Matrix Free Mg^{2+} Changes with Metabolic State in Isolated Heart Mitochondria†

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ABSTRACT: The concentration of free Mg^{2+} in the matrix of isolated heart mitochondria has been monitored by using the fluorescent probe furaptra (mag-fura-2). Beef heart mitochondria respiring in a KCl medium in the absence of external Mg^{2+} maintain free matrix Mg^{2+} near 0.50 mM. Addition of P_i under these conditions decreases free Mg^{2+} by 0.12–0.17 mM depending on the substrate. This decrease in free Mg^{2+} appears to reflect changing ligand availability in the matrix. The decrease is prevented when the P_i transporter is blocked by mersalyl. Addition of ADP to initiate state 3 respiration causes a marked increase in free matrix Mg^{2+} (0.1–0.2 mM) that persists as long as ATP formation is taking place; free Mg^{2+} then returns to the base level. This cyclic change is blocked by oligomycin and carboxyatractyloside and appears to reflect to a large extent the decrease in matrix P_i that accompanies oxidative phosphorylation. Exchange of external ADP for matrix ATP may also contribute to the increase in free matrix Mg^{2+}. Addition of an uncoupler promotes anion efflux and increases free matrix Mg^{2+}. Similar changes in free Mg^{2+} on addition of P_i, ADP, or uncoupler are seen when extramitochondrial Mg^{2+} is buffered from 0.5 to 2 mM, but the basal free matrix Mg^{2+} increases as external Mg^{2+} concentration increases in this range. Free matrix Mg^{2+} also increases when total mitochondrial Mg^{2+} is increased by respiration-dependent uptake in the presence of P_i. It is concluded that matrix free Mg^{2+} changes significantly with changing ligand availability and that such changes may contribute to the regulation of Mg^{2+}-sensitive matrix enzymes and membrane transporters.

It has long been recognized that the concentration of free Mg^{2+} in a cell may contribute to the regulation of metabolism and transport [see Garfinkel et al. (1986) or Grubbs and Maguire (1987) for reviews]. Garfinkel et al. (1986) concluded that intracellular $[Mg^{2+}]$[1] is low (on the order of 0.4 mM) and varies with time and conditions. These conclusions appear to be substantiated by recent estimates of $[Mg^{2+}]$ in heart cells (Murphy et al., 1989; Rotevatn et al., 1989) and changes in this component in response to changing extracellular ion concentrations. On the other hand, Corkey et al. (1986) concluded that Mg^{2+} is an unlikely short-term regulator of metabolic processes because it is highly buffered in both the cytosol and mitochondria of hepatocytes and is present in virtually identical concentrations in the two compartments. Corkey et al. (1986) also pointed out, however, that the high concentration and low affinity of the available Mg^{2+} binding sites will result in significant changes in $[Mg^{2+}]$ when there is a change in total Mg^{2+}.

There are also numerous indications that $[Mg^{2+}]$ may have regulatory functions in the matrix of mitochondria. Kohn and Garfinkel (1983), for example, concluded that matrix $[Mg^{2+}]$ is an important regulator of fatty acid metabolism by virtue of its effects on the aconitase equilibrium and the control of citrate synthase by matrix citrate. In addition, it has been proposed that mitochondrial volume homeostasis is maintained by a K^+/H^+ antiport and an anion uniport that are regulated by matrix $[Mg^{2+}]$ and which come into play when matrix $[Mg^{2+}]$ is decreased by changing ligand availability or by matrix dilution (Garlid, 1980, 1988; Garlid & Beavis, 1986). This would require that matrix $[Mg^{2+}]$ change sufficiently to be recognized by one or more regulatory sites and that it be sufficiently independent of $[Mg^{2+}]$ in the cytosol to reflect the changing volume of the matrix compartment.

It has been shown that heart mitochondria can take up and release Mg^{2+} by respiration-dependent reactions, but that the flux of Mg^{2+} is low under conditions that approach those in situ [Brierley et al. (1987); see also Johnson and Pressman (1969)]. However, recent electron probe studies indicate that mitochondria in situ can show increases in total Mg^{2+} in response to hormone treatment (Bond et al., 1987). In this regard, Jung and Brierley (1986) have reported that the matrix $[Mg^{2+}]$ of isolated heart mitochondria increases nearly linearly with increasing total Mg^{2+}. In order to establish whether changes in matrix $[Mg^{2+}]$ can indeed contribute to the regulation of mitochondrial enzymes or transporters it would appear necessary to establish whether there are significant changes in matrix $[Mg^{2+}]$ under conditions that may be encountered by mitochondria in situ. In addition, because cytosol $[Mg^{2+}]$ does appear to vary with conditions (Murphy et al., 1989), it would be desirable to establish the extent to which matrix $[Mg^{2+}]$ reflects cytosolic $[Mg^{2+}]$.

Resolution of many of these issues has been hampered by the lack of appropriate methodology for determining matrix $[Mg^{2+}]$ and for monitoring changes in its concentration. The recent introduction of the Mg^{2+}-sensitive fluorescent probe furaptra (Raju et al., 1989; Murphy et al., 1989) and the development of techniques for loading analogous probes into the mitochondrial matrix (Davis et al., 1987; Jung et al., 1989; Lukacs & Kapus, 1987; Gunter et al., 1988; McCormack et al., 1989; Reers et al., 1989) make it possible to estimate matrix $[Mg^{2+}]$ and to monitor changes in this component as a function of metabolic state. The present communication describes the calibration of furaptra fluorescence in terms of matrix $[Mg^{2+}]$ and reports that there are significant changes

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[1] Abbreviations: $[Mg^{2+}]$, concentration of free Mg^{2+} ions; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; CCCP, carbonyl cyanide (*m*-chlorophenyl)hydrazone; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; BCECF, 2,4-bis(carboxyethyl)-5(6)-carboxyfluorescein.

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in matrix [Mg^{2+}] when respiring mitochondria are challenged with P_i, ADP, or uncoupler. A preliminary report of these findings has been made (Jung et al., 1990).

Materials and Methods

Mitochondria. Beef heart mitochondria were prepared as described by Brierley et al. (1984) and suspended in sucrose (0.25 M) containing TES (10 mM, pH 7.4) at 25 mg of protein/mL. Pig heart mitochondria were prepared from surgically removed hearts that were perfused with cold cardioplegic solution and stored overnight in the cold prior to preparation of mitochondria as described for beef heart.

Furaptra (or mag-fura-2) and its acetoxymethyl ester were obtained from Molecular Probes, Inc. (Eugene, OR) and dissolved in water or dimethyl sulfoxide, respectively. The probe was loaded into mitochondria by incubating 1 mL of the mitochondrial suspension for 20 min at 25 °C in the presence of NaCl (20 mM), ATP (2 mM), EGTA (0.2 mM), and furaptra ester (15 μM). At this point 6 mL of ice cold sucrose–TES was added and the mitochondria were isolated by centrifugation (13000g × 10 min) and resuspended in fresh sucrose–TES (1 mL, 25 °C). After an additional 5-min incubation the mitochondria were again diluted in cold sucrose–TES, reisolated, and suspended at 25 mg/mL. Control mitochondria were carried through an identical procedure with furaptra omitted. This loading procedure depletes the mitochondria of Ca^{2+}, which is present in sufficient amounts to affect furaptra fluorescence when Na^+ is not added during the loading step. The addition of ATP during loading helps preserve the adenine nucleotide content of the mitochondria.

