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Magnesium Transport by Mitochondria

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The pathways for the uptake and extrusion of Mg^{2+} by mitochondria are not well defined. the present evidence suggests that uptake occurs by nonspecific diffusive pathways in response to elevated membrane potential. There is disagreement as to some of the properties of Mg^{2+} efflux from mitochondria, but the reaction resembles K^+ efflux in many ways and may occur in exchange for H^+. Matrix free magnesium ion concentration, $[Mg^{2+}]$, can be measured using fluorescent probes and is set very close to cytosol $[Mg^{2+}]$ by a balance between influx and efflux and by the availability of ligands, such as P_i. There are indications that matrix $[Mg^{2+}]$ may be under hormonal control and that it contributes to the regulation of mitochondrial metabolism and transport reactions.

INTRODUCTION

Mitochondria can both take up and extrude Mg^{2+} by respiration-dependent reactions. Although the massive respiration-dependent accumulation of Mg^{2+} by heart mitochondria was reported over 30 years ago (Brierley *et al.*, 1962, 1963) and respiration-dependent extrusion of Mg^{2+} was first described in 1976 (Crompton *et al.*, 1976), the pathways for uptake and release of Mg^{2+} by mitochondria remain obscure. Interest in these reactions has been renewed by recent reports that mitochondrial Mg^{2+}, as well as total cell Mg^{2+}, may be altered rapidly and reversibly in response to hormone signals (Romani and Scarpa, 1992; Romani *et al.*, 1991, 1993a,b). There are many indications that the concentration of free magnesium ions, designated $[Mg^{2+}]$ throughout this discussion, may affect cellular and mitochondrial enzymes and transporters and that both cycosol $[Mg^{2+}]$ and matrix $[Mg^{2+}]$ may be important regulatory components in the cell (see Murphy *et al.*, 1991, White and Hartzell, 1989, or Romani and Scarpa, 1992, for reviews). Changes in $[Mg^{2+}]$ in either or both compartments in response to hormone messages would constitute powerful evidence for such a regulatory role for $[Mg^{2+}]$ (see Maguire, 1990). The present brief review summarizes the current state of work on mitochondrial Mg^{2+} transport and the evaluation of matrix $[Mg^{2+}]$. Collections of reviews on various aspects of Mg^{2+} metabolism can be found in recent volumes edited by Birch (1993) and Sigel and Sigel (1990). The transport of Mg^{2+} by mitochondria was last reviewed by Diwan (1988).

Mg^{2+} CHEMISTRY AND Mg^{2+} TRANSPORT

The chemical properties of Mg^{2+} present challenges to transport systems that are not encountered with Na^+, K^+, or Ca^{2+} (Maguire, 1993). The Mg^{2+} ion is small and has a high charge density at its surface that results in a very large hydrated cation. The small Mg^{2+} accommodates six oxygen ligands to form a stable $Mg(H_2O)_6^{2+}$ hydrate. These water molecules exchange very slowly with bulk water with exchange rates three orders of magnitude less than those for Ca^{2+}, K^+, or Na^+. The hydrated Mg^{2+} (the form encountered by membrane transport systems) has 350 to 400 times the volume of the unhydrated ion as opposed to comparable values of 5- to 35-fold for the hydrated to unhydrated forms of Ca^{2+}, Na^+ and

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K^+ (Maguire, 1993). A hydrated cation can readily move through a large pore, such as the PTP[2] which is 2–3 nm in diameter, but to pass through a smaller pore or to interact with a mobile carrier, at least some of the water of hydration must be replaced by groups from the transporter. Although hydration–dehydration reactions may be faster in channels than in free solution (Hille, 1984), the slow rate of removal of water of hydration is the major rate-limiting factor controlling the ability of Mg^{2+} to react with membrane transport systems (Williams, 1993). For a cation to traverse even in a short pore, several water molecules must be removed and replaced with pore ligands. In the case of Mg^{2+} this process could result in slow movement of the cation and inhibition of the flux of other ions through the pore (Flatman, 1993). Accordingly, Mg^{2+} transport systems must recognize and coordinate the binding of a hydrated ion that is seven times the radius and 370 times the volume of the unhydrated ion and also accommodate a slow ligand substitution rate during transport.

It is therefore likely that transport systems for Mg^{2+} will provide unusual and distinctive approaches to membrane transport (Maguire, 1993). The initial binding site for Mg^{2+} on such a transporter is likely to be larger and more highly charged than a corresponding site for Ca^{2+}, and the diameter of the pore may be smaller than those for other cations. Maguire (1993) has described high-affinity ATP-dependent Mg^{2+} transport systems found in bacteria that discriminate well between Ca^{2+} and Mg^{2+}. This is in contrast to most available probes and chelators. Romani *et al.* (1993a,b) have reported that hepatocytes and cardiac myocytes can take up Mg^{2+} from a low $[Mg^{2+}]$ medium containing high $[Ca^{2+}]$ when protein kinase C is activated by carbachol or other reagents. This would indicate that high-affinity transporters that discriminate between Mg^{2+} and Ca^{2+} are not only present in these cells, but subject to hormone regulation.

