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On the relationship between matrix free Mg^{2+} concentration and total Mg^{2+} in heart mitochondria

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Abstract

The matrix free magnesium ion concentration, $[Mg^{2+}]_m$, estimated using the fluorescent probe furaptra, averaged 0.67 mM in 15 preparations of beef heart mitochondria containing an average of 21 nmol total Mg^{2+} per mg protein. $[Mg^{2+}]_m$ was compared with total Mg^{2+} during respiration-dependent uptake and efflux of Mg^{2+} and during osmotic swelling. In the absence of external P_i these mitochondria contain about 32 nmol/mg non-diffusible Mg-binding sites with an apparent K_d of 0.34 mM. $[Mg^{2+}]_m$ depends on both the size of the total Mg^{2+} pool and the ability of matrix anions to provide Mg-ligands. P_i interacts strongly with Mg^{2+} to decrease $[Mg^{2+}]_m$ and, in the absence of external Mg^{2+}, promotes respiration-dependent Mg^{2+} efflux and a decrease in $[Mg^{2+}]_m$ to very low levels. The uptake of P_i by respiring mitochondria converts ΔpH to membrane potential ($\Delta\Psi$) and provides additional Mg-binding sites. This permits large accumulations of Mg^{2+} and P_i with little change in $[Mg^{2+}]_m$. Nigericin also converts ΔpH to $\Delta\Psi$ in respiring mitochondria and induces a large and rapid increase in both total Mg^{2+} and $[Mg^{2+}]_m$. Mersalyl increases the permeability of the mitochondrial membrane to cations and this also induces a marked increase in both total Mg^{2+} and $[Mg^{2+}]_m$. These results suggest that mitochondria take up Mg^{2+} by electrophoretic flux through membrane leak pathways, rather than via a specific Mg^{2+} transporter. Mitochondria swollen by respiration dependent uptake of potassium phosphate show decreased $[Mg^{2+}]_m$, whereas those swollen to the same extent in potassium acetate do not. This suggests that $[Mg^{2+}]_m$ is well-buffered during osmotic volume changes unless there is also a change in ligand availability.

Keywords: Mitochondria; Furaptra; Mitochondrial Mg^{2+}; Mg^{2+} metabolism

1. Introduction

It is well established that isolated mitochondria can both take up and extrude Mg^{2+} by respiration-dependent processes (see [1,2] for reviews). There is also evidence that mitochondrial Mg^{2+} levels may be altered in response to hormonal messages ([3], for example) and that the concentration of free Mg^{2+}, $[Mg^{2+}]_m$, in the mitochondrial matrix can be adjusted to regulate Mg^{2+}-sensitive reactions. These possibilities have not yet been subjected to rigorous experimental scrutiny because the methodology to monitor $[Mg^{2+}]_m$ under the necessary conditions has not been available.

In a recent study [4] we examined the use of the fluorescent probe furaptra to report $[Mg^{2+}]_m$. It was

Abbreviations: Furaptra is also known as mag-fura-2; $[Mg^{2+}]_m$, the matrix concentration of free magnesium ions; AN, total adenine nucleotides; $\Delta\Psi$, mitochondrial membrane potential; AM, acetoxymethyl ester; TES, *N*-[tris(hydroxymethyl)methyl-2-amino]ethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TEA^+, tetraethylammonium ion

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concluded that the fluorescence intensity of this probe in the 380 nm_{ex} region reports increases and decreases in $[Mg^{2+}]_m$ in an acceptable and useful way. There is some uncertainty with regard to its quantitation in terms of absolute $[Mg^{2+}]_m$, due to possible secondary equilibria when furaptra is sequestered in the mitochondrial matrix. However, estimates of cytosol $[Mg^{2+}]$ in heart cells obtained using furaptra fluorescence closely match values obtained by other methods [4]. It was also concluded that a K_d for Mg-furaptra determined in vitro is more reliable than in situ estimates because combinations of ionophores fail to equilibrate external $[Mg^{2+}]$ with that of the matrix [4,5].

Our first investigation of $[Mg^{2+}]_m$ using furaptra fluorescence [6] revealed that $[Mg^{2+}]_m$ increases and decreases in parallel with changes in total mitochondrial Mg^{2+}, that it responds to changes in ligand availability and that it decreases when matrix volume is increased by hypotonic swelling. Rutter et al. [7] also reached several of these conclusions, although their use of in situ estimates of the K_d placed their values for $[Mg^{2+}]_m$ higher than ours [6].