Analytical Procedures. Total Mg^{2+} was estimated by atomic absorption of acid extracts of centrifuged mitochondria using the cold KCl stop and wash procedure (Brierley et al., 1987). Mitochondrial Ca^{2+} was monitored by fura-2 fluorescence (Davis et al., 1987; Jung et al., 1989). Respiration was followed with an oxygen electrode. Adenine nucleotides were quantitated by HPLC of acid extracts (Geisbuhler et al., 1984). The [Mg^{2+}] in Mg^{2+}-buffered suspending media was calculated by use of the program described by Fabiato (1988). The concentration of furaptra in mitochondria was estimated by fluorescence after centrifugation of the mitochondria and lysis with Triton X-100.

Fluorescence Measurements. Fluorescence was measured as emission at 510 nm by using a Perkin-Elmer LS-5B with slits set at 10 nm. The fluorometer was interfaced with a computer using an application program for fura-2 supplied by Perkin-Elmer and modified in this laboratory using QuickBASIC (Microsoft Corp.). For kinetic measurements this program drives the excitation monochromator to provide a 340- and 380-nm reading every 7 s.

The excitation spectra of furaptra dissolved in the KCl medium used in these protocols show a maximum at 340 nm when saturated with Mg^{2+} or Ca^{2+} and a broad maximum at 380 nm in the absence of Mg^{2+} and in the presence of 2 mM EDTA (Figure 1). The control mitochondria show significant background or autofluorescence in this excitation range and the autofluorescence at 340 and 380 nm is unequal (Figure 1). The fluorescence of furaptra can be calibrated in terms of matrix [Mg^{2+}] by use of the following expression (Raju et al., 1989; Grynkiewicz et al., 1985):

$$[Mg^{2+}] = K_D \frac{R - R_{min}}{R_{max} - R} \left(\frac{Sf_2}{Sb_2} \right) \quad (1)$$

where K_D is the dissociation constant (1.5 mM), R is the ratio of the fluorescence at the excitation wavelengths of 340/380 nm, and Sf_2 and Sb_2 are the fluorescence intensities at 380 nm for furaptra with zero and excess Mg^{2+}, respectively.

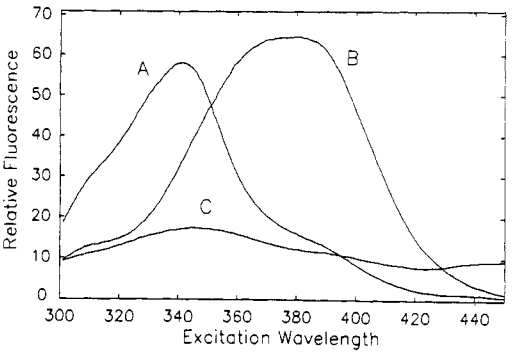

FIGURE 1: Excitation spectra of furaptra in the absence of Mg^{2+} and when saturated with Mg^{2+} or Ca^{2+}. The probe was dissolved at a final concentration of 118 nM in KCl (0.1 M) containing HEPES (10 mM, pH 7.4) and rotenone (3 μg/mL). A. Saturating Mg^{2+} or Ca^{2+}. EGTA (10 μM) and Ca^{2+} (1 mM) or Mg^{2+} (20 mM) were added. B. No divalent cation; EGTA was also present at 2 mM. C. Fluorescence of control mitochondria (incubated as for furaptra-loaded mitochondria, but with the probe omitted) suspended at 0.5 mg/mL in the KCl medium.

R_{min} was determined by treating furaptra-loaded mitochondria with succinate followed by the ionophore BrA23187 in the presence of 2 mM EGTA to deplete endogenous Mg^{2+} (Figure 2A). The fluorescence was recorded with excitation at 340 and 380 nm. A parallel incubation was then carried out with control (nonloaded) mitochondria with fluorescence recorded at both wavelengths. The fluorescence at 380 and 340 nm was then corrected by subtracting control fluorescence for each corresponding point. The ratio of 340/380 fluorescence was then computed from these net values (Figure 2A). R_{max} was estimated by allowing mitochondria respiring with succinate to accumulate Ca^{2+} until the sequestered furaptra was saturated (Figure 2B). This procedure was chosen since high concentrations of Mg^{2+} are not effectively equilibrated across the membrane by ionophores [see Raju et al. (1989)] and tend to aggregate isolated mitochondria as well. Again the fluorescence at 340 and 380 nm was corrected for the background autofluorescence of a parallel incubation of control mitochondria and the 340/380 ratio calculated (Figure 2B). The R_{min} and R_{max} values are used to calculate matrix [Mg^{2+}] as a function of time in these protocols (Figure 2A) using eq 1. R_{max} values were typically near 11 and R_{min} near 0.4 as shown in Figure 2 and a typical value for the Sf_2/Sb_2 term was ~12.

Results

Use of Furaptra Fluorescence To Follow Matrix [Mg^{2+}] with Changing Metabolic State. Virtually all of the furaptra in a suspension of probe-loaded mitochondria appears to be localized in the matrix compartment. More than 95% of the fluorescence is lost when the mitochondria are removed by centrifugation and there is little or no fluorescence response upon the addition of Mg^{2+} to the mitochondrial suspension. The loaded mitochondria contain an average of 0.3 nmol of furaptra/mg of protein, or ~0.3 mM.

Furaptra-loaded beef heart mitochondria respiring with succinate in the absence of external Mg^{2+} show changes in fluorescence at 340- and 380-nm excitation in response to the addition of P_i, ADP, and uncoupler (Figure 3A). The fluorescence of control mitochondria also changes with these additions when incubated in parallel (Figure 3A). Because points at both wavelengths can be acquired every 7 s, it is possible to subtract the autofluorescence from the experimental