METHODS FOR THE STUDY OF MITOCHONDRIAL Mg^{2+} TRANSPORT

An adequate analysis of a transport mechanism requires the determination of both unidirectional transport flux and the ionic concentration gradients that drive or maintain transport. Transporters for Mg^{2+} respond to $[Mg^{2+}]$, rather than the total Mg^{2+} present, and our limited understanding of mitochondrial Mg^{2+} transport has been blamed on the paucity of methods for the estimation of $[Mg^{2+}]$. The fluorescent probes that are now becoming available seem to offer an attractive solution to this dilemma, but they are not without their own problems. More extensive discussions of methods available for estimation of total Mg^{2+} and $[Mg^{2+}]$ can be found in reviews by London (1991), Romani and Scarpa (1992), Mota de Freitas and Dorus (1993), or McGuigan *et al.* (1993).

The uptake and release of Mg^{2+} by isolated mitochondria is readily followed by atomic absorption-spectroscopy after separation of mitochondria from the suspending medium by centrifugation or filtration. This method is quite specific and gives reliable estimates of total Mg^{2+}. Where small differences in mitochondrial Mg^{2+} are seen, it is essential to analyze both pellet and supernate and to maintain constant ionic background in all samples. It is possible to calculate $[Mg^{2+}]$ from total Mg^{2+} data using Mg-dependent enzyme equilibria, such as the citrate/isocitrate ratio, if estimates of these components are available (Veloso *et al.*, 1973).

Use of $^{28}Mg^{2+}$

A major obstacle in studying Mg^{2+} transport is the lack of a readily available and affordable Mg^{2+} isotope (Grubbs *et al.*, 1989). $^{28}Mg^{2+}$ is the only isotope usable for measuring Mg^{2+} fluxes. It emits high-energy electrons and gamma radiation and can be detected by Cerenkov counting. However, it has only a 21.3 h half-life and is expensive. $^{28}Mg^{2+}$ may be considered an "orphan" isotope and is available in the U.S. only from Brookhaven National Laboratories where current policies dictate that it must be produced on a cost effective basis. At this writing the isotope costs $700 per 50 μCi unit, but is produced only when a minimum of 6 to 8 units is ordered. Brookhaven Laboratories produced no $^{28}Mg^{2+}$ in 1993. The University of Munich is also a possible source for $^{28}Mg^{2+}$ (see Grubbs *et al.*, 1989).

Fluorescent Probes

The first fluorescent probes used to measure $[Mg^{2+}]$ were derivatives of APTRA (*o*-aminophenol-*N*,*N*,*O*-triacetate), an EDTA analogue, modified to

[2] Abbreviations used: $[Mg^{2+}]$, concentration of free magnesium ion; PTP, the mitochondrial permeability transition pore; AM, acetoxymethyl esters of fluorescent probes; $\Delta\Psi$, mitochondrial membrane potential.

produce fluorescent molecules (Raju *et al.*, 1989; London, 1991). Although called "magnesium probes", these indicators all show considerably higher affinity for $[Ca^{2+}]$ than for $[Mg^{2+}]$ (Raju *et al.*, 1989; Haugland, 1992). Currently available probes with apparent K_d values for Mg^{2+} and Ca^{2+}, respectively (Haugland, 1992), include furaptra or mag-fura-2 (1.5 mM, 53 μM), mag-fura-5 (2.6 mM, 25 μM), mag-indo-1 (2.7 mM, 23 μM), Magnesium Green (0.9 mM, 4.8 μM), Magnesium Orange (3.5 mM, 12 μM), and Magnesium-Fura-Red (4.6 mM, 17 μM). The high Ca^{2+} affinity of these probes presents a problem because Ca^{2+} uptake, even from low extramitochondrial $[Ca^{2+}]$ media, results in matrix $[Ca^{2+}]$ above 1 μM. This may cause fluorescence changes that could be mistaken for $[Mg^{2+}]$. Ideally, a parallel estimation of $[Ca^{2+}]$ with a Ca^{2+}-specific probe would allow a separation of $[Mg^{2+}]$ movements from those of $[Ca^{2+}]$ (London, 1991). We have loaded two probes (fura-2 and cSNARF-1) into mitochondria and measured matrix $[Ca^{2+}]$ and pH simultaneously (Baysal *et al.*, 1994), so it should be possible to follow $[Mg^{2+}]$ and $[Ca^{2+}]$ at the same time using complementary short- and long-wavelength probes.

