Energy-dependent Release of Magnesium from Beef Heart Submitochondrial Particles*

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SUMMARY

A reversible association of magnesium with beef heart submitochondrial particles was monitored with the use of the fluorescent chelate probe, chlorotetracycline, and direct measurement of the magnesium content of the membranes under various conditions. Addition of an oxidizable substrate, such as succinate, or ATP to the submitochondrial particles caused an energization of the membranes and the release of up to 50% of the magnesium from the particles. The release of magnesium was prevented by the addition of uncoupler, antimycin A, or malonate, each of which prevented the establishment of the energized state of the mitochondrial membranes. Following the energized release of magnesium from the particles, the addition of compounds leading to de-energization caused a rapid, nearly complete reassociation of magnesium with the membranes. Although energization of the submitochondrial particles was not dependent upon either a high level of membrane-associated magnesium or the actual release of magnesium from the membrane, the rapid release of magnesium was dependent upon the establishment of the high energy state. It was also apparent that the magnesium content of the membranes determined the amount of chlorotetracycline which was bound to the membrane, i.e. the greater the magnesium content, the greater the amount of chelate probe which was bound to the particles. The membrane-associated magnesium which was capable of being released from the particles upon energization was not accessible to polar chelators such as ethylenediaminetetraacetate, but was accessible to the lipophilic chelator, o-phenanthroline.

These studies suggest that the ratio of bound to free magnesium within the mitochondrion may be regulated by an energy-dependent, reversible association of magnesium with the mitochondrial membranes.

The activity of the pyruvate dehydrogenase multienzyme complex derived from mammalian sources is regulated by a phosphorylation-dephosphorylation mechanism as shown by Reed and his associates (1-4) and by Wieland and his co-workers (5, 6). By this mechanism a specific protein kinase, requiring ATP and free magnesium, may catalyze the phosphorylation and inactivation of pyruvate dehydrogenase while a magnesium-dependent phosphatase may re-activate the enzyme by cleaving the phosphoryl moiety from the inactive enzyme. Huchet et al. (4) have proposed that the differential regulation of the pyruvate dehydrogenase-linked kinase and phosphatase may result as a consequence of the different sensitivities of these two enzymes to magnesium. That this modulation of pyruvate dehydrogenase occurs in intact metabolic systems has been implicated by studies employing isolated heart (7, 8), liver (9), and adipose tissue (10) mitochondria as well as by studies in which the phosphorylation state and activity of pyruvate dehydrogenase have been assessed in intact tissues under various metabolic and hormonal conditions (11-13).

Evidence obtained in this laboratory suggested that the availability of free intramitochondrial magnesium was an important consideration for the regulation of the pyruvate oxidase function of isolated cardiac mitochondria (7, 8). It was concluded from these studies that the regulation of the phosphorylation state and the activity of pyruvate dehydrogenase represents an example of an enzymatic process involved in energy generation which may be regulated by the reversible binding and release of magnesium to site(s) in or on the mitochondrial membrane. The purpose of the present study was to attempt to characterize the binding site(s) for magnesium on mitochondrial membranes and to elucidate factors which may regulate the reversible binding of magnesium to these sites. Data will be presented suggesting that the energy state of mitochondrial membranes plays an important role in the regulation of the ratio of free to bound magnesium in mitochondrial systems. Also, direct evidence will be presented showing that the fluorescent chelate probe, chlorotetracycline, represents an effective means for the visualization of changes in the level of membrane-associated divalent cations (14).

MATERIALS AND METHODS

Heavy beef heart mitochondria were prepared from fresh hearts according to the procedure of Löw and Vallin (15). Mitochondria were frozen at -80°C in small aliquots for storage prior to the preparation of submitochondrial particles by means of a slight modification of the procedure of Racker (16). Three types of submitochondrial particles were prepared from beef heart mitochondria: ETPn,Mg2+, ETPn,Mg2+ and ETPn,EDTA.