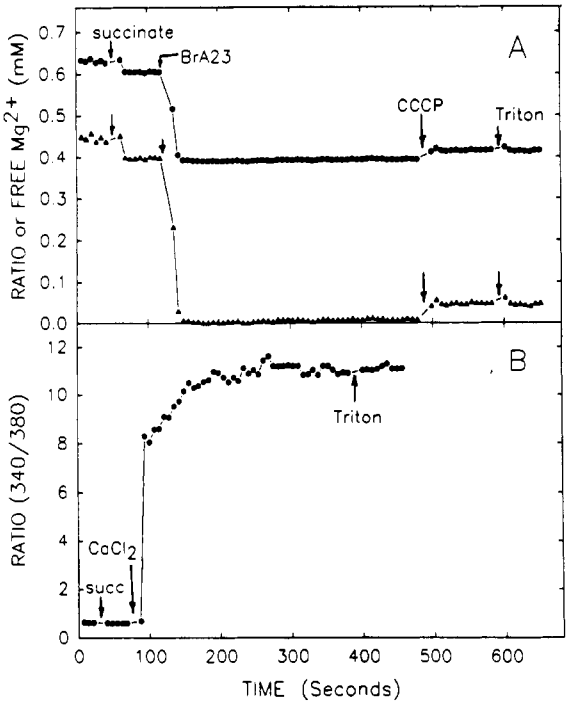

FIGURE 2: Estimation of R_{min} and R_{max} for furaptra-loaded beef heart mitochondria. A. R_{min}. Furaptra-loaded mitochondria were suspended at 0.5 mg of protein/mL in a medium of KCl (0.1 M), HEPES (10 mM, pH 7.4), rotenone 3 μg/mL, and EGTA (2 mM). Fluorescence was recorded with excitation at 340 and 380 nm (Materials and Methods) at 25 °C. Where indicated, succinate (2 mM) and BrA23187 (BrA23; 1 μM) were added to deplete endogenous divalent cations. A parallel determination of fluorescence of control mitochondria was also made and the autofluorescence was subtracted from the furaptra fluorescence at each wavelength before calculating ratios for use in eq 1. R_{min} is the ratio after fluorescence has reached a steady state. (●) 340/380 ratio after subtraction of autofluorescence. (▲) computed value for $[Mg^{2+}]$. B. R_{max}. Furaptra-loaded mitochondria respiring with succinate under the conditions of A (EGTA reduced to 0.03 mM) were treated with $CaCl_2$ (1 mM) and later with Triton X-100 (0.07%). In some preparations the ratio increases on Triton addition and the higher ratio was used as R_{max}. A parallel incubation with control mitochondria was also recorded and the 340/380 ratio calculated after the subtraction of autofluorescence.

value at each point, if the additions for the two runs are made on the same time scale. These corrected fluorescence points (experimental minus autofluorescence) are shown in Figure 3A as 340_{net} and 380_{net}. It should be noted that the 340 record shows little change after autofluorescence is subtracted (340_{net}) whereas the 380 record is amplified (380_{net}) because the autofluorescence often changes in the direction opposite to that of furaptra at this wavelength. These net values are then used to compute the 340/380 ratio and the $[Mg^{2+}]$ computed from eq 1 can be plotted as a function of time with changing metabolic state (Figure 3B).

The initial values for matrix $[Mg^{2+}]$ vary from preparation to preparation and may increase or decrease somewhat in the absence of respiration. After the addition of substrate, $[Mg^{2+}]$ increases slightly in the experiment shown in Figure 3B. The value for matrix $[Mg^{2+}]$ in respiring beef heart mitochondria under these conditions is quite reproducible, with a mean of 0.48 mM (Table I).

Matrix $[Mg^{2+}]$ decreases following the addition of P_i to respiring mitochondria (Figure 3B) with a new steady state established near 0.30 mM. Most of the decrease appears to be due to increased ligand availability as P_i enters the matrix on the phosphate transporter. The P_i-induced decline in $[Mg^{2+}]$ averages 0.17 for succinate respiration (Table I) and is abolished by levels of mersalyl or *N*-ethylmaleimide that

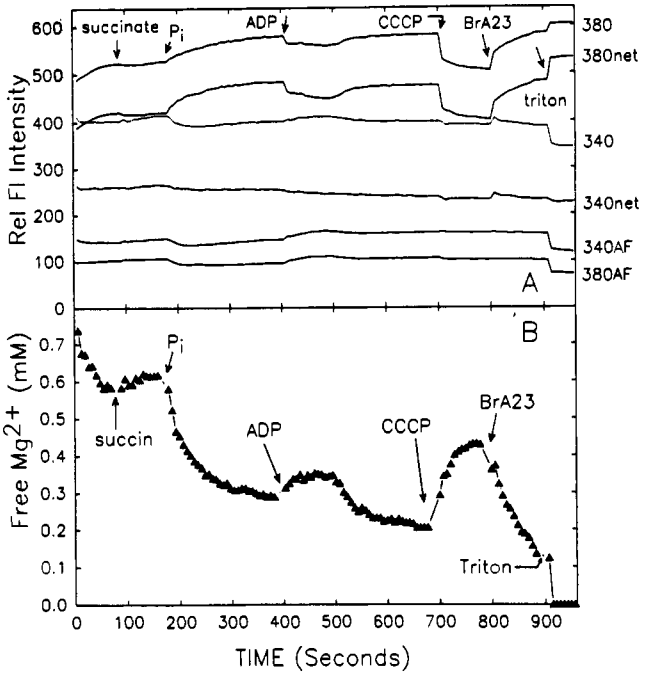

FIGURE 3: Change in $[Mg^{2+}]$ in the matrix of beef heart mitochondria respiring in a KCl medium. Furaptra-loaded mitochondria were suspended at 0.5 mg/mL in KCl (0.1 M) containing HEPES (10 mM, pH 7.4) rotenone (3 μg/mL), and EGTA (2 mM). The temperature was maintained at 25 °C. Where indicated, succinate (2 mM) was added followed by potassium phosphate (2 mM), ADP (0.1 mM), CCCP (1.5 μM), BrA23187 (1 μM), and finally Triton X-100 (0.07%). A. Fluorescence intensity with excitation at 340 and 380 nm is shown as a function of time. Also shown is the autofluorescence from a parallel incubation of control mitochondria (labeled 340AF and 380AF) and the net fluorescence (experimental minus autofluorescence). The lines are drawn through data points collected 7 s apart. B. Computed matrix $[Mg^{2+}]$ concentration as a function of metabolic state. Ratios calculated from the net fluorescence values in A are used to calculate matrix $[Mg^{2+}]$ by using eq 1.

Table I: Matrix $[Mg^{2+}]$ of Respiring Heart Mitochondria: Changes with Changing Ligand Availability[a]

	succinate	glutamate + malate
initial $[Mg^{2+}]$ (mM)	0.48 ± 0.07 (13)	0.49 ± 0.10 (7)
decrease in $[Mg^{2+}]$ with P_i addition (mM)	0.17 ± 0.06 (12)	0.12 ± 0.03 (6)
increase in $[Mg^{2+}]$ with ADP (mM)	0.09 ± 0.02 (9)	0.12 ± 0.03 (6)
$[Mg^{2+}]$ after CCCP (mM)	0.53 ± 0.06 (7)	0.51 ± 0.13 (7)

[a] Values tabulated are means ±SD for the number of preparations given in parentheses. The protocol of Figures 3 and 4 was followed with CCCP added after 10 min of incubation.

Table II: Changes in Matrix $[Mg^{2+}]$ of Respiring Heart Mitochondria with Changes in Ligand Availability[a]

conditions	initial $[Mg^{2+}]$ (mM)	change in matrix $[Mg^{2+}]$ (mM) +P_i	+ADP	+CCCP
control	0.70	−0.15	+0.13	+0.31
+rotenone (3 μg/mL)	0.61	+0.02	−0.03	+0.13
+oligomycin (2 μg/mL)	0.63	−0.11	−0.06	+0.30
+carboxyatractyloside (0.1 mM)	0.65	−0.14	−0.02	+0.36
+mersalyl (20 nmol/mg of protein)	0.67	0	0	

[a] Data from a typical experiment in which mitochondria were suspended in buffered KCl containing glutamate and malate (5 mM each) and Mg^{2+} (0.6 mM free). The other conditions were identical with those of Figure 4. Carboxyatractyloside was added 50 s prior to ADP and mersalyl 50 s before P_i. All other reagents were present initially or added on the time sequence shown in Figure 4.