The fluorescent probes are available as membrane-permeable AM esters. The esters diffuse into the mitochondrial matrix where they are hydrolyzed to the highly charged and nonpermeant free acids by endogenous esterases (Jung *et al.*, 1989). Isolated heart mitochondria are readily equilibrated with furaptra and mag-indo-1 (Jung *et al.*, 1990; Rutter *et al.*, 1990), but liver mitochondria are more difficult to load with such probes (Gunter *et al.* 1988). We have also found mag-fura-5 and Magnesium Green to be satisfactory probes for $[Mg^{2+}]$ in heart mitochondria. The latter cannot be ratioed, but holds promise for $[Mg^{2+}]$ estimation by fluorescence lifetime measurements (Szmacinski and Lakowicz, 1993).

When fluorescent probes such as furaptra are equilibrated into intact cells, as the AM esters, a significant portion of the probe is localized in the mitochondria matrix compartment (Davis *et al.*, 1987; Silverman *et al.*, 1994). This along with the response of the probe to changes in mitochondrial $[Ca^{2+}]$, as well as $[Mg^{2+}]$, may have resulted in misleading fluorescence responses and inappropriate interpretation of $[Mg^{2+}]$ changes in the cytosol in some of the studies available in the literature. However, Silverman *et al.* (1994) did not see evidence of a major $[Ca^{2+}]$ artefact in myocytes in which $[Mg^{2+}]$ was estimated using mag-indo-1 fluorescence. Techniques such as confocal microscopy (Chacon *et al.*, 1994, for example) may be useful in resolving the problem of mitochondrial $[Mg^{2+}]$ analysis in intact cells in the future.

ESTIMATION OF $[Mg^{2+}]$ BY NUCLEAR MAGNETIC RESONANCE

Cellular $[Mg^{2+}]$ has been estimated by chemical shift differences between the β and α resonances of ATP (Gupta *et al.*, 1984), and matrix ATP is visible in ^{31}P-NMR scans of suspensions of isolated mitochondria (Masiakos *et al.*, 1991). This suggests that matrix $[Mg^{2+}]$ could be estimated by ^{31}P-NMR. However, over 90% of mitochondrial ATP appears to be bound to Mg^{2+} (Hutson *et al.*, 1989) and, if matrix $[Mg^{2+}]$ is between 0.5 and 1.0 mM as other estimates suggest, the apparent K_d of 50 μM for the Mg-ATP complex means that such estimates will be at the upper limit of sensitivity. Because this approach uses endogenous ATP as the $[Mg^{2+}]$ indicator, it requires very concentrated suspensions of mitochondria in which it is difficult to maintain a given metabolic condition. The ^{19}F-derivatives of APTRA that are available (London, 1991) may be more useful in this regard because analogous probes load well into heart mitochondria as the AM esters. These fluorinated probes have K_d values for $[Mg^{2+}]$ in the physiological range (0.6–3.1 mM) and sufficient sensitivity to require less dense mitochondrial suspensions (London, 1991; Levy *et al.*, 1988). The ^{19}F-NMR probes do not appear to have been used with mitochondria as yet, however.

Additional methods applicable to mitochondria Mg^{2+} transport studies include electron probe x-ray microanalysis, used to measure changes in total Mg in intact tissues (Bond *et al.*, 1987); metallochromic indicators that undergo absorbance change on Mg^{2+} binding (Scarpa, 1979); and $[Mg^{2+}]$-selective electrodes (McGuigan *et al.*, 1993) that may be used to follow changes in $[Mg^{2+}]$ in the extramitochondrial medium.

ESTIMATION OF MATRIX $[Mg^{2+}]$

Corkey *et al.* (1986) used a null-point procedure to estimate the matrix $[Mg^{2+}]$ of isolated liver mitochondria at 0.35 ± 0.01 mM. Jung and Brierley (1986), using a similar approach, found that matrix

$[Mg^{2+}]$ increased linearly by about 20 μM for each nmol total Mg^{2+} mg^{-1} protein in the range from 10 to 50 nmol · mg^{-1}. Below 10 nmol · mg^{-1}, free $[Mg^{2+}]$ changed very little with changing total Mg^{2+}.

More recently, Jung *et al.* (1990) and Rutter *et al.* (1990) have used furaptra and mag-indo-1 to follow changes in matrix $[Mg^{2+}]$. These studies agree that matrix $[Mg^{2+}]$ increases or decreases in parallel with changes in total mitochondrial Mg^{2+}. There are also indications that matrix $[Mg^{2+}]$ changes with ligand availability (Jung *et al.*, 1990). Matrix $[Mg^{2+}]$ decreased when P_i was equilibrated into the matrix and increased in parallel with state 3 respiration when ADP was added to respiring mitochondria. However, the two studies differ in their estimates of the matrix $[Mg^{2+}]$ maintained by respiring heart mitochondria. Rutter *et al.* (1990) placed this value between 0.8 and 1.5 mM, whereas the study from our laboratory concluded that matrix $[Mg^{2+}]$ averaged 0.5 mM (Jung *et al.*, 1990).