1 The abbreviations used are: ETPn, electron transport particles, ANS, 1-anilino-8-naphthalenesulfonic acid.
During the preparation of the ETP$_H$-Mg$^{2+}$ and ETP$_H$-EDTA particles, frozen beef heart mitochondria were diluted in 0.25 M sucrose containing either 7.5 mM magnesium chloride or 7.5 mM potassium EDTA prior to the preparation of submitochondrial particles. It should be noted that both the magnesium- and EDTA-treated particles were washed twice with 0.25 M sucrose containing neither magnesium chloride nor EDTA during subsequent steps in the preparative scheme. All experiments were performed with freshly prepared, unfrozen submitochondrial particles or with particles which had been frozen at $-80^\circ$C for no more than 48 hours.

Fluorescence measurements with submitochondrial particles treated with the chelate probe, chlorotetracycline, were accomplished using a Perkin-Elmer MFP-2A spectrophotometer according to the procedure of Caswell (14). An excitation wave length of 410 nm and an emission wave length of 500 nm were used for the chlorotetracycline-treated particles throughout the present studies. For experiments employing the membrane probe, 1-anilino-8-naphthalenesulfonic acid, the wave length pair 380 → 480 nm was used (17). Light scattering (90$^\circ$) was measured by setting both excitation and emission wave lengths of the fluorimeter at 560 nm.

In experiments requiring the determination of magnesium levels of the submitochondrial particles, samples (2 ml) were withdrawn from the reaction vessel (volume, 10 ml), the particles were sedimented (15 min at 48,000 × g at 0$^\circ$C), and the resultant pellets were hydrolyzed in 11.6 M HCl for 24 hours at 25$^\circ$. Magnesium and calcium were determined directly with the use of an atomic absorption spectrophotometer with appropriate standards. Magnesium or calcium levels reported in the figures and tables represent the level of the divalent ion remaining within or attached to the submitochondrial particles. Incubations of submitochondrial particles were performed with the use of a reaction mixture containing either sucrose, 310 mM, and Tris-chloride, 20 mM, at pH 6.7, or potassium chloride, 180 mM, and Tris-chloride, 20 mM, at pH 6.7. A maximal decrease in the chlorotetracycline-associated fluorescence following energization of the submitochondrial particles with succinate was observed with the use of reaction mixtures with a pH of 6.7. The reaction volume was 2.0 ml except when samples were taken for the analysis of magnesium in the particles. The temperature at which the experiments were performed was 25$^\circ$. Submitochondrial particle protein concentrations were estimated by means of a biuret procedure (18).

The binding of chlorotetracycline to submitochondrial particles was measured in the following manner. With the reaction conditions noted in the figure legends, submitochondrial particles at various protein concentrations were incubated at room temperature, samples (2.0 ml) were withdrawn and centrifuged for 30 min at 48,000 × g at 0$^\circ$, an aliquot (1.0 ml) of the supernatant solution was mixed with 1.0 ml of ethanol (95%) containing 25 mM MgCl$_2$, and the fluorescence of this mixture was determined with the use of the wave length pair 390 → 520 nm. These fluorescence measurements were quantitated with standard solutions of chlorotetracycline and magnesium in ethanol.

Chlorotetracycline and 1-anilino-8-naphthalenesulfonic acid were purchased from Nutritional Biochemicals Corporation. Solutions of these two membrane probes were made fresh each day. The uncoupler p-trifluoromethoxyphenylhydrazone of carbonyl cyanide was the generous gift of Dr. P. G. Heytler of the E. I. DuPont de Nemours Co. m-Phenanthrolene was the generous gift of Dr. Cyrl Parkanyi, Department of Chemistry, University of Texas at El Paso. All other compounds and reagents were of reagent grade and were purchased from common commercial suppliers.

### RESULTS

The objective of the present study was to elucidate factors affecting the binding or transport of magnesium in mitochondrial systems. An attempt was made to minimize possible compartmentation complexities in this study by utilizing submitochondrial particles isolated from beef heart to characterize the interaction of magnesium with mitochondrial membranes. In order to visualize interactions between magnesium and the submitochondrial membranes, the fluorescent chelate probe, chlorotetracycline, was employed (14). Caswell had demonstrated that the interaction of magnesium with chlorotetracycline in a lipophilic environment, such as might be found within membranes, leads to the formation of a highly fluorescent complex (19, 20). In fact, it has been shown that chlorotetracycline can be effectively utilized to visualize divalent ion movements in intact liver mitochondria (14) and in sarcoplasmic reticulum (21).