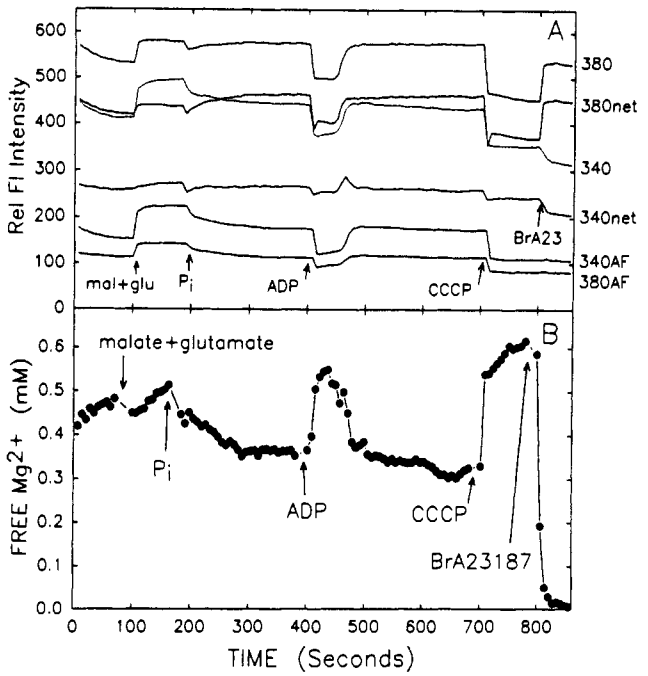

FIGURE 4: Changes in matrix $[Mg^{2+}]$ of beef heart mitochondria respiring with glutamate and malate (5 mM each). The conditions and measurements were identical with those of Figure 3 except that rotenone was omitted and 0.16 mM ADP was added. The respiratory control ratio was 6.6 with this substrate as opposed to 1.5 for succinate. A. Records of fluorescence intensity. B. Computed values for matrix $[Mg^{2+}]$.

block the P_i transporter (Table II). The effect is maximal at 0.1 mM P_i and remains nearly constant as P_i is increased to 5 mM.

Addition of ADP after P_i (Figure 3) induces state 3 respiration, a low-amplitude matrix contraction, and an increase in matrix $[Mg^{2+}]$ that persists as long as ATP is being formed. When state 4 respiration resumes, $[Mg^{2+}]$ rapidly returns to its original value (Figure 3B). The magnitude of this cyclic change in $[Mg^{2+}]$ amounts to 0.1–0.2 mM, depending on substrate. The duration of the elevation in matrix $[Mg^{2+}]$ is dependent on the amount of ADP added. The increase in $[Mg^{2+}]$ following ADP addition is eliminated by oligomycin and by carboxyatractyloside at concentrations that block oxidative phosphorylation and adenine nucleotide transport, respectively (Table II).

Addition of an uncoupler (CCCP in Figure 3) results in a rapid increase in matrix $[Mg^{2+}]$. Addition of the ionophore BrA23187 results in release of matrix Mg^{2+} to the Mg^{2+}-free suspending medium (Figure 3). The matrix $[Mg^{2+}]$ declines to an average value of 0.26 ± 0.03 mM (n = 7) over 10 min of incubation with the additions shown in Figure 3B and is increased to 0.53 mM (Table I) by addition of the uncoupler.

When heart mitochondria respire with a pyridine nucleotide linked substrate, such as glutamate plus malate as shown in Figure 4, the correction for autofluorescence is particularly important, since fluorescence at both 340 and 380 nm shows changes that often roughly parallel those of furaptra (Figure 4A). When the fluorescence is corrected and $[Mg^{2+}]$ calculated (Figure 4B), it is apparent that matrix $[Mg^{2+}]$ decreases following addition of P_i and that there is a marked increase in $[Mg^{2+}]$ on addition of ADP (mean values shown in Table I). It should be noted that the state 3 respiration rate, as well as the respiratory control ratio (state 3/state 4), is much higher with glutamate + malate than with succinate in beef heart mitochondria in the KCl medium (6.6 vs 1.5 for the respiratory control ratios).

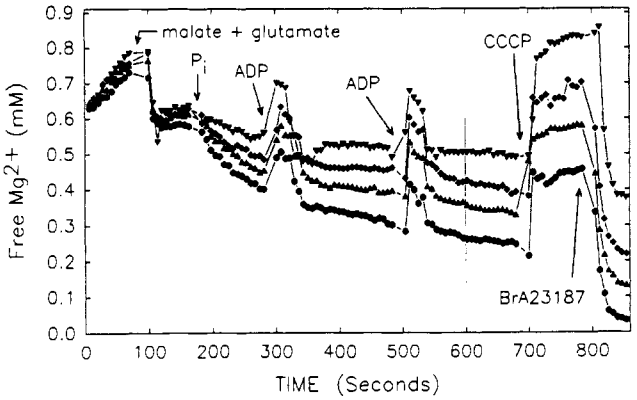

FIGURE 5: Changes in matrix $[Mg^{2+}]$ as a function of metabolic state at different levels of extramitochondrial Mg^{2+}. Beef heart mitochondria were incubated as for Figure 4 in media containing $[Mg^{2+}]$ buffered initially at 0 (●), 0.6 (▲), 1.2 (◆), and 2.3 (▼), mM $[Mg^{2+}]$ (Fabiato, 1988). These buffers were made by using 0, 0.7, 1.4, and 2.8 mM $MgCl_2$ and 2, 2.17, 2.33, and 2.67 mM EGTA, respectively. The addition of glutamate, malate, P_i, and ADP decreases the external $[Mg^{2+}]$ to 0, 0.37, 0.73, and 1.46 mM, respectively (estimated by using furaptra free acid). Values for matrix $[Mg^{2+}]$ after 600 s in the above protocol are 0.25, 0.35, 0.40, and 0.50 mM, respectively, as the external $[Mg^{2+}]$ increases. The total mitochondria Mg^{2+} (atomic absorption analysis) of identical parallel incubations was increased over the initial value of 20.6 ± 1.6 nmol/mg by 0, 1.2, 5.5, and 10.5 nmol/mg, respectively. Note that the furaptra fluorescence shows no difference between 0 and 2 mM Mg^{2+} when the incubation is initiated.

Mitochondria respiring with α-ketoglutarate or β-hydroxybutyrate maintain matrix $[Mg^{2+}]$ at close to 0.50 mM and show changes on addition of P_i, ADP, and CCCP that are of similar magnitude to those shown in Table I. Beef heart mitochondria show a marked decrease in matrix $[Mg^{2+}]$ when pyruvate is added, either in the presence or absence of malate. These mitochondria maintain matrix $[Mg^{2+}]$ at 0.26 ± 0.05 mM (n = 5, conditions of Figure 4) and show decreases when P_i is added of 0.08 mM and increases with added ADP that are comparable to those shown in Table I. The decrease in matrix $[Mg^{2+}]$ seen on addition of P_i and the cyclic increase with ADP addition are both prevented when respiration is blocked with rotenone (Table II).