The present study uses furaptra fluorescence to examine the relationship between $[Mg^{2+}]_m$ and total Mg^{2+} in more detail. Changes in $[Mg^{2+}]_m$ during respiration-dependent uptake and loss of total Mg^{2+} and during osmotic swelling were examined. It is concluded that $[Mg^{2+}]_m$ is highly dependent on the anion composition of the suspending medium, as well as on the total Mg^{2+} available. The respiration-dependent efflux of Mg^{2+} from mitochondria [8] requires P_i, but otherwise the pathway for this reaction remains obscure. New information on the mechanism of respiration-dependent Mg^{2+} uptake [9] is also presented.

2. Materials and methods

Furaptra AM was obtained from Molecular Probes, Eugene, OR. Beef heart mitochondria were prepared and suspended in sucrose (0.25 M) containing TES (10 mM, pH 7.4) at 25 mg protein/ml as described by Brierley et al. [10]. The mitochondria were equilibrated with furaptra in the presence of ATP, Na^+, EGTA and furaptra AM as described by Jung et al. [5,6]. Furaptra-loaded mitochondria were suspended in sucrose-TES at 25 mg/ml for further use. Control mitochondria were subjected to the same protocol with the omission of Furaptra AM.

Fluorescence was measured routinely using a Perkin Elmer LS-5B fluorimeter interfaced with a computer [6]. Unless otherwise indicated, all fluorescence data reported have been corrected for autofluorescence as described by Jung et al. [5,6]. Matrix $[Mg^{2+}]$ was calculated as described [6] using the ratios equation of Grynkiewicz et al. [11] and a K_d for Mg-furaptra at 25°C of 2.1 mM [4]. Such a calculation assumes that the ionic strength of the matrix is equivalent to that of 100 mM KCl (see [4]). Fluorescence intensity at 340 nm_{ex} rarely shows a significant change [4,6] and effectively serves as an isosbestic in these protocols. Retention of furaptra by mitochondria during these incubations was estimated from the excitation spectra of supernates after removing the mitochondria by centrifugation.

Furaptra-loaded mitochondria were suspended at 0.5 mg/ml in a medium of sucrose (0.25 M), HEPES (15 mM, neutralized to pH 7.2 with TEA hydroxide), Tris EGTA (1 mM), Tris succinate (10 mM), rotenone (5 μg/ml) and oligomycin (1.5 μg/ml). Initial values for total Mg^{2+} were obtained by immediately centrifuging the mitochondria at 0–4°C for 2 min in a Sorvall SE-12 rotor at 18 000 rpm. The tubes were blotted dry and the pellets extracted with 2 ml of 1 N perchloric acid. After protein removal the extract was diluted with an equal volume of water and Mg^{2+} determined by atomic absorption using a Varian Spectra AA-20. Initial values for $[Mg^{2+}]_m$ were obtained by furaptra fluorescence [6] in a parallel sample at 25°C.

When Mg^{2+} was present in the suspending medium, total Mg^{2+} values were corrected for occluded extramitochondrial Mg^{2+} in the pellet. Mitochondria were suspended in the sucrose-HEPES medium at 0–4°C with zero and increasing concentrations of external Mg^{2+}. The tubes were centrifuged immediately and Mg^{2+} determined. The pellet Mg^{2+} increases linearly with increasing external Mg^{2+} and the difference between the zero Mg^{2+} and any given external $[Mg^{2+}]$ was taken as the occluded extramitochondrial Mg^{2+}. In the absence of mitochondrial volume changes, corrections obtained in this way correspond well to those in which non-matrix water and solutes are determined by isotope distribution

[12,13]. External free [Mg^{2+}] was established using Mg^{2+}/EGTA buffers and the computer program of Brooks and Storey [14].

Excitation spectra were recorded in a SLM 8100 fluorimeter as described in [4] and corrected for differences in source intensity with wavelength. Mitochondrial swelling was followed by the change in absorbance at 520 nm using a Brinkman PC801 probe colorimeter with a 2 cm light path. Incubation conditions and any changes in the composition of the suspending medium are described with the individual experiments reported.