The initial experiments in the present study were designed to elucidate the relationship between the energy state of mitochondrial membranes and the binding or transport of the divalent metal ion magnesium. By means of beef heart submitochondrial particles prepared in the presence of magnesium, ETP$_H$-Mg$^{2+}$, Fig. 1A shows that the addition of an oxidizable substrate such as succinate (2.5 mM) caused a marked decrease in fluorescence measured with the use of the wave length pair 410 → 560 nm. It is proposed that upon energization the decrease in the fluorescence of the chlorotetracycline-treated submitochondrial particles represents either a decrease in the interaction of magnesium with the chelate probe or an actual release of the magnesium-chlorotetracycline complex from the lipophilic environment of the membrane. Further information pursuant to this latter possibility will be presented later. No changes in the light-scattering properties of the suspension were observed upon energization of the particles with succinate. The rate and the magnitude of the fluorescence decrease caused by succinate

![Fig. 1. The effect of oligomycin, uncoupler, or malonate on the chlorotetracycline-associated fluorescence of energized beef heart submitochondrial particles (ETP$_H$-Mg$^{2+}$).](http://www.jbc.org/.../fig1.png)

The incubation medium contained sucrose, 0.31 M, and Tris-Cl, 0.02 M, pH 6.7. The additions noted in the figure were to final concentrations of A, succinate, 2.5 mM; B, oligomycin, 2.5 μg per ml; C, p-trifluoromethoxyphenylhydrazone of carbonyl cyanide (FCCP), 10 μM; and D, malonate, 5.0 mM. The submitochondrial particles (ETP$_H$-Mg$^{2+}$) were added to a protein concentration of 0.67 mg per ml, and the final concentration of chlorotetracycline was 25 μM.)
addition was increased by the inclusion of oligomycin (2.5 µg per ml) in the reaction mixture, suggesting that the proposed release of magnesium from the particles was dependent upon the generation of a high energy state as a result of the oxidation of succinate (Fig. 1B). The inclusion of the uncoupler, p-trifluoromethoxyphenylhydrazone of carbonyl cyanide, in the incubation medium prevented the generation of the high energy situation and prevented the release of magnesium from the particles (decrease in the fluorescence of chlorotetracycline) (Fig. 1C). The decrease in fluorescence or release of magnesium can also be prevented by inclusion of malonate (5 mM), a competitive inhibitor of succinate dehydrogenase, in the incubation mixture (Fig. 1D). It will be shown later that inhibitors of electron transfer, such as antimycin A, which prevent the energization of the submitochondrial particle membranes, prevent the release of magnesium as monitored either by the response of the fluorescent chelate probe or by actual measurement of the magnesium retained in the particles.

If the apparent release of magnesium from the particles upon energization was prevented by compounds or conditions which de-energized the membranes, it was appropriate to determine whether this effect could be reversed once the magnesium had been released during energization. Fig. 2 demonstrates that the magnesium-treated particles in the presence of oligomycin were capable of cyclic changes in the chlorotetracycline-associated fluorescence when small amounts of succinate (25 µM) were added repeatedly to the particles. Also, pertinent to the question of the reversibility of magnesium binding, which was dependent upon the energetic state of the membrane, are the data shown in Fig. 3A. Magnesium was released from the particles upon energization with succinate plus oligomycin while the addition of malonate, p-trifluoromethoxyphenylhydrazone of carbonyl cyanide, or antimycin A caused an increase in the fluorescence of the chlorotetracycline, indicating a reassociation of magnesium with the particles upon de-energization. That the submitochondrial particles were indeed being energized upon substrate addition and de-energized upon the addition of the inhibitors or uncouplers was apparent from the data shown in Fig. 3B. Addition of the fluorescent membrane probe 1-anilino-8-naphthalenesulfonic acid to the magnesium-treated submitochondrial particles indicated that upon substrate addition the particles became energized (increase in fluorescence at 380 → 480 nm) while addition of malonate, antimycin A, or p-trifluoromethoxyphenylhydrazone of carbonyl cyanide caused the ANS fluorescence to decrease markedly, indicating the de-energization of the membranes.