Changes in Matrix $[Mg^{2+}]$ with Extramitochondrial Mg^{2+}. The changes in $[Mg^{2+}]$ with metabolic state shown in Figures 3 and 4 were obtained in the absence of external Mg^{2+}. Under these conditions there is some net loss of total Mg^{2+} from respiring heart mitochondria (Crompton et al., 1976; Brierley et al., 1987) so that a portion of the decline in matrix $[Mg^{2+}]$ seen over the incubation period may reflect a decreased total Mg^{2+}. Beef heart mitochondria as isolated contain 30–40 nmol of total Mg^{2+}/mg of protein (Brierley et al., 1987). The furaptra-loaded preparations used in this study contain 20.6 ± 1.6 nmol/mg following the incubation and washing steps used to introduce the probe into the matrix. The furaptra-loaded mitochondria lose from 0 to 3 nmol of Mg^{2+}/mg over a 10-min incubation with substrate and P_i in a Mg^{2+}-free KCl medium (conditions of Figure 3 or 4).

When furaptra-loaded heart mitochondria are incubated in a KCl medium containing buffered $[Mg^{2+}]$ at 0.6, 1.2, and 2.3 mM, the changes due to addition of P_i, ADP, and uncoupler parallel those in a Mg^{2+}-free medium (Figure 5). The decrease in matrix $[Mg^{2+}]$ upon addition of P_i is less as external $[Mg^{2+}]$ increases, going from 0.17 mM in the Mg^{2+}-free medium to 0.08 mM in the presence of 2 mM external Mg^{2+} (Figure 5). However, the increase seen upon addition of ADP remains the same at each concentration of external Mg^{2+}. After the ADP has been converted to ATP the $[Mg^{2+}]$ returns to a base level and a second addition of ADP produces another

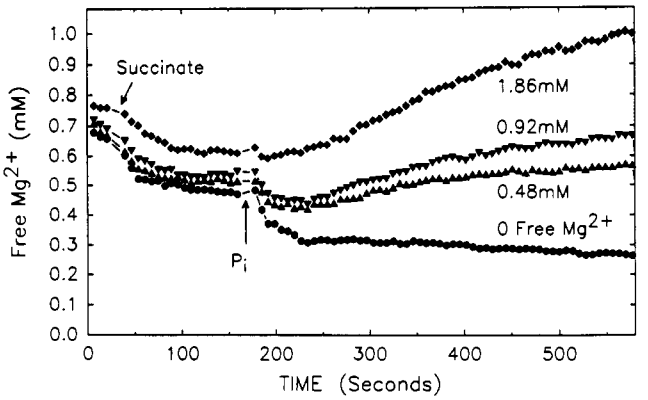

FIGURE 6: Increase in matrix $[Mg^{2+}]$ with increasing total Mg^{2+} in the matrix. Beef heart mitochondria were incubated under the conditions of Figure 3 with external $[Mg^{2+}]$ buffered at 0, 0.6, 1.2, and 2.3 mM. Addition of succinate (3.3 mM) and P_i (3.3 mM) decreases the external $[Mg^{2+}]$ to 0, 0.48, 0.92, and 1.86 mM as shown. After 10 min of incubation with the additions shown, a parallel incubation was centrifuged and Mg^{2+} determined by atomic absorption. The total mitochondrial Mg^{2+} after 10 min showed a decrease of 1.0 nmol/mg for zero external Mg^{2+}, no change for 0.6 and 1.2 mM external Mg^{2+}, and an increase of 8 nmol/mg for 2.3 mM $[Mg^{2+}]$.

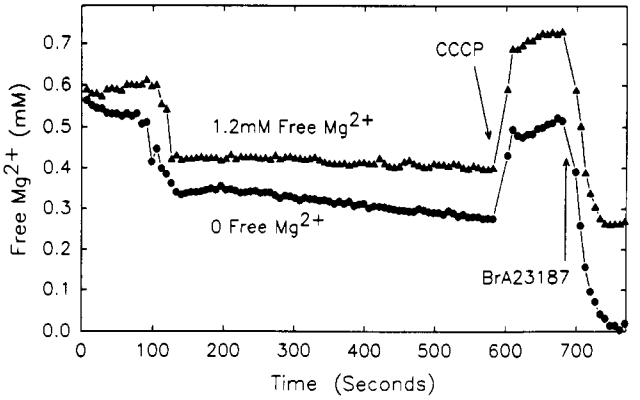

FIGURE 7: Matrix $[Mg^{2+}]$ as a function of extramitochondrial $[Mg^{2+}]$ for mitochondria in state 4. Beef heart mitochondria loaded with furaptra were added to a cuvette containing glutamate + malate (5 mM each), ADP (0.5 mM), and P_i (3.0 mM) with other additions as in Figure 4. External $[Mg^{2+}]$ was buffered initially at 0 or 1.2 mM. Addition of substrate, ADP, and P_i decreased the external $[Mg^{2+}]$ to 0 and 0.73 mM, respectively. The decline in matrix $[Mg^{2+}]$ at ~100 s of incubation corresponds to the state 3–4 transition.

cycle of $[Mg^{2+}]$ increase and decrease (Figure 5). The matrix $[Mg^{2+}]$ maintained after 10 min of incubation under these conditions is a near-linear function of external $[Mg^{2+}]$, increasing from 0.25 mM at zero external Mg^{2+} to 0.5 mM at 2 mM Mg^{2+} (Figure 5). The matrix $[Mg^{2+}]$ after addition of an uncoupler under these conditions also increases linearly with external Mg^{2+}, going from 0.45 to 0.85 mM as external $[Mg^{2+}]$ increases from zero to 2 mM (Figure 5). Total mitochondrial Mg^{2+} assayed after 10 min of incubation also increases as external Mg^{2+} is increased from zero to 2 mM (Figure 5) with a net increment of 10.5 nmol/mg seen at the highest $[Mg^{2+}]$.

The decrease in matrix $[Mg^{2+}]$ following the addition of P_i lessens markedly with increasing external $[Mg^{2+}]$ in mitochondria respiring with succinate (Figure 6). In this case the decrease due to P_i is 0.17 mM in the absence of external Mg^{2+}, but only 0.03 mM when 2 mM external Mg^{2+} is present. There is a slow increase in matrix $[Mg^{2+}]$ with continued incubation in the presence of external Mg^{2+} (Figure 6). Beef heart mitochondria have been reported to increase their total Mg^{2+} content under these conditions (Brierley et al., 1987). In the presence of 2 mM extramitochondrial $[Mg^{2+}]$, matrix $[Mg^{2+}]$ increases from 0.6 to 1.0 mM and total mitochondrial Mg^{2+} increases by 8 nmol/mg (Figure 6).