These fluorescent probes are well suited for following changes in matrix $[Mg^{2+}]$, but calculation of the absolute value for matrix $[Mg^{2+}]$ involves a number of uncertainties. The excitation spectrum of matrix sequestered furaptra is strongly quenched in the 340 nm region when it reacts with Mg^{2+} (Jung and Brierley, 1992). This suggests that the probe may interact with mitochondrial components or self-associate at the high concentrations (200 μM) seen in the mitochondrion. Self-association of the analogous probe mag-indo-1 at concentrations above 10 μM has been demonstrated (Morelle *et al.*, 1994). There are also indications that ionophores, such as BrA23187 or ionomycin, may not completely equilibrate the matrix with external $[Mg^{2+}]$ (Raju *et al.*, 1989; Jung and Brierley, 1992; but see Silverman *et al.*, 1994) and this may compromise *in situ* calibration of the probe. Lastly there is the issue of the apparent K_d for the Mg-furaptra complex in the matrix environment. Jung *et al.* (1990) used the value of 1.5 mM reported by Raju *et al.* (1989) to calculate matrix $[Mg^{2+}]$ by a ratioing procedure taking the fluorescence seen after massive Ca^{2+} accumulation as the value for R_{max}. In contrast, Rutter *et al.* (1990) evaluated the apparent K_d of matrix-sequestered furaptra from fluorescence intensity using ionophores. The differences in apparent K_d alone can account for much of the discrepancy in matrix $[Mg^{2+}]$ in the two reports (Rutter *et al.*, 1990; Jung and Brierley, 1992).

We have recently re-examined the spectral properties of furaptra and its apparent K_d for Ca^{2+} and Mg^{2+} in aqueous solution and when sequestered in either phospholipid vesciles or the mitochondrial matrix (Jung *et al.*, manuscript in review). The vesicle studies indicate that BrA23187, in combination with nigericin and an uncoupler, equilibrates up to 2 mM $[Ca^{2+}]$ and 10 mM $[Mg^{2+}]$ across the membrane. The apparent K_d of furaptra was found to be temperature dependent (see also Lattanzio and Bartschat, 1991) and to depend on the concentration of probe. The apparent K_d for the K^+ salt of furaptra measured at 25° in dilute aqueous solution (100 nM probe) was found to be 26 μM for $[Ca^{2+}]$ and 2.1 ± 0.1 ($n = 6$) for $[Mg^{2+}]$. Corresponding values for the probe sequestered in phospholipid vesciles at concentrations equivalent to those found in probe-equilibrated mitochondria (200 μM) were 10 μM $[Ca^{2+}]$ and 0.8 mM $[Mg^{2+}]$. The apparent K_d for $[Ca^{2+}]$ for furaptra sequestered in the matrix of heart mitochondria was 34 μM $[Ca^{2+}]$. Direct evaluation of the K_d for Mg-furaptra in the matrix was not possible due to an interference from the ionophores BrA23187 and ionomycin. However, in the absence of the ionophore, increases or decreases in total mitochondrial Mg were accompanied by changes in the excitation spectrum that were indicative of parallel changes in matrix $[Mg^{2+}]$. The excitation spectrum for sequestered Mg-furaptra did not differ from that of Ca-furaptra in the absence of ionophores. It is concluded that it is not possible to obtain a meaningful K_d for matrix-sequestered furaptra using ionophores and that a value of at least 2.1 mM $[Mg^{2+}]$ should be used in such calculations. If the K_d of Mg-furaptra parallels that of Ca-furaptra in the mitochondrial matrix, a value as high as 2.7 mM $[Mg^{2+}]$ can probably be justified in such calculations. These values would put our estimates of matrix $[Mg^{2+}]$ between 0.7 and 0.9 mM $[Mg^{2+}]$ instead of the 0.5 mM originally reported (Jung and Brierley, 1990). Only about 3% of total mitochondrial Mg^{2+} is present as the free ion.

The uptake of Mg^{2+}, like that of other cations, appears to be an electrophoretic response to the mitochondrial $\Delta\psi$. Even though mitochondria take up Mg^{2+} electrophoretically, Mg^{2+} does not come to electrochemical equilibrium across the coupling membrane. Matrix $[Mg^{2+}]$ appears to be maintained at very nearly the same level as cytosol $[Mg^{2+}]$ (Corkey *et al.*, 1986). The flux of $^{28}Mg^{2+}$ across the inner membrane of isolated heart mitochondria appears to be very slow, about 0.25 nmol min^{-1} mg^{-1} at 25° under conditions approaching those encountered *in situ* (Brierley *et al.*, 1987). However, studies

of $^{28}Mg^{2+}$ flux in isolated cardiac myocytes have shown that the specific activity of the mitochondrial fraction is the same as that for whole cells over an extended time range (Altschuld *et al.*, 1994). This would indicate that exchange of $^{28}Mg^{2+}$ between cytosol and matrix is at least as fast as the net uptake of label across the sarcolemma. These observations imply that there is Mg^{2+} uptake by mitochondria *in situ* and that, regardless of the nature of the transport pathway, there must be a mechanism for extrusion of entering Mg^{2+}. It seems likely that matrix $[Mg^{2+}]$ is maintained by a balance between these uptake and extrusion transport pathways, in conjunction with the availability of Mg ligands in the matrix and competition between Mg^{2+}, Ca^{2+}, K^+, and H^+ for potential anionic binding sites.

