF RESPIRATORY CONTROL IN EDTA PARTICLES

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1. Introduction

Participation of thiol function in oxidative phosphorylation has been proposed by several workers [1–3]. The study of the involvement of -SH in the energy transformation mechanism is, however, complicated by multi-directional effects of SH-reagents on mitochondrial function. Low concentrations of mercaptide-forming agents stimulate the ATPase activity of intact mitochondria, while the increase of inhibitor concentrations causes an inhibition of ATP hydrolysis [4]. The binding of membrane sulfhydryl inhibits phosphate transport across the intramitochondrial membrane [5–7]. Further, low concentrations of SH-reagents induce a non-specific permeability of mitochondrial membrane for certain ions [8,9]. Both dehydrogenases tightly-bound to the respiratory chain i.e. succinate dehydrogenase [10] and NAD·H dehydrogenase [11] are also shown to be sensitive to SH-reagents.

Lee and Ernster have demonstrated that oligomycin causes an inhibition of respiration in nonphosphorylating EDTA particles and this inhibition can be completely prevented by uncouplers [12]. The same phenomenon has been observed in the case of another specific inhibitor of energy transformation - DCCD* [13,14].

The induction of 'respiratory control' in non-phosphorylating submitochondrial particles provides a valuable tool for further investigation of the energy transformation mechanism in relatively simple systems.

The present paper deals with the effect of a mercurysubstituted nitrophenol (CMNP) on the energy-transducing system of intact mitochondria and non-phosphorylating submitochondrial particles. It is found that similarly to oligomycin, CMNP inhibits the ATPase activity and causes an inhibition of NAD-H oxidation in EDTA particles which is relieved by an uncoupler.

2. Materials and methods

'Heavy' beef-heart mitochondria were isolated as described by Crane et al. [15]. EDTA particles from heavy beef-heart mitochondria were prepared according to Lee and Ernster [16]. The preparation which had an average respiratory control index of 3 in case of NAD·H oxidase was used. The soluble mitochondrial ATPase (factor F_1) was prepared from acetone powder according to Selwyn [17] with minor modifications [18].

The ATPase activity of mitochondria, EDTA particles and soluble ATPase was measured either colorimetrically [19] (inorganic phosphate liberation) or with a hydrogen ion-sensitive glass electrode [20]. Both methods gave identical results. The ratio of hydrogen ions released to inorganic phosphate formed was found to be 0.9 under the conditions used.

Particle-bound protein was determined by the biuret method [21] and the soluble protein (F_1) by the Lowry procedure [22].

Oxygen consumption was measured polarographically with platinum – Ag/AgCl electrodes.

CMNP was prepared as described by McMurray and Trentham [23]; all other chemicals were the purest commercially available.

Abbreviations: DCCD, dicyclohexyl carbodiimide; CMNP, 2-chloromercuri-4-nitrophenol; p-CMB, *p*-chloromercuribenzoate; CCCP - chloromethoxycarbonylphenylhydrazone.

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3. Results

Among many other sulfhydryl group reagents CMNP has two advantageous properties. The change of its spectrum after binding to the sulfhydryl depends on the environment [23], so the reagent can be used as a 'reporter group'. Secondly, CMNP is a close structural analog of the classical uncoupler – DNP; therefore it might be expected that the reagent can readily penetrate into the hydrophobic area of mitochondria. Fig.1 shows the effect of CMNP on the ATPase activity of mitochondria as compared to p-CMB. Both reagents are almost equally efficient in inhibiting uncoupler-stimulated ATPase. It was found that the low concentrations of CMNP as well as p-CMB stimulate several times the ATPase activity over that without uncoupler added to the reaction medium (not shown).

The basic difference between the two SH-reagents used is that the inhibitory effect of p-CMB is prevented by the presence of detergent, while the inhibition induced by CMNP is not.