Data were also obtained (not shown) indicating that the addition of ATP to unenergized submitochondrial particles resulted in an extensive decrease in the chlorotetracycline-associated fluorescence. This fluorescence decrease was prevented by the inclusion of either uncoupler or oligomycin.

At this point it was considered crucial to demonstrate unequivocally that a decrease in the fluorescence of the chelate probe, chlorotetracycline, corresponded to a release of bound or sequestered magnesium from the submitochondrial particle membranes. The data shown in Fig. 4 demonstrate that the fluorescence decrease caused by energization of the particles with succinate plus oligomycin was dependent upon the method of preparation of the three types of submitochondrial particles. The magnesium-treated particles (ETPn-Mg2+) showed a larger decrease in fluorescence upon energization than the untreated particles (ETPn) or the EDTA-treated particles (ETPn-EDTA), which exhibited little, if any, change in chlorotetracycline-associated fluorescence upon energization. Although it is not apparent in Fig. 4, the initial levels of chlorotetracycline-associated fluorescence in the three types of particles decreased in the order ETPn-Mg2+ > ETPn > ETPn-EDTA. Fig. 4B merely indicates that each of the three types of particles was capable of being energized upon addition of succinate plus oligomycin with the use of ANS-associated fluorescence as a monitor of membrane energization. This experiment implies that significant variations in the magnesium level did not affect the energization of the particles upon addition of an oxidizable substrate. Also, it is apparent from these experiments that although

![Fig. 2. The effect of the repeated addition of succinate on the chlorotetracycline-associated fluorescence of beef heart submitochondrial particles (ETPn-Mg2+). The incubation medium as well as the chlorotetracycline and oligomycin concentrations were the same as described in the legend for Fig. 1. The submitochondrial particles were present in a protein concentration of 1.35 mg per ml, and the successive additions of succinate were each to a concentration of 25 µM.](http://www.jbc.org/)

![Fig. 3. The effect of antimycin A, malonate, or uncoupler on chlorotetracycline-associated (A) or ANS-associated (B) fluorescence changes in beef heart submitochondrial particles (ETPn-Mg2+). The experiments were performed in the sucrose medium described in the legend for Fig. 1, and oligomycin, 2.5 µg per ml, was present in each incubation. The final concentrations of the other components in these experiments were succinate, 2.5 mM; p-trifluoromethoxyphenylhydrazone of carbonyl cyanide (FCCP), 10 µM; malonate, 5 mM; antimycin A, 20 µg per ml. The final concentration of both chlorotetracycline and ANS was 25 µM. The submitochondrial particles were present in a protein concentration of 0.71 mg per ml.](http://www.jbc.org/)
an energy-induced conformation change occurred in all three
types of particles, a chlorotetracycline-associated fluorescence
change occurred exclusively in particles which were enriched
with respect to the divalent metal ion, magnesium (see Table I).
These observations also suggest that both the initial level of
fluorescence of the chelate probe-treated particles and the change
in fluorescence of the probe upon energization were functions of
the content of magnesium in the particles. In the present study
no such correlation may be made for calcium, the other divalent
cation of interest in this system. It is also of interest to note
that magnesium apparently displaced calcium in the magnesium-
treated particles, e.g. compare the magnesium levels of 15.4
nmoles per mg of protein and 34.3 nmoles per mg of protein in
the ETPH and ETPH-Mg++ particles, respectively, with the
calcium levels of 14.9 nmoles per mg of protein and 2.3 nmoles
per mg of protein in the same particles.

Further evidence indicating a correspondence between the mag-
nesium content of the particles and the relative initial levels of
chlorotetracycline-associated fluorescence may be discerned
from the chlorotetracycline-binding data for the three types of
particles shown in Fig. 5. These data indicate that the amount
of chlorotetracycline bound to the three types of particles was
dependent upon the magnesium content. The amount of
chlorotetracycline bound to the particles at any given protein
concentration decreased in the order ETPH-Mg++ > ETPH
> ETPH-EDTA, again correlating with the magnesium con-
tent of the respective particle preparations.