When furaptra-loaded heart mitochondria are added to the KCl medium containing glutamate, malate, ADP, and P_i, the matrix $[Mg^{2+}]$ is stabilized at just over 0.5 mM in the absence of external Mg^{2+} and at ~0.6 mM when 1 mM external Mg^{2+} is present (Figure 7). When these mitochondria consume the available ADP and the transition from state 3 to state 4 occurs, matrix $[Mg^{2+}]$ drops by ~0.2 mM in each case (Figure 7). The matrix $[Mg^{2+}]$ stabilizes at 0.4 mM in the presence of external Mg^{2+} and declines slowly to 0.3 mM in the absence of added Mg^{2+} (Figure 7). The level of matrix $[Mg^{2+}]$ maintained increases with increasing external Mg^{2+} to a saturation point near 2 mM external free Mg^{2+} (not shown). The matrix $[Mg^{2+}]$ of uncoupled mitochondria also reflects increasing external $[Mg^{2+}]$. It should be noted that BrA23187 does not equilibrate internal Mg^{2+} with the suspending medium under these conditions.

Changes in Matrix $[Mg^{2+}]$ of Pig Heart Mitochondria. Mitochondria prepared from surgically removed pig hearts are less likely to have been subjected to extensive ischemia than the beef preparations from slaughterhouse hearts. The pig heart mitochondria contain roughly twice the total adenine nucleotides of beef heart mitochondria (8.8 ± 2.1 nmol/mg, $n = 8$, as opposed to 4.3 ± 1.2, $n = 14$) and might be expected to differ in matrix $[Mg^{2+}]$. However, these mitochondria maintain quite comparable matrix $[Mg^{2+}]$ of 0.63 ± 0.02 mM ($n = 3$) when respiring under the conditions of Figure 3 and show comparable decreases when challenged with P_i (0.3 mM Mg^{2+}) and increases with ADP (0.14 mM Mg^{2+}).

Response of $[Mg^{2+}]$ to Osmotic Swelling and Contraction of the Matrix. It is well-known that the matrix compartment of isolated mitochondria expands when the osmotic strength of the suspending medium is decreased [Stoner and Sirak (1968) for example]. When beef heart mitochondria are exposed to a series of buffers with KCl set from 150 mM to zero (~350 to ~50 mosmol), the matrix $[Mg^{2+}]$ as reported by furaptra fluorescence decreases from 0.60 to 0.18 mM with a nearly linear decrease as a function of decreasing osmolality (Figure 8). The decrease in matrix $[Mg^{2+}]$ with osmotic swelling is reversible. Addition of KCl (150 mM final concentration) to hypotonically swollen beef heart mitochondria contracts the matrix and increases matrix $[Mg^{2+}]$ by ~0.3 mM (Figure 8).

Discussion

These studies have established that the free Mg^{2+} concentration in the matrix of isolated heart mitochondria can be monitored continuously by using furaptra fluorescence and that significant changes in $[Mg^{2+}]$ occur in response to changing ligand availability. The addition of P_i decreases matrix $[Mg^{2+}]$ whereas adding ADP to initiate state 3 respiration increases $[Mg^{2+}]$. The matrix $[Mg^{2+}]$ decreases with a state 3 to 4 transition and increases when an uncoupler is added (Figures 3 and 4). These studies also establish that matrix $[Mg^{2+}]$ increases with increasing extramitochondrial $[Mg^{2+}]$ (Figure 5) and when the total mitochondrial Mg content is increased by respiration-dependent accumulation with P_i (Figure 6). In addition, $[Mg^{2+}]$ decreases when the matrix is expanded by hypotonic swelling and increases when the matrix is contracted osmotically (Figure 8).

Furaptra as a Probe for Matrix $[Mg^{2+}]$. Furaptra fluorescence calibrated as described here indicates that the matrix $[Mg^{2+}]$ of respiring heart mitochondria is initially maintained near 0.5 mM. The concentration of furaptra in the matrix is estimated to be 0.3 mM and is therefore quite

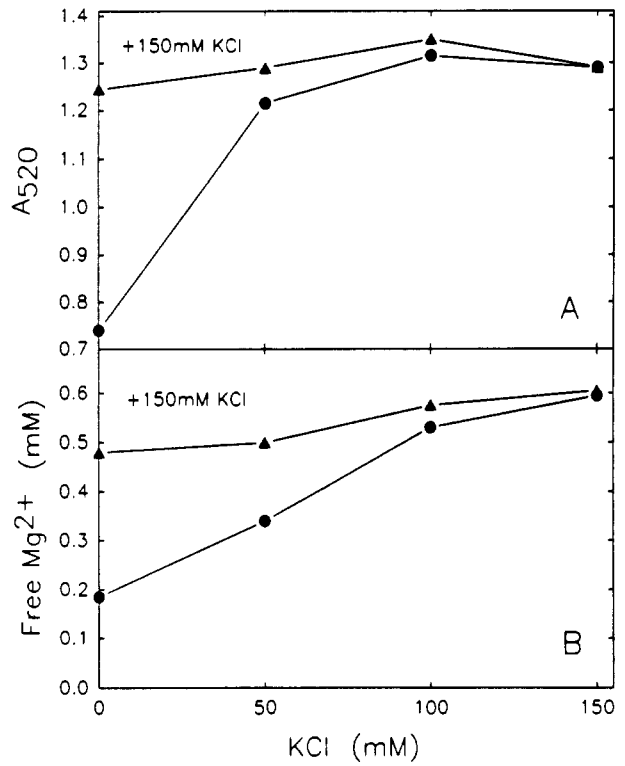

FIGURE 8: Decrease in matrix [Mg^{2+}] with hypotonic swelling of beef heart mitochondria. Furaptra-loaded mitochondria were suspended at 0.5 mg/mL in HEPES buffer (10 mM, pH 7.4) containing rotenone (3 μg/mL), EGTA (2 mM), and the indicated concentration of KCl. Absorbance at 520 nm was read as a measure of swelling using a Brinkman PC801 probe colorimeter and matrix [Mg^{2+}] was estimated as shown in Figure 3. The triangles indicate the change in response to increasing KCl to a final concentration of 150 mM.

close to the level of [Mg^{2+}] present. As pointed out by Raju et al. (1989) this situation should result in little buffering of matrix [Mg^{2+}] by the added furaptra. The presence of furaptra in the matrix has no effect on the total Mg content of beef heart mitochondria and the probe does not alter either state 3 respiration or the respiratory control index (data not shown).

Addition of Mg^{2+} to the suspending medium of most preparations of furaptra-loaded mitochondria produces little or no fluorescence response (see Figure 5, for example). This indicates that little of the probe is exposed to the suspending medium. Other preparations (Figure 6, for example) show fluorescence equivalent to about a 0.1 mM increase in matrix [Mg^{2+}] when external [Mg^{2+}] is increased from zero to 2 mM. This indicates that ~5% of the fluorescence in these preparations results from furaptra that is in contact with or has leaked into the suspending medium. Incubation of respiring mitochondria in state 4 for 10 min at 25 °C produces little change in matrix [Mg^{2+}] reported by furaptra (Figure 7) and this can be taken as evidence that there is little leakage of probe from the matrix during such incubations. Some loss of furaptra from the matrix occurs on prolonged standing in the cold [see Davis et al. (1987)]. Large-amplitude osmotic swelling also releases internalized furaptra, especially at elevated pH (records not shown), and with this exception, the method appears quite satisfactory for the continuous monitoring of matrix [Mg^{2+}].