Mg^{2+} UPTAKE BY MITOCHONDRIA

Heart mitochondria accumulate massive amounts of Mg^{2+} when suspended in sucrose containing P_i and high external $[Mg^{2+}]$ (Brierley *et al.*, 1962, 1963). The reaction requires respiration and is sensitive to uncouplers, but not to oligomycin. Such accumulations reached as high as $1600\,nmol \cdot mg^{-1}$ at 37° as the result of deposition of insoluble phosphate salts in the matrix. A lesser accumulation of Mg^{2+} can be supported by exogenous ATP in an oligomycin-sensitive reaction. It is now recognized that the uptake of Mg^{2+}, like that of Ca^{2+} and monovalent cations, is an electrophoretic response to the $\Delta\psi$ component of the electrochemical $[H^+]$ gradient (Mitchell, 1966, 1968). The uptake of Mg^{2+} is strongly inhibited by addition of ADP to initiate oxidative phosphorylation (Brierley *et al.*, 1963). It appears that the uptake of Mg^{2+} represents an alternative mode of utilization of $\Delta\psi$ and that transport of this cation, unlike Ca^{2+} transport, does not compete well with oxidative phosphorylation.

When P_i is added to respiring mitochondria, its distribution results in a partial conversion of ΔpH to $\Delta\psi$ which would favor the electrophoretic uptake of cations. The rapid electrophoretic uptake of Ca^{2+} under such conditions occurs via the ruthenium red-sensitive Ca^{2+} uniport which provides a specific pathway for this cation (see Gunter and Pfeiffer, 1990, for a review). It is not yet clear whether such a specific transport mechanism is present in the membrane for Mg^{2+} uniport. If such a uniport is present, it must be regulated in such a way that its activity is very limited under most conditions. The uptake of Mg^{2+} shows saturation kinetics (Diwan *et al.*, 1979), but has no distinctive inhibitor profile that suggests a specific transport pathway. The respiration-dependent uptake of Mg^{2+} by heart mitochondria is not inhibited by ruthenium red (Crompton *et al.*, 1976; Brierley *et al.*, 1987), so it appears that Mg^{2+} does not enter the matrix via the Ca^{2+}-uniport.

The electrophoretic permeability of the mitochondrial membrane to H^+ and monovalent cations shows a nonohmic increase with increasing $\Delta\psi$ (Brown and Brand, 1986). It seems possible that Mg^{2+} uptake may result from a diffusive leak in response to the increase in $\Delta\psi$ produced by P_i. The uptake of Mg^{2+} is preceded by binding of the cation to sites on the membrane surface in nonionic media, such as sucrose (Schuster and Olson, 1974; Brierley *et al.*, 1987). This surface binding places Mg^{2+} in a position to move through the membrane readily when $\Delta\psi$ increases. Both surface binding and Mg^{2+} uptake are inhibited by K^+ and other monovalent cations (Brierley *et al.*, 1963; 1987). This inhibition by ionic media would be in line with Mg^{2+} uptake by diffusive leaks, as would the rather nonspecific inhibitor profile. The uptake of Mg^{2+} is not inhibited by diltiazem, is only partially sensitive to dicyclohexylcarbodiimide, and is inhibited by Ba^{2+} when this cation has access to the matrix. The uptake of Mg^{2+} is activated by Zn^{2+}, Cd^{2+}, and mercurials (Brierley *et al.*, 1967) and these reagents seem to increase the non-specific permeability of the membrane. Diwan (1988) has pointed out the close similarity of the inhibition and activation profiles for Mg^{2+} uptake and K^+ influx.

We have recently seen large increases in both total Mg^{2+} and matrix $[Mg^{2+}]$ when heart mitochondria suspended in a sucrose median containing 1 mM $[Mg^{2+}]$ are treated with nigericin. Under these conditions nigericin converts ΔpH to $\Delta\psi$ with the loss of endogenous K^+ by K^+/H^+ exchange. The rapid uptake of Mg^{2+} in response to nigericin is inhibited by K^+ (at concentrations that do not affect $\Delta\psi$) and other monovalent cations and by spermine. Ruthenium red, quinine, and DCCD produce only a partial inhibition of this reaction. These observations, like those noted above, are quite consistent with Mg^{2+} entry into the mitochondrion by diffusive leak pathways (Jung *et al.*, manuscript in preparation).