The correspondence between the fluorescence change of the
submitochondrial particles treated with the chelate probe,
chlorotetracycline, and the magnesium content of the sub-
mitochondrial particles is shown in Fig. 6. The fluorescence
trace at the top of this figure indicates the decrease in fluores-
cence when the particles were energized with succinate plus
oligomycin. Addition of antimycin A approximately 5 min
after energization caused a nearly complete reversal of the flu-
orescence trace. Samples were taken for magnesium analyses
at the points indicated in the figure (S1, S2, and S3). The mag-
nesium content of the particles at the various time points is

![Fig. 4. Chlorotetracycline-associated (A) and ANS-associated
(B) fluorescence changes upon energization of three different types
of beef heart submitochondrial particles. The sucrose-Tris reaction
medium described in the legend to Fig. 1 was employed. Succinate
was added to a final concentration of 2.5 mM and oligo-
mycin to a concentration of 2.5 µg per ml. For the traces shown
in A, the chlorotetracycline concentration was 25 nM, and for the
traces shown in B, the ANS concentration was 25 nM. The protein
concentration in all cases was 0.6 mg per ml.](image1)

![Fig. 5. The binding of chlorotetracycline to various types of
beef heart submitochondrial particles. Chlorotetracycline-
binding studies were performed as described under “Materials and
Methods” at the protein concentrations indicated. The chloro-
tetracycline concentration was 12.5 µM in all incubations. These
studies were performed in the sucrose-Tris medium described in
the legend for Fig. 1.](image2)

![Fig. 6. The relationship between the magnesium content and
the chlorotetracycline-associated fluorescence changes upon
energization and de-energization of beef heart submitochondrial
particles (ETP-H-Mg++). The sucrose incubation medium was
described in the legend to Fig. 1. Other additions to the incuba-
tions were to final concentrations of succinate, 2.5 mM; oligomycin,
2.5 µg per ml; and antimycin A, 20 µg per ml. Samples were taken
as noted, and magnesium remaining in the submitochondrial
particles was determined by the technique described under “Mate-
rials and Methods.” The submitochondrial particles were added
to a protein concentration of 0.44 mg per ml.](image3)

| TABLE I Comparison of magnesium and calcium content of various types of beef heart submitochondrial particles |
|---------------------------------------------------------------|
| Submitochondrial particle preparation | Magnesium (nmoles/mg protein) | Calcium (nmoles/mg protein) |
| ETPH | 15.4 | 14.9 |
| ETPH-Mg++ | 34.3 | 2.3 |
| ETPH-EDTA | 3.8 | 3.5 |
Comparison of magnesium content of beef heart submitochondrial particles incubated under energized or de-energized conditions

| Incubation number | Incubation medium | Addition            | Incubation time | Magnesium content of particles |
|------------------|------------------|---------------------|-----------------|-------------------------------|
| 1                | Sucrose, 0.31 M  | None                | 30 min          | 29.7 nmoles/mg protein        |
| 2                | Sucrose, 0.31 M  | Succinate, 5.0 mM   | 30 min          | 17.0 nmoles/mg protein        |
| 3                | Sucrose, 0.31 M  | Succinate, 5.0 mM; antimycin A, 20 μg/ml | 30 min | 20.8 nmoles/mg protein        |
| 4                | KCl, 0.18 M      | None                | 30 min          | 19.8 nmoles/mg protein        |

*All experiments contained oligomycin, 2.5 μg per ml; chlorotetracycline, 25 μM; and Tris-chloride, 20 mM. The protein concentration in all experiments was 1.32 mg per ml.*

shown on the lower portion of Fig. 6. The magnesium content decreased from 22.9 nmoles per mg of protein in the untreated state to 17.3 nmoles per mg of protein following energization with substrate and returned to 20.4 nmoles per mg of protein following de-energization, indicating a reasonable correspondence of the directional change of the chlorotetracycline fluorescence with the level of magnesium in the ETPH-Mg2+ particles. As long as the time of the incubation ranged in length from 5 to 10 min, the changes in magnesium levels were on the order of 20 to 30% of the total magnesium even though the total magnesium content of the particles ranged from 20 to 35 nmoles of magnesium per mg of protein. When the time of incubation was increased to 30 min, the data represented in Table II were obtained. Incubation of the ETPH-Mg2+ particles in a sucrose-Tris medium for 30 min without additions resulted in a loss of very little magnesium, e.g. the control value for magnesium prior to incubation of the ETPH-Mg2+ particles. Addition of antimycin A in the presence of succinate substantially decreased the release of magnesium. In the final experiment described in Table II, a release or leaking of magnesium in the KCl-Tris medium was also observed as a gradual fluorescence decrease of the chlorotetracycline-treated particles.