The simplest explanation for the data presented in fig.1 is that, according to several authors [5–7], p-CMB inhibits phosphate transport across the intramitochondrial membrane, and its effect depends on the existence of a permeability barrier; the effect of CMNP can hardly be due only to the inhibition of the phosphate transport, since this reagent inhibits ATPase even when the membranes are destroyed by the detergent. Therefore it seemed likely that the effect of CMNP is directed to the ATPase system itself. The data presented in fig.2 is in good agreement with this assumption.

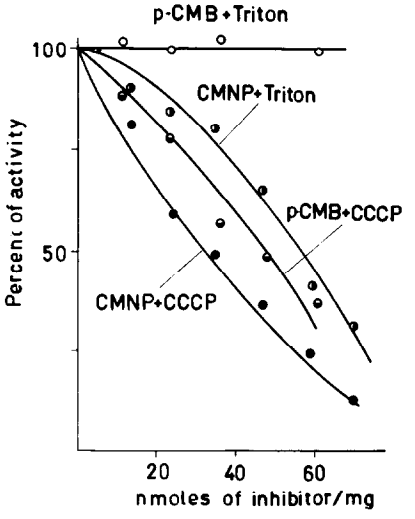

Fig.1. Effect of SH-reagents on ATPase activity of intact mitochondria. Mitochondria (1.1 mg of protein per ml) were incubated in mixture containing 0.25 M sucrose; 5 mM Tris–HCl buffer (pH 7.8), 20 mM KCl; different concentration of SH-reagent; 1.2 μM CCCP; 0.08% Triton-X100 (where indicated); 2 mM ATP and 1 mM $MgCl_2$ in a total vol 4 ml at room temperature.

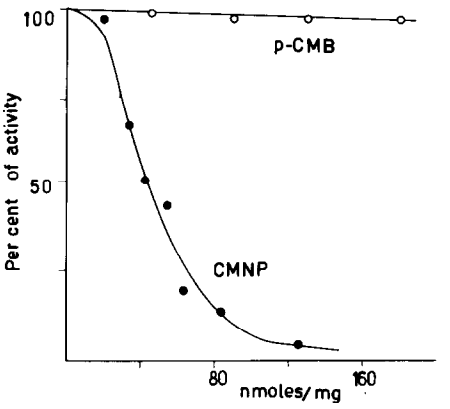

Fig.2. Effect of SH-reagents on ATPase activity of EDTA-particles. The activity was measured as indicated in fig.1, except uncoupler and detergent were not added and protein content was 0.8 mg per ml.

The comparison of p-CMB and CMNP effects on the ATPase of EDTA-particles, which are believed to be 'inside out', shows that under the condition when ATPase is up to 90 per cent inhibited by CMNP, p-CMB is not effective at all even when its concentration is twice as high.

Special experiments showed that both p-CMB and CMNP do not inhibit the soluble ATPase (factor F_1), which is in a good agreement with the data of Racker's group [24].

The mode of CMNP action has a feature in common with that of oligomycin: it inhibits particle-bound ATPase and has no effect on the soluble enzyme. It seemed to be of interest to compare CMNP and oligomycin with respect to their effects on NAD·H oxidase in non-phosphorylating submitochondrial particles. Such a study is complicated by the fact that the sulfhydryl reagents inhibit NAD·H dehydrogenase.

Table 1
Effect of mercaptoethanol on CMNP induced inhibition of NAD·H oxidase and ATPase activities of EDTA-particles[a]

	NAD·H oxidase	ATPase
	μmoles/min per mg	
Control	0.55	0.80
CMNP inhibited	0.06	0.00
CMNP inhibited, treated with mercaptoethanol	0.48	0.00

[a]EDTA-particles were incubated 2 min in the presence of 180 nmol of CMNP per mg of protein, mercaptoethanol (20-fold excess) was then added where indicated and NAD·H oxidase and ATPase after 5 min were measured. NAD·H oxidase was measured polarographically in mixture containing 0.25 M sucrose; 5 mM Tris–HCl buffer (pH 7.8); 20 mM KCl, 1 mM NAD·H; 1 μM CCCP; 1 mg of protein in a total vol 2 ml at room temperature. ATPase was measured in mixture containing 0.25 M sucrose; 5 mM Tris–HCl buffer (pH 7.8); 20 mM KCl; 2 mM ATP; 1 mM $MgCl_2$; 1 μM CCCP; 0.7 mg of protein in a total vol 4 ml at room temperature.