Heart mitochondria that have not been treated with Na^+ to deplete endogenous [Ca^{2+}] show Na^+-dependent changes in furaptra fluorescence that can be ascribed to Ca^{2+} efflux. These mitochondria also show furaptra fluorescence changes equivalent to a decrease of [Mg^{2+}] with CCCP addition, rather than an increase as seen in low-Ca^{2+} mitochondria (Figure 3). It is clear that [Ca^{2+}] must be kept low in these protocols if it is not to interfere with the estimation of [Mg^{2+}].

Heart mitochondria that have been loaded with fura-2 in the presence of Na^+ show matrix [Ca^{2+}] of 80–100 nM (using a K_D of 0.31 μM for fura-2 in eq 1). The fura-2 fluorescence shows essentially no change in [Ca^{2+}] with P_i or ADP addition in the protocol of Figure 3, but the endogenous Ca^{2+} is released with BrA23187 (records not shown). These experiments assure that the changes seen in furaptra fluorescence (Figures 3 and 4, for example) are due to changing [Mg^{2+}] and not to interference by [Ca^{2+}].

The fluorescent pH indicator BCECF shows changes in matrix pH when P_i, ADP, and CCCP are added to respiring heart mitochondria (Davis et al., 1987; Jung et al., 1989). However, the pH of the matrix remains well above the region in which furaptra responds to [H^+] (Raju et al., 1989) and all three of these additions produce matrix acidification. In contrast, furaptra fluorescence shows a decrease in [Mg^{2+}] as P_i enters the matrix and increased [Mg^{2+}] when ADP or CCCP are added (Figures 3 and 4). It therefore seems that furaptra is not simply reporting a change in ligand availability due to changing matrix pH.

Changes in Matrix [Mg^{2+}] with Changing Ligand Availability. The value of 0.5 mM obtained with furaptra for matrix [Mg^{2+}] of respiring heart mitochondria is somewhat higher than the 0.35 mM found by Corkey et al. (1986) for liver mitochondria using a null-point procedure. Jung and Brierley (1986) using a similar null-point approach with beef heart mitochondria found that total matrix Mg^{2+} below ~10 nmol/mg was largely in a bound form and that [Mg^{2+}] increased with increasing total Mg^{2+} above this value. When total Mg^{2+} was 20 nmol/mg, as with the furaptra-loaded mitochondria used in the present study, the [Mg^{2+}] was ~0.3 mM [see Figure 9 of Jung and Brierley (1986)]. It is clear that the furaptra fluorescence values found in the present work are consistent with previous estimates of matrix [Mg^{2+}] obtained by other methods.

It is also clear from all of these studies that the vast majority of matrix Mg^{2+} is bound (Grubbs & Maguire, 1987) and that the value for [Mg^{2+}] and the relative amounts of free and bound Mg^{2+} will vary with ligand availability in the matrix. Corkey et al. (1986) found a 35% decrease in [Mg^{2+}] of liver mitochondria when the matrix was loaded with citrate. In this study matrix [Mg^{2+}] also decreased when mitochondrial Ca^{2+} was increased (Corkey et al., 1986). In the present study, the addition of P_i decreases matrix [Mg^{2+}], and since the decrease is blocked by mersalyl and rotenone, reagents that prevent the net uptake of P_i on the phosphate transporter, it seems likely that most of this decrease represents increased interaction of matrix Mg^{2+} with P_i. In addition, P_i uptake may also promote conversion of endogenous ADP to ATP and, depending on extramitochondrial [Mg^{2+}], Mg^{2+} efflux (Crompton et al., 1976). These processes would also decrease matrix [Mg^{2+}]. Addition of ADP to initiate state 3 respiration (Chance & Williams, 1955) results in an increase in [Mg^{2+}], which decays back to the original level when state 4 respiration resumes. The change in matrix [Mg^{2+}] on ADP addition is sensitive to carboxyatractyloside and could possibly reflect changing ATP/ADP ratios in the matrix as ATP^{4-} is exchanged out for ADP^{3-} on the adenine nucleotide transporter. The affinity of ATP^{4-} for Mg^{2+} is approximately 6-fold greater than that of ADP^{3-} (Sillen & Martel, 1964). However, the process is also sensitive to oligomycin (Table II), a reagent that prevents oxidative phosphorylation but has no effect on adenine nu-

cleotide transport. Oligomycin also prevents the low-amplitude volume change that accompanies the conversion of ADP and P_i to ATP. This change in matrix $[Mg^{2+}]$ seen on addition of ADP under our conditions seems more likely to depend on a decrease in matrix P_i and less availability of ligands for free $[Mg^{2+}]$. It must be remembered that isolated beef heart mitochondria have a low adenine nucleotide content (see above). The relative contribution of ADP^{3-}/ATP^{4-} exchange to matrix $[Mg^{2+}]$ may be much greater in mitochondria having a higher adenine nucleotide content, and we are currently investigating this possibility. The basis for the drop in matrix $[Mg^{2+}]$ on addition of pyruvate is not known as yet but may result from increasing citrate in the matrix. It is clear that changes in matrix $[Mg^{2+}]$ with changing ligand availability can be quite significant, with fluctuations of as much as 0.3 mM seen under various conditions (Figures 3–7).

Changes in Matrix $[Mg^{2+}]$ with External $[Mg^{2+}]$. The study of Corkey et al. (1986) concluded that there is no significant gradient of $[Mg^{2+}]$ between the cytosol and matrix compartments in hepatocytes and that cytosol $[Mg^{2+}]$ was 0.37 mM. Cytosol $[Mg^{2+}]$ of heart cells has been estimated at 0.4 mM by Garfinkel et al. (1986) using computer simulation methods that also suggest that matrix and cytosol $[Mg^{2+}]$ are unequal under many conditions. Murphy et al. (1989) have used furaptra to determine $[Mg^{2+}]$ in cultured chick heart cells to be 0.48 mM and Rotevatn et al. (1989) found 0.56 mM in the same cells by ^{19}F NMR. Other estimates of cytosol $[Mg^{2+}]$ in heart cells range from 0.4 (Blatter & McGuigan, 1986) to 0.8 mM (Gupta et al., 1983). The present work shows that mitochondria suspended in media buffered near 0.5 mM $[Mg^{2+}]$ maintain matrix $[Mg^{2+}]$ very near the external $[Mg^{2+}]$, but that changes on the order of 0.2 mM can be superimposed by changing ligand availability (Figure 5).

It is well-known that heart mitochondria can accumulate Mg^{2+} by a respiration-dependent process (Brierley et al., 1963). The uptake of Mg^{2+} is probably secondary to P_i accumulation and the reaction proceeds very slowly in a KCl medium and when adenine nucleotides are present (Brierley et al., 1987). Heart mitochondria also extrude Mg^{2+} when they respire in a Mg^{2+}-free medium (Crompton et al., 1976). These authors reported that there is no net movement of Mg^{2+} into or out of mitochondria in the presence of 2.5 mM external $[Mg^{2+}]$, a result that was confirmed in a recent study in this laboratory (Brierley et al., 1987). In the present study a net increase in mitochondrial Mg^{2+} of 8 nmol·mg^{-1} was seen in the study shown in Figure 6 and 11 nmol·mg^{-1} in Figure 5 when external $[Mg^{2+}]$ was buffered at 2 mM. These values for a 10-min incubation are consistent with rates of uptake from 0.25 to 1.0 nmol·mg^{-1}·min^{-1} reported by Brierley et al. (1987) for higher external Mg^{2+} under slightly different conditions. Beef heart mitochondria initially contain 30 nmol of Mg^{2+}/mg (or more) and average 20.6 nmol·mg^{-1} after the furaptra loading process. Because some matrix Mg^{2+} is lost during the incubation with the probe, it seems likely that these mitochondria may contain an excess of potential binding sites for Mg^{2+}.