The experiment described in Fig. 7 shows that the amount of chlorotetracycline which was bound to ETPH-Mg2+ particles under energized and unenergized conditions, e.g. plus and minus succinate, was considerably different. This observation supports the contention that the amount of magnesium residing in the membranes determined the amount of the chelate probe which was bound or retained by the particle. This chlorotetracycline-binding experiment was performed in two ways. First, the data shown in Fig. 7 were obtained in an experiment in which chlorotetracycline was added to two individual series of incubation mixtures at different particle concentrations; one series contained succinate (energized), and the other series contained no succinate (unenergized). Samples were withdrawn from each series to determine the extent of binding of chlorotetracycline. Nearly identical data were obtained when chlorotetracycline was added to a single series of incubation mixtures at different particle concentrations. Samples to determine the extent of chlorotetracycline binding were withdrawn prior to energization and again following addition of succinate. This latter experiment indicated that the unenergized particles with their magnesium component intact bound relatively large amounts of chlorotetracycline. Upon energization both magnesium and the chelate probe were released from the particles. It is tempting to conclude from this data that the decrease in chlorotetracycline-associated fluorescence observed upon energization of ETPH-Mg2+ particles was due to the release of both magnesium and the chelate probe from the lipophilic environment of the membrane.

The final consideration of interest in this study of the relationship between the energetic status of submitochondrial membranes and the magnesium-binding properties of the membrane was the accessibility of the bound magnesium to various types of divalent metal ion chelators. The fluorescence trace denoted A in Fig. 8 demonstrates that the addition of the polar chelator, EDTA, did not affect the chlorotetracycline-associated fluorescence and, it may be presumed, EDTA treatment did not result in the release of magnesium from the particles. On the other hand, the addition of a lipophilic, divalent metal chelator such as o-phenanthroline caused an extensive decrease in the chlorotetracycline-associated fluorescence, indicating that the o-phenanthroline had access to the magnesium pool associated with the chelate probe, chlorotetracycline. Addition of succinate to the particles following o-phenanthroline treatment did not result in a further decrease in fluorescence. When o-phenanthroline was added in the presence of EDTA the fluorescence decrease was even more extensive, indicating the possibility that the inclusion of o-phenanthroline made magnesium available to the EDTA. The fluorescence trace B of Fig. 8 indicates that the addition of o-phenanthroline following energization of the particles with succinate caused a slight but transient increase in the fluorescence suggestive of de-energization. When the particles were treated with antimycin A, the characteristic increase in chlorotetracycline fluorescence previously observed upon de-energization was not observed (see Fig. 3). This experiment suggests that the reassociation of magnesium to the submitochondrial particle membranes following de-energization was prevented by a chelator which had
access to the magnesium pool capable of reassociating with the membranes. Addition of large amounts of EDTA following energization of the particles with succinate (not shown) had no apparent effect on the fluorescence trace.

Fig. 5C indicates that the addition of the nonchelating analogue of o-phenanthroline, m-phenanthroline, following energization with succinate, caused a rapid and extensive increase in fluorescence suggestive of a de-energization of the membranes. Addition of antimycin A following m-phenanthroline did not increase further the chlorotetracycline fluorescence, confirming the suggested de-energization by m-phenanthroline. Addition of m-phenanthroline prior to succinate (not shown) caused no change in the fluorescence associated with the chelate probe. The experiments described in Fig. 9 merely confirm the suggested de-energizing effects of both o- and m-phenanthroline as measured by the fluorescence changes in ANS-treated particles. It is interesting to note the dual effects of the lipophilic chelator, o-phenanthroline, on this system. On one hand, o-phenanthroline seemed to have access as a chelator to the membrane-associated magnesium and on the other hand, o phenanthroline acted as an uncoupler or de-energizer of the membrane, a process which has been shown to lead to a reassociation of magnesium with the membrane.

