THE DEPENDENCE OF OXIDATIVE PHOSPHORYLATION AND ATP-ASE OF LIVER MITOCHONDRIA ON BOUND Mg^{2+}

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SUMMARY: The concentration of hydrophobic proton donor type uncouplers necessary to induce maximal ADP dependent ejection of bound mitochondrial Mg^{2+} coincides with the minimal concentration of these agents required to uncouple oxidative phosphorylation. DNP stimulated ATP-ase activity of mitochondria and ADP + DNP dependent ejection of bound Mg^{2+} exhibit an inverse relationship, i.e. ATP-ase activity depends on bound mitochondrial Mg^{2+}. It is concluded that bound mitochondrial Mg^{2+} is an obligatory component of the mitochondrial energy transducing apparatus.

In spite of the large amount of experiments performed with weak acid type of uncouplers of oxidative phosphorylation, no generally acceptable mechanism of action of these agents on energy transduction is presently available (1, 2). It seems that uncoupling by these agents can be eliminated if the uncouplers are removed (e.g. by adsorption on BSA) within a relatively short period after their addition to mitochondria (3). In other words, the effect of short (1 to 5 minutes) contact of mitochondria with uncouplers appears to be reversible. In contrast to this reversible effect, we have previously been able to demonstrate an ADP + DNP induced irreversible ejection of inner membrane bound mitochondrial Mg^{2+}. This takes place upon incubation of mitochondria with DNP (or other weak acid uncouplers), and ADP for 10 to 15 minutes (4, 5). Analyses of this process revealed that the rate of Mg^{2+} ejection depends on the concentration of both ADP and DNP. It became apparent that DNP + ADP dependent Mg^{2+} ejection exhibits characteristics of a catalytic system, hence ADP and DNP interact with a bound Mg^{2+} containing mitochondrial site (5). Since stimulation of mitochondrial oxidation of NAD^+ dependent substrates by DNP disappeared at the same rate as Mg^{2+} was ejected, we proposed that bound Mg^{2+} in the inner membrane may be closely related to the function of the energy transducing mitochondrial mechanism. It was also postulated that exposure of mitochondria to ADP + DNP initiates an artificial operation of the energy transducing system, and Mg^{2+} ejection from the inner mitochondrial membrane is the result of the uncoupler induced malfunction of this mitochondrial apparatus (4, 5). If this assumption is correct, then various reactions which are components of the mitochondrial energy

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conversion system should be inhibited by the same amounts of uncouplers as required for Mg^{2+} ejection.

The following report deals with the experimental testing and confirmation of this postulate. Uncoupler sensitivity of oxidative phosphorylation itself and of a generally acknowledged partial reaction to this process, namely DNP-activated ATP-ase, were correlated with uncoupler requirement of the Mg^{2+} ejection system. It was found that both oxidative phosphorylation and ATP-ase activity require bound Mg^{2+}. Furthermore, the same amounts of uncouplers were needed to disrupt energy coupling as required to eject bound Mg^{2+}.

MATERIALS AND METHODS

Preparation of rat liver mitochondria and the technique of ADP dependent Mg^{2+} ejection by uncouplers as employed in the present experiments have been published in preceding papers (4, 5). Analytically pure DNP and pentachlorophenol (PCP) were commercial samples, while 4,5,6,7-tetrachlorotrifluoromethylbenzimidazole (TTFB) (cf. 6, 7) was a gift received from Dr. B. Chance. Uncoupling by 2,6-dichlorophenol-indophenol (DCIP) was described by Löw et al., (8). An analytical sample of this dye (General Biochemicals) was used in present studies.

Oxidative phosphorylation was measured with a Gilson Polarograph (Oxygraph Model K) fitted with a Clark electrode. The reaction was carried out in 5 ml. 0.15 M KCl, 0.05 M Tris, 0.01 M phosphate (both pH 7.3) with 5 to 8 mg rat liver mitochondria. Constant temperature (30^0) and magnetic stirring was maintained during short term (5 to 10 minutes) assays. Our rat liver mitochondria showed no appreciable O_2 uptake in the presence of 0.01 M glutamate unless ADP (0.2 mM) or uncouplers were added. Titration of the effectivity of uncouplers was readily accomplished by recording rates of O_2 uptake at increasing concentrations of uncouplers until further addition of ADP failed to augment the rate of O_2 consumption. This method permits the estimation of the lowest concentration of uncoupling agents sufficient to produce complete uncoupling.

The effect of ADP + DNP induced Mg^{2+} ejection on ATP-ase was assayed as follows. Mitochondria were incubated aerobically for 10 minutes (Dubnoff shaker, 30^0) in the presence of 2.3 mM ADP and varying concentrations of DNP. At the end of 10 minutes 2.3 mM ATP was added and the initial rate of orthophosphate liberation was determined after further 2 minutes incubation. The reactions were stopped with $HClO_4$ (final concentration 0.6 N) and orthophosphate was determined colorimetrically (9) after removal of perchlorate as the K^+ salt.