Rat liver mitochondria show respiration-dependent Mg^{2+} uptake, but the rate and extent of accumulation are small relative to the reaction in heart mitochondria (Diwan, 1988). Unidirectional Mg^{2+} fluxes have

been measured with $^{28}Mg^{2+}$ in liver mitochondria (Diwan *et al.*, 1979) and both influx and efflux shown to the respiration dependent. Influx shows saturation kinetics with a K_m of 0.7 mM in a sucrose medium and is inhibited competitively by Tl^+.

An alternative pathway for Mg^{2+} uptake, the $Mg^{2+}ATP^{4-}/P_i^{2-}$ antiport, is available in mitochondria from some tissues, but does not appear to be present in heart (Aprille, 1993). This pathway is electroneutral and reversible, derived by the substrate gradients, activated by $[Ca^{2+}]$, and may be involved in the modulation of the adenine nucleotide content in response to hormonal stimulation. It is a specific transport mechanism separate from the adenine nucleotide translocase since it is not inhibited by atractyloside, but is inhibited by trifluoperazine (Aprille, 1993; Nosek *et al.*, 1990). Although this pathway moves Mg^{2+} across the mitochondrial membrane, it would be expected to have little effect on $[Mg^{2+}]$ because it is cotransported with the ATP^{4-} ligand.

EFFLUX OF Mg^{2+} FROM MITOCHONDRIA

It is now well established that isolated heart mitochondria can extrude Mg^{2+} in a respiration-dependent reaction (Crompton *et al.*, 1976; Akerman, 1981; Brierley *et al.*, 1987, 1988). This extrusion of endogenous Mg^{2+} is sensitive to uncouplers and amounts to 10 nmol Mg^{2+} mg^{-1}, or less, of the 30 nmol · mg^{-1} normally found in heart miotchondria. Unlike the Mg^{2+} uptake reaction, Mg^{2+} efflux is quite insensitive to the composition of the external medium and otherwise shows an inhibitor profile markedly different from that of the Mg^{2+} accumulation reaction (Brierley *et al.*, 1987). This suggests that mitochondria have separate pathways available for Mg uptake and release. Since the $Mg^{2+}ATP^{4-}/P_i^{2-}$ mechanism operates in both directions (Aprille, 1993) it could possibly mediate P_i-dependent Mg^{2+} efflux.

The amount of respiration-dependent loss is more variable from preparation to preparation than most mitochondrial transport reactions There is also considerable difference in the reaction properties reported by various groups. Some of the difficulties appear to result from the need to correct respiration-dependent Mg^{2+} efflux for the contribution of passive efflux pathways. In other cases it appears that conditions have ben established that result in opening of the PTP with nonspecific loss of matrix Mg^{2+}.

Crompton *et al.* (1976) reported that net efflux of 30–40% of the endogenous Mg^{2+} occurred when external $[Mg^{2+}]$ was less than 2.5 mM and that net uptake was seen with higher $[Mg^{2+}]$ in the suspending medium. A similar balance point between influx and efflux was found in our later study (Brierley *et al.*, 1987) although it is clear that the balance point is highly dependent on the composition of the suspending medium. Akerman (1981) showed that valinomycin stimulated Mg^{2+} extrusion from heart mitochondria and that nigericin prevented the reaction. This was taken as evidence that efflux depends on the ΔpH component of protonmotive force. There is also evidence to suggest that Mg^{2+} efflux may occur via the K^+/H^+ antiport (Diwan, 1988; Brierley *et al.*, 1988).

Crompton *et al.* (1976) suggested that P_i may be necessary for respiration-dependent Mg^{2+} efflux. They found efflux to be inhibited by ADP, but the inhibition could be removed by inclusion of P_i or addition of P_i after several minutes of incubation. Oligomycin and atractyloside prevented but did not reverse the inhibition by ADP, and ADP inhibition was ascribed to its ability to remove endogenous P_i by ATP formation. However, Brierley *et al.* (1987) found inhibition of Mg^{2+} efflux by ADP in the presence or absence of P_i that was largely prevented by oligomycin. This pattern was also seen in a more recent series of determinations (Altschuld *et al.*, 1994). In contrast, Romani *et al.* (1991) have reported that ADP stimulates the loss of Mg^{2+} from isolated liver mitochondria respiring in the presence of P_i and that the stimulation is prevented by the inhibitors of adenine nucleotide transport atractyloside and bonkrekic acid. The ADP responses parallel those to cAMP in liver mitochondria and digitonin-permeabilized hepatocytes (Romani *et al.*, 1991; Romani and Scarpa, 1992). However, a recent study failed to replicate these results (Altschuld *et al.*, 1994), and the basis for the discrepancy has not yet been resolved. An enhanced loss of mitochondria Mg^{2+} in the presence of P_i was confirmed by both groups (Romani *et al.*, 1991; Altschuld *et al.*, 1994). It seems likely that unrecognized factors, such as the hormonal status of the animal prior to mitochondrial isolation, other cytosolic regulatory factors, or the redox status of membrane thiols, could be responsible for the different observations. It is clear that further work will be necessary to resolve these issues.