Discussion

The divalent metal ion, magnesium, serves as an essential constituent of a variety of enzymatic reactions (see review by Mildvan (22)). One crucial but poorly defined facet of cellular metabolism is whether the ratio of bound to free magnesium may be involved in the regulation of various metabolic pathways. An implication that magnesium serves as a regulator of certain enzymatic steps requires documentation that the level of available magnesium changes over a reasonable concentration range in a specific cellular compartment during or preceding a regulatory event. Since inorganic metal ions, unlike other types of effector molecules, are not readily synthesized or interconverted in biological systems, three plausible mechanisms may be postulated for the alteration of cellular or compartmentalized magnesium concentrations. (a) Changes in the concentration of magnesium might be caused by an increased flux of magnesium into or out of the tissue, depending upon the concentration of magnesium in the blood. (b) Changes in the magnesium concentration of a cell or of subcellular organelles may result from selective changes in the permeability of the cellular membranes to magnesium. (c) Changes in the free magnesium level may result from alterations in the binding or release of magnesium from binding site(s) within each cell.

It is unlikely that changes in the tissue concentration of magnesium result from alterations in the magnesium level of the perfusing blood. Various studies of magnesium deficiency states have shown that deprivation of dietary magnesium in various species results in a decrease in the blood level of magnesium to as little as 20% of the normal level over a prolonged period without significant changes in the magnesium content of heart, liver, kidney, or other soft or muscular tissues (23).

That the transport of magnesium from one cellular compartment to another, e.g., from the cytosol to the mitochondrion, may represent a reasonable mechanism for the alteration of compartmentalized magnesium levels was suggested from the studies of Brerley et al. (24). These authors demonstrated an energy-dependent accumulation of magnesium and inorganic phosphate by isolated beef heart mitochondria. Also, it was shown that the accumulation (25-27) and exchange (28) of magnesium in liver mitochondria were energy-dependent processes. The critical problem concerning these magnesium uptake studies is the uncertainty of whether or not magnesium uptake can be equated with membrane binding or whether an actual change in the level of intramitochondrial free magnesium occurred during the energized uptake of magnesium. A technique is needed which can independently monitor differences in the distribution of magnesium between its free and bound forms in various membranous systems. Rose (29) proposed an approach to this problem utilizing the dependence of the adenylyl kinase equilibrium on the concentration of free magnesium in its environment. He demonstrated that the free magnesium concentration of intact or lysed erythrocytes was...
sufficiently low to indicate that a significant proportion of the ATP and ADP of the cells was in the uncomplexed form. In addition, the sum of the magnesium bound to nucleotides and that which was free only equaled half of the total magnesium of the cell. The question of the nature of the ligand responsible for the binding of the remaining half of the cellular magnesium remains unanswered; however, that the erythrocyte membrane may possess binding sites for magnesium has not been either demonstrated or refuted. The procedure suggested by Rose (29) has not been effectively extended to studies on the distribution of magnesium in mitochondrial systems.

The physical distribution of magnesium in the various compartments of liver and heart mitochondria was reported by Bogucka and Wojtczak (30). These authors suggested that approximately 90% of the mitochondrial magnesium was contained in the intermembrane or matrix compartments. The membrane fractionation procedures used in these studies may have obscured the actual distribution of magnesium between the membranes and soluble compartments.

Our previous studies have indicated that the "mobilization" of magnesium from within isolated cardiac mitochondria in the presence of elevated ATP levels caused a nearly complete inhibition of pyruvate oxidation (7, 8). This mobilization or release of intramitochondrial magnesium was effected by the incubation of the mitochondria with uncouplers and ADP and was originally reported by Kun and his associates (31-33). Lee et al. (34) have postulated that the origin of the magnesium released from the mitochondria upon incubation with dinitrophenol plus ADP originated as magnesium bound to the inner mitochondrial membrane without presenting data to this effect. This release of magnesium from the mitochondria may be prevented by the inclusion of a cytoplasmic metabolic factor in the mitochondrial incubation.