Two conclusions emerge from the present studies with respect to matrix $[Mg^{2+}]$ and its relationship to extramitochondrial $[Mg^{2+}]$. The first is that matrix $[Mg^{2+}]$ increases with increasing external $[Mg^{2+}]$ in the range from 0 to 2 mM (Figure 5). Matrix $[Mg^{2+}]$ does not increase immediately when the mitochondria are exposed to external $[Mg^{2+}]$, however (Figure 5), and the two compartments do not equilibrate or reach electrochemical equilibrium over the 10-min incubations used in this study. The second is that $[Mg^{2+}]$ increases with increasing total Mg^{2+} in these mitochondria (Figure 6). In this case, since there is little response of matrix $[Mg^{2+}]$ to the addition of P_i in the presence of 2 mM external $[Mg^{2+}]$, it is possible that sufficient Mg^{2+} enters the matrix with P_i to prevent the decrease in $[Mg^{2+}]$ seen in a Mg^{2+}-free suspending medium. It is also possible that the loss of matrix $[Mg^{2+}]$ is prevented by increasing external $[Mg^{2+}]$. Further work will be necessary to establish this point.

Respiration in vivo usually is poised at values between state 3 and state 4 and may be regulated by $[Ca^{2+}]$ control of citric cycle dehydrogenases [see Denton and McCormack (1985) for example]. The present studies (Figures 5 and 7) suggest that matrix $[Mg^{2+}]$ under near-physiological conditions will be set by a combination of matrix P_i availability, the matrix ratio of ATP and ADP, and the extramitochondrial $[Mg^{2+}]$ of the cell. It is therefore quite possible for matrix $[Mg^{2+}]$ to have secondary effects on Mg^{2+}-sensitive enzymes or the availability of substrates [as suggested by Kahn and Garfinkel (1983) for example]. It is also clear that matrix $[Mg^{2+}]$ does not meet the criteria advanced by Grubbs and Maguire (1987) for a regulatory cation, principally because changes in $[Mg^{2+}]$ appear to be secondary to anion translocation rather than adjusted by a Mg^{2+} transport system. Although not evaluated in this study, changes in matrix $[Ca^{2+}]$ may also affect the interaction of Mg^{2+} with ligands and alter $[Mg^{2+}]$ [see Murphy et al. (1989)].

Matrix $[Mg^{2+}]$ and Mitochondrial Volume Changes. Mitochondrial swelling in situ probably involves P_i uptake, as this anion tends to accumulate under conditions of hypoxia or other toxic conditions that alter the cytosol phosphorylation potential. This, combined with declining adenine nucleotide levels (Jennings & Steenbergen, 1985), would appear to provide conditions that might cause the type of decrease in matrix $[Mg^{2+}]$ seen with P_i addition in Figures 3 and 4. In the absence of external $[Mg^{2+}]$ or adenine nucleotides the addition of P_i to respiring heart mitochondria has been shown to produce a transient increase in matrix volume and to activate the exchange of matrix $^{42}K^+$ with external K^+ (Chavez et al., 1977). This increase in $^{42}K^+/K^+$ exchange has been ascribed to activation of the mitochondrial K^+/H^+ antiport and increased influx/efflux cycling of K^+ [see Brierley and Jung (1988)]. The decrease in matrix $[Mg^{2+}]$ observed on addition of P_i in the present study is consistent with Garlid's (1980) hypothesis that matrix $[Mg^{2+}]$ provides a negative regulation of the K^+/H^+ antiport, since matrix $[Mg^{2+}]$ decreases by as much as 50% under conditions of optimal activation of $^{42}K^+/K^+$ exchange (Figures 3 and 4).

Under conditions that are closer to those that may be encountered by mitochondria in situ (external $[Mg^{2+}]$ buffered from 0.5 to 1.0 mM) the response of matrix $[Mg^{2+}]$ to P_i addition is reduced (Figures 5 and 6). However, these conditions produce a smaller matrix expansion and less enhancement of $^{42}K^+/K^+$ exchange (Chavez et al., 1977), so the relationship between K^+/H^+ activity and matrix $[Mg^{2+}]$ appears to be maintained here as well.

The mitochondria K^+/H^+ antiport is also activated by swelling in a hypotonic medium (Garlid, 1978), and this has been ascribed to a dilution of matrix $[Mg^{2+}]$ (Garlid, 1980). The studies shown in Figure 8 are consistent with this hypothesis, because matrix $[Mg^{2+}]$ decreases with hypotonic swelling and increases when the matrix is contracted osmotically by addition of salt. This protocol appears to meet the criteria of Grubbs and Maguire (1987) for regulation of a process by $[Mg^{2+}]$ in that the K^+/H^+ antiport has been shown to be sensitive to $[Mg^{2+}]$, the $[Mg^{2+}]$ of the matrix can be altered by the change in tonicity of the suspending medium,

the Mg^{2+} appears to be retained in the matrix compartment (because $[Mg^{2+}]$ returns to nearly the original value when the matrix contracts; Figure 8), and K^+/H^+ activity is seen after the initial volume change (Garlid, (1980). However, it is well to keep in mind that these in vitro manipulations are far from physiological and comparisons of the K^+/H^+ activity with matrix $[Mg^{2+}]$ suggest that the latter would have to be decreased to values below 150 μM for activation of the K^+/H^+ antiport (Jung and Brierley, 1986). A direct comparison of $^{42}K^+/K^+$ exchange vs matrix $[Mg^{2+}]$ monitored by furaptra is currently in progress in an attempt to obtain a direct experimental assessment of this relationship.

Estimation of Cytosolic $[Mg^{2+}]$ Using Furaptra Fluorescence. The present studies also establish that furaptra loads very effectively into mitochondria. It therefore seems quite likely that a portion of the probe will be sequestered in the mitochondrial matrix when heart cells are exposed to furaptra ester [Murphy et al. (1989) for example] and that the fluorescence obtained from such cells will reflect the $[Mg^{2+}]$ concentration in both the matrix and cytosol compartments. This appears to be the case for fura-2 (Davis et al., 1987). In addition, it appears that if mitochondria are exposed to elevated Ca^{2+}, they are quite capable of taking up enough of this cation to affect furaptra fluorescence (see Figure 2B). Raju et al. (1989) have pointed out that normal in situ levels of free Ca^{2+} should not interfere with Mg^{2+} estimation using furaptra fluorescence. However, if cytosol Ca^{2+} is elevated and there is significant furaptra in the mitochondria of intact cells, it is possible that the uptake and release of mitochondrial Ca^{2+} could give fluorescence changes that might be incorrectly attributed to $[Mg^{2+}]$.

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