Masini *et al.* (1983) have reported that liver

mitochondria extrude as much as 10 nmol $Mg^{2+} \cdot mg^{-1}$ when ADP is added to initiate state 3 respiration and that the extruded Mg^{2+} is reaccumulated when state 4 is resumed. These observations have not been replicated or adequately explained. We have suggested that the apparent Mg^{2+} movements are artefacts due to volume changes in liver mitochondria during these transitions (Brierley *et al.*, 1987), but this remains to be established.

Several reports indicate that Mg^{2+} movements in liver (Kun, 1976; Siliprandi *et al.*, 1978) or brain mitochondria (Rugolo and Zoccarato, 1984) are sensitive to ruthenium red and related to Ca^{2+} cycling. These reports now seem best explained in terms of Ca^{2+} effects on the PTP (Gunter and Pfeiffer, 1990). Mg^{2+} released from mitochondria that have undergone the transition would not be taken up readily by competent mitochondria, whereas Ca^{2+} would be reaccumulated and the cycle would continue until Mg^{2+} loss from the population was complete. This would account for the differential release of Ca^{2+} and Mg^{2+} seen during Ca^{2+} cycling. There are indications that the PTP may operate in mitochondria *in situ* (Altschuld *et al.*, 1992). If this is indeed the case, the opening and closing of the pore could provide a pathway for equilibration of Mg^{2+} and K^+ between matrix and cytosol (see the discussion in Gunter and Pfeiffer, 1990).

DOES $[Mg^{2+}]$ REGULATE MITOCHONDRIAL REACTIONS?

The available methodology indicates that both cytosol and matrix $[Mg^{2+}]$ are set between 0.5 and 1 mM in most cells and that there is little concentration gradient maintained between the two compartments. These levels of $[Mg^{2+}]$ are in the appropriate range to regulate many enzymes and transporters *in situ*, but clear evidence that $[Mg^{2+}]$ actually functions in such a role is lacking. To establish that matrix $[Mg^{2+}]$ regulates matrix enzymes or membrane components on the matrix aspect of the inner membrane, one must show that the reactions are sensitive to $[Mg^{2+}]$, that $[Mg^{2+}]$ is varied through the sensitive range by transport reactions, and that changes in $[Mg^{2+}]$ and the Mg^{2+}-sensitive process occur in a sequential and integrated way (Maguire, 1990). Because the intermembrane space appears to equilibrate with the cytosol, it is possible for transporters that span the inner membrane to be affected by either cytosol or matrix $[Mg^{2+}]$. The question of whether matrix $[Mg^{2+}]$ can be altered by transport reactions on a time scale consistent with regulatory function remains to be answered.

Electron probe x-ray analysis has shown that mitochondrial Mg^{2+} increases in liver cells when rats are injected with glucagon or vasopressin (Bond *et al.*, 1987). This treatment released Ca^{2+} from the rough endoplasmic reticulum but did not produce significant changes in mitochondria Ca^{2+}. As mentioned above, β-agonists have been reported to induce a net loss in cellular Mg^{2+} from hepatocytes and from perfused liver (Romani *et al.*, 1991). The loss of cellular Mg^{2+} was related to decreases in mitochondrial Mg^{2+} because cAMP induced Mg^{2+} efflux from isolated mitochondria and from digitonin-lysed hepatocytes that maintain an intact mitochondrial population (Romani *et al.*, 1991). Romani *et al.* (1991) propose that cAMP reacts with the mitochondrial adenine nucleotide transporter and changes its substrate specificity so that it permits Mg-ATP efflux. Marfella *et al.* (1994) have recently reported that cAMP binds to the adenine nucleotide transporter with a K_m of 40 nM and that atractyloside inhibits Mg^{2+} efflux without affecting the binding of cAMP. Studies from our laboratory show that Mg^{2+} traffic across the membrane of isolated mitochondria is slow under conditions approaching those *in situ* (Brierley *et al.*, 1987), but this conclusion may have to be modified if the reported mobilization of mitochondrial Mg^{2+} by hormones can be substantiated.