The present study was initiated to characterize processes which may be involved in the alteration of intramitochondrial magnesium levels under various metabolic conditions. Submitochondrial particle membranes prepared in the presence of magnesium were shown to release significant amounts of magnesium upon energization of the membranes either by adding an oxidizable substrate or ATP. Our studies also demonstrate that this magnesium release from the particles was either prevented or completely reversed upon the inclusion in the incubation mixture of uncouplers or inhibitors of the respiratory process. The dynamic dissociation-reassociation of magnesium from the submitochondrial particles implies that the mitochondrial membrane possesses a binding capacity for magnesium which can be regulated by the energetic status of the membrane.

An important consideration in the present study is the justification of the use of the fluorescent chelate probe, chlorotetracycline, as an accurate indicator of membrane-associated magnesium. Evidence was presented demonstrating that both the initial level of chlorotetracycline-associated fluorescence and the changes in this fluorescence upon energization were consistent in both degree and direction to the change in the actual magnesium content of the submitochondrial particles during an experiment (see Tables I and II and Figs. 4 and 6). In addition, evidence was presented indicating that the observed changes in chlorotetracycline-associated fluorescence were distinct from energy-dependent changes in the conformation of the mitochondrial membranes. Fig. 3 indicated that the direction of the changes in chlorotetracycline fluorescence upon energization and subsequent de-energization were opposite to the changes in fluorescence seen with ANS. That opposite changes in the polarity of the membrane may occur around the binding sites for chlorotetracycline and for ANS, seems unlikely relative to the distribution of these effects by Radka and Vanderkooi (35) and Azzi and Santato (36). On the basis of our data it is probable that the two membrane probes measure independent variables. Again, it should be noted that the baseline fluorescence level and the energy-dependent changes in fluorescence associated with chlorotetracycline correlated well with the magnesium content of the particles while the ANS-associated fluorescence showed no such correlation (see Fig. 3B and Table I). Further evidence indicating that chlorotetracycline is not a probe for membrane energization may be seen in the data presented in Figs. 8 and 9. The addition of the chelator, o-phenanthroline, caused a decrease in chlorotetracycline fluorescence which was nearly identical with the fluorescence change observed upon energization with substrate, yet Fig. 9 demonstrates that o-phenanthroline resulted in membrane energization as monitored by ANS fluorescence changes.

It is important to consider the question of whether chlorotetracycline treatment represents a significant perturbation of the magnesium association with the submitochondrial particle membranes. That the presence of chlorotetracycline was not necessary for the energy-dependent release of magnesium from the membranes was indicated by two lines of evidence. First, the experiments regarding the reversibility of the magnesium release shown in Fig. 6 and the effects of substrate and antimycin addition on membrane magnesium content during a 30-min incubation seen in Table II were performed both in the presence and absence of chlorotetracycline with nearly identical results. Second, it was shown in Fig. 7 that chlorotetracycline was not retained or bound by the particles if the mitochondrial membranes were depleted of magnesium by energization with succinate prior to the addition of the chelate probe. In addition, it was demonstrated in Fig. 5 that chlorotetracycline was not retained by submitochondrial particles which were prepared under conditions which depleted them of magnesium, e.g. ATP$_{i}$-EDTA. These data clearly indicate that the processes of release and uptake of magnesium by the mitochondrial membranes were independent of the presence of chlorotetracycline. It is not apparent at this time whether the state of intramembrane magnesium is altered upon introduction of the chelate probe into the membrane.

Whether the metabolic energy-generating functions of cellular systems are regulated by the ratio of free to bound magnesium in a rapid, reversible fashion remains to be established. Initial questions regarding the "state" or "set" of the intracellular free magnesium must be considered under a variety of metabolic conditions before any definitive statements can be made. However, it seems reasonable from our studies that a significant pool of magnesium is associated with the mitochondrial membrane which may be alternately released or bound depending upon the energetic state of the membrane. Further investigations will be designed to elucidate enzymatic processes which may be regulated by alterations in the flux of magnesium from binding sites in or on the mitochondrial membrane.

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