RESULTS

Comparison of the effectivities of four uncoupling agents on the rate of Mg^{2+} release and on oxidative phosphorylation is summarized in the Table. The second vertical column shows the concentration range of uncouplers required to initiate (indicated as $0>\%$, i.e. "more than 0%") and cause maximal ($\sim$100%) ejection of

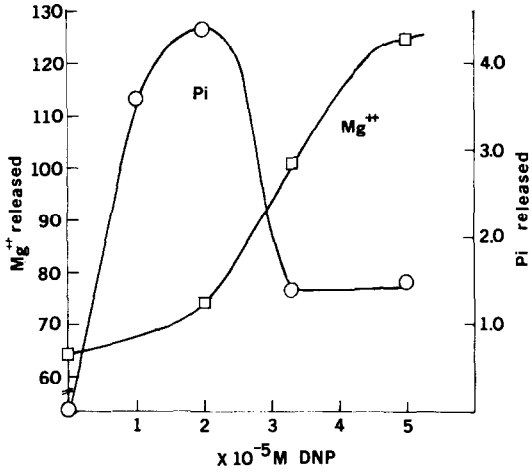
TABLE

CONCENTRATIONS OF UNCOUPLERS REQUIRED FOR ADP DEPENDENT Mg^{2+} EJECTION AND FOR DISRUPTION OF OXIDATIVE PHOSPHORYLATION

Uncouplers	Concentration of uncouplers (in M) required for:			
	0< Mg^{2+} ejection ~100%			Uncoupling
DNP	2	to	5×10^{-5}	2.7×10^{-5}
PCP	2	to	8×10^{-5}	2.5×10^{-5}
TTFB	2	to	10×10^{-7}	3.0×10^{-7}
DCIP	2	to	5×10^{-5}	4.0×10^{-5}

For abbreviations, see Methods. Ejection of Mg^{2+} was measured at 30° in the presence of 2.3 mM ADP and varying amounts of uncouplers (see Methods) and oxidative phosphorylation (at 30°) polarographically with glutamate as substrate (see Methods).

FIGURE



DNP-concentration dependence of ATP-ase (upper curve) of mitochondria and induced Mg^{2+} ejection (lower curve). Conditions are described in Methods.

bound Mg^{2+} at a constant level (2.3 mM) ADP. This notation of Mg^{2+} ejection efficiency of various uncouplers appeared feasible for the following practical reasons. As shown earlier, there is a sigmoidal relationship between the rates of Mg^{2+} ejection and uncoupler concentration (cf. 5). Since accurate determination of inflexion points of sigmoidal curves of varying shapes is technically difficult and linearization (e.g. by log function) is equally inaccurate, the 0 to 100% act-

ivity range, as expressed by limiting concentrations of uncouplers appears to be experimentally more realistic. On the other hand, the minimum concentrations of uncouplers which completely abolish the stimulatory effect of ADP on O_2 consumption can be determined with greater accuracy ($\pm$ 50%), as shown in the third vertical column. Within the experimental limits of accuracy, the two sets of concentrations of uncouplers fall within the same range. The dependence of DNP-activated ATP-ase on bound mitochondrial Mg^{2+} is illustrated in the Figure. The left ordinate shows the rates of Mg^{2+} release (in mμ moles per 3 ml per 10 minutes) induced by increasing concentrations of DNP (abscissa), while the right ordinate indicates the initial rates of ATP hydrolysis (in μ moles per 2 minutes; see Methods). It is evident that as soon as bound Mg^{2+} is ejected (above 2 x 10^{-5} M DNP), the rate of ATP-ase activity drops.

DISCUSSION

The effect of externally added Mg^{2+} on various mitochondrial functions is generally attributed to Mg^{2+} requiring enzyme systems. The cofactor role of Mg^{2+} requiring reactions would be expected to be generally a reversible one. The known Mg^{2+} requirement for DNP-activated ATP-ase (cf. 1, 2), appeared to be similar to other Mg^{2+} activated enzymatic reactions until it was discovered (4, 5) that the effect of added Mg^{2+} was a protective one exerted by stabilization of a bound form of Mg^{2+}. It is shown in this paper that bound mitochondrial Mg^{2+} is required for DNP-activated ATP-ase activity, as well as for oxidative phosphorylation. We have found (4), that the bound form of Mg^{2+}, comprising about 50-60% of total mitochondrial Mg^{2+}, is present in the inner membrane, isolated by the digitonin method of Schnaitman and Greenwalt (10). Although the chemical nature of the bound Mg^{2+} containing system is as yet unknown, we consider it highly probable that macromolecular components of the inner membrane are involved as ligands. The bound Mg^{2+} is specifically labilized by reagents which interfere with energy coupling (5), while the diffusible Mg^{2+} is insensitive to these agents and can presumably participate in orthophosphate dependent ion flux, as shown by Johnson and Pressman (11). The most significant difference between Mg^{2+} requiring mitochondrial enzyme systems and the bound Mg^{2+} containing system is that uncoupler induced Mg^{2+} loss in the latter is irreversible. Induced Mg^{2+} ejection (e.g. by DNP + ADP) can only be prevented (4, 5) but not reversed. High, clearly unphysiological concentrations of added Mg^{2+} (5 to 10 mM) temporarily protect bound Mg^{2+} against the effect of Mg^{2+} labilizing agents (4). Since, in most biochemical experiments involving isolated mitochondria, relatively high levels of Mg^{2+} were customarily employed, it is not surprising that the existence and function of the bound Mg^{2+} containing membrane system remained unrecognized.

The superimposable DNP profiles of both rates of O_2 uptake (Fig. 6A of Ref. 4) and of rates of ATP hydrolysis (see Figure) illustrate identical quantitative

dependence of these processes and of oxidative phosphorylation (see Table) on bound mitochondrial Mg^{2+}. On the basis of this -- as yet circumstantial -- evidence, it seems reasonable to propose that Mg^{2+} in a specific, probably energy dependent association with macromolecular membrane components, is part of the energy transfer system.

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