Romani *et al.* (1993a) have also reported a rapid and reversible loss of cellular Mg^{2+} when cardiac myocytes are suspended in a Mg-free medium and challenged with a β-agonist. Altschuld *et al.* (1994) confirmed that there is a loss of cell Mg^{2+} under these conditions, but related the loss to increased myocyte fragility in the Mg-free medium. These authors were also not able to confirm a net uptake of Mg^{2+} in response to carbachol and other activators of protein kinase C reported by Romani *et al.* (1993a,b). However, stimulation of Mg^{2+} uptake by these reagents has been seen in other cell systems (Grubbs and Maguire, 1986; Zhang and Melvin, 1992). A decrease in cellular Mg^{2+} in response to β-agonists has also been reported in other cell systems (Wolf *et al.*, 1994; Gunther and Vorman, 1992, for example). Large increases in heart cell $[Mg^{2+}]$ in response to β-agonists have been seen by ^{31}-P-NMR (Headrick and Willis, 1991), but McGuigan *et al.* (1993) found no change in $[Mg^{2+}]$ in ferret hearts

treated with these reagents. These experimental conflicts need to be resolved before a clear picture of the role of $[Mg^{2+}]$ in metabolic regulation can be drawn.

One of the most specific proposals for the regulation of a mitochondrial reaction by $[Mg^{2+}]$ is the carrier brake hypothesis for the control of the K^+/H^+ antiport (Garlid, 1980). A latent anion uniport is also sensitive to $[Mg^{2+}]$, and these two transporters appear to act in concert to maintain mitochondrial volume homeostasis (Garlid, 1988). It is proposed that as the matrix volume increases, matrix $[Mg^{2+}]$ decreases and Mg^{2+} diffuses from negative regulatory sites to activate the transporters to extrude ions and produce osmotic contraction. As the volume decreases, matrix $[Mg^{2+}]$ would increase and re-equilibrate Mg^{2+} with the regulation sites to inhibit net ion efflux. Jung *et al.* (1990) have shown that matrix $[Mg^{2+}]$ decreases with hypotonic swelling and returns to the original value when the mitochondria are recontracted by KCl addition. However, it has still not been confirmed that matrix $[Mg^{2+}]$ can be varied through the concentration range that affects these transporters under conditions that approach those seen by mitochondria *in situ* (see Maguire's 1990 criteria).

The $[Mg^{2+}]$ of cells and mitochondria appears to be strongly buffered by virtue of the high concentration of total Mg^{2+} and the presence of a variety of binding sites (Corkey *et al.*, 1986). These authors predicted that $[Mg^{2+}]$ would be nearly constant in both matrix and cytosol unless total Mg^{2+} changed. In recent studies (Jung *et al.*, in preparation) we have shown that mitochondria contain 8–10 nmol Mg^{2+} mg^{-1} that is tightly bound and does not appear to contribute to matrix $[Mg^{2+}]$. Tight binding of Mg^{2+} to high-affinity sites in the membrane (Bogucka and Wojtczak, 1971), to the F_0F_1 ATPase (Senior, 1979), and to cytochrome oxidase (Lin *et al.*, 1993) has been reported. Above this range, matrix $[Mg^{2+}]$ determined by furaptra fluorescence shows a linear relationship to total Mg^{2+}, increasing by 60–70 μM for each nmol $\cdot$ mg^{-1} increase in total Mg^{2+}. However, if exogenous P_i is present, both total Mg^{2+} and matrix P_i can increase to very high levels with little further increase in $[Mg^{2+}]$. These considerations emphasize the importance of determining the $[Mg^{2+}]$ value directly and the range of $[Mg^{2+}]$ that affects a putative Mg-regulated reaction before a regulatory role for $[Mg^{2+}]$ can be assigned.

In this regard, Rutter *et al.* (1990) point out that matrix $[Mg^{2+}]$ in the 1 mM range can be expected to decrease the inhibition of pyrophosphatase by $[Ca^{2+}]$. The $K_{0.5}$ for inhibition increases from less than 1 μM to 10 μM $[Ca^{2+}]$ in the presence of 1 mM $[Mg^{2+}]$. Matrix $[Mg^{2+}]$ near 1 mM also prevents activation of NAD^+-isocitrate dehydrogenase at $[Ca^{2+}]$ levels that affect the pyruvate dehydrogenase complex and α-ketoglutarate dehydrogenase. Conversely, the presence of $[Ca^{2+}]$ also brings the $K_{0.5}$ for activation of isolated pyruvate dehydrogenase phosphatase down to the 1 mM range (Rutter *et al.*, 1990).

There are many indications that $[Mg^{2+}]$ plays a major role in the regulation of cellular and mitochondrial reactions. Firm evidence that it functions as a primary on/off signal, as does $[Ca^{2+}]$, is still not available, however. Even if $[Mg^{2+}]$ cannot be shown to function in such a role, it seems likely that it may act as a static or chronic regulator of cell and mitochondrial function, establishing the "set point" of many Mg^{2+}-dependent reactions. It therefore seems important to define in detail the factors that contribute to changes in both cytosol and matrix $[Mg^{2+}]$ and their effects on cell and mitochondria function.

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