However, conditions were found where the effect of CMNP on the energy-transducing system could be analyzed without interference by the NAD·H dehydrogenase inhibition. As seen from table 1, short-term incubation of CMNP-treated EDTA particles with mercaptoethanol (20-fold excess on the basis of molar ratio) restores the NAD·H oxidase activity up to 90 per cent while the ATPase activity remains completely inhibited. The data obtained provided an opportunity to find out whether CMNP is able, like oligomycin, to induce a state of 'respiratory control'. The data presented in table 2 show a comparsion of the effects of CMNP, oligomycin and p-CMB on NAD·H oxidation in EDTA-particles. In the presence of mercaptoethanol, CMNP-inhibited NAD·H oxidation is activated 3-fold by uncoupler; p-CMB, which does not inhibit the ATPase activity of EDTA particles (fig.2), is not able to induce a state of 'respiratory control'. The presence of mercaptoethanol does not change the effect of oligomycin. It has to be pointed out that the effect found is not due to the partial inhibition of NAD·H dehydrogenase (13 per cent inhibition); experiments showed that partial inhibition of NAD·H oxidation by rotenone does not induce 'respiratory control'. This result is in good agreement with the kinetic studies on the effect of oligomycin on the respiratory chain of EDTA-particles [25].

Table 2
Comparison of the effects of oligomycin and SH-reagents on the induction of respiratory control in EDTA-particles[a]

Treatment	NAD·H oxidase μmoles/min per mg		Respiratory control ratio
	−CCCP	+CCCP	
None	0.51	0.82	1.6
Oligomycin	0.25	0.87	3.5
CMNP	0.27	0.85	3.1
p-CMB	0.64	0.88	1.4

[a]EDTA-particles were preincubated with SH-reagent or oligomycin (160 nmol and 1 μg per mg of protein respectively) 2 min; mercaptoethanol (2 mM) was then added and after 2 min NAD·H oxidase was measured as indicated in Table 1.

4. Discussion

Very little is known at present about the chemical groups involved in the coupling between electron and energy transfer in the respiratory chain. The tyrosine residue has been recently found to participate in the hydrolytic activity of F_1 [26]. There is some indirect evidence that the carboxylic group is also involved in the activity of the soluble ATPase [27]. No sensitivity of F_1 to the sulfhydryl reagent was observed [24].

Observation of different sensitivities of soluble and membrane-bound ATPase to the inhibition together with the data on the reconstitution of oxidative phosphorylation [28] strongly suggest that a multienzyme system is involved in the energy transformation mechanism.

Two components of this system, factor B [29], which is identical to factor F_2 [30], and factor D [31], were shown to be sensitive to organic mercurials. The functional importance of SH-groups in these factors was shown under conditions where purified factors were preincubated with the sulfhydryl reagents [29,31]. In present paper the involvement of SH-groups in the coupling mechanism is demonstrated using a functionally active system (oligomycin-like effect on the respiration). The data obtained support the original proposal of Fluharthy and Sanadi on the involvement of SH-groups at the level of oligomycin sensitive site [1]. It is of interest to note that both oligomycin, presumably containing the aldehyde group in its

molecule [32], and carbodiimides [33] have a potential reactivity towards the sulfhydryl.

The chemical properties of CMNP, marked differences between the effects of p-CMB and CMNP on the ATPase activity (fig.2), and the inability of mercaptoethanol to reverse the CMNP-induced inhibition of ATPase (table 1) taken together clearly indicate the specific environment (presumably hydrophobic) of the SH-groups attacked. The simplest proposal on the mechanism of CMNP action is that this compound inhibits a proton channel of the ATP-synthase complex. Whatever the chemical mechanism of CMNP inhibition is, this reagent, due to its specific spectral properties, may be a useful tool for further fractionation of the energy transfer system.

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