3. Results

3.1. Respiration-dependent Mg^{2+} efflux

Heart mitochondria respiring in a Mg^{2+}-free sucrose medium lose little endogenous Mg^{2+} in the absence of P_i (Fig. 1A). In the experiment shown, mitochondrial Mg^{2+} decreased from about 25 to 22 nmol/mg protein in the first 3 min and then changed little in 27 min at 25°C. A slow and nearly linear decrease in $[Mg^{2+}]_m$ is reported by furaptra fluorescence during this incubation (Fig. 1B). This value drops from 1.25 to about 0.9 mM in 30 min under these conditions. This decrease in $[Mg^{2+}]_m$ is slightly over estimated due to the loss of small amounts of the sequestered probe (less than 10%) to the Mg-free medium over this extended period of incubation.

When this protocol is repeated in the presence of P_i, the loss of total Mg^{2+} is much more extensive (Fig. 1A). Under these conditions, total Mg^{2+} falls to 13 nmol/mg in a typical experiment, a net loss of 12 nmol/mg in 30 min. This decrease is also reflected in $[Mg^{2+}]_m$ which decreases to about 0.2 mM in a 30-min incubation (Fig. 1B). The accelerated loss of total Mg^{2+} from mitochondria respiring in a Mg-free medium supplemented with P_i is in line with earlier reports [3,8,12]. It should be noted that the loss of total Mg^{2+} from furaptra-loaded mitochondria exactly parallels that from preparations treated in the same way but which contain no probe (not shown). In the experiment shown in Fig. 1B, about 13% of the probe was lost to the Mg^{2+}-free medium in 30 min in the presence of P_i. This may reflect some loss of mitochondrial integrity, but if it is assumed to be simply a leak of the probe from the matrix, the 30 min value of 0.2 mM should be corrected upward to about 0.27 mM $[Mg^{2+}]_m$.

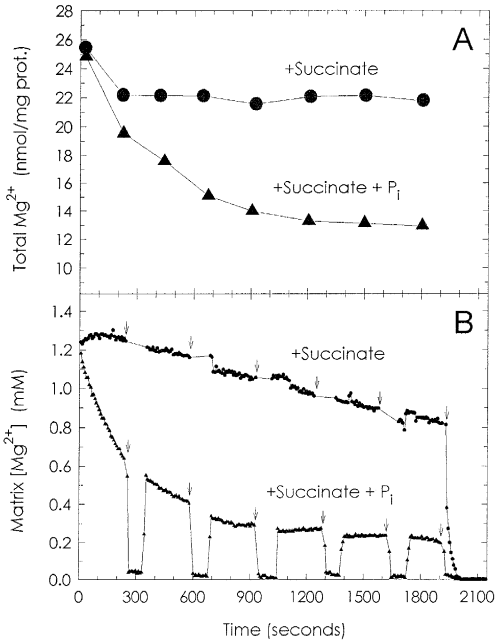

Fig. 1. Respiration-dependent extrusion of total Mg^{2+} (A) and decrease in $[Mg^{2+}]_m$ (B) in heart mitochondria suspended in a Mg-free medium. Beef heart mitochondria were loaded with furaptra [6] and suspended at 0.5 mg/ml in 130 ml of a medium of sucrose (0.20 M) containing HEPES (15 mM, neutralized to pH 7.2 with TEA hydroxide), rotenone (1 μg/ml), oligomycin (1.5 μg/ml), Tris EGTA (1 mM), and Tris succinate (10 mM) at 25°C in a stirred, water-jacketed beaker. At the indicated times in A, a sample (6 ml) was removed, centrifuged and Mg^{2+} determined by atomic absorption. At the start of the incubation a 3 ml sample was also removed to a cuvette and $[Mg^{2+}]_m$ estimated from furaptra fluorescence [6]. The experiment was then repeated in the presence of Tris P_i (3.3 mM). At the points indicated by the arrows in B, BrA23187 (2 μM) was added to deplete $[Mg^{2+}]_m$ and a new sample was placed in the fluorimeter. This was done to ensure that estimates of $[Mg^{2+}]_m$ corresponded as closely as possible to those of total Mg^{2+} taken from the large volume incubation. The results plotted are typical of three complete replications.

3.2. Respiration-dependent Mg^{2+} uptake

When heart mitochondria respire in the sucrose medium of Fig. 1 containing P_i and with external [Mg^{2+}] buffered at 2 mM, total mitochondrial Mg^{2+} increases from 22 to 67 nmol/mg protein in 12 min in a typical experiment (not shown). There is only a

small increase in mitochondrial Mg^{2+} in the absence of P_i under these conditions. These results agree well with previous reports from this laboratory [12]. A parallel determination in the fluorimeter shows a marked increase in $[Mg^{2+}]_m$, both in the presence and absence of P_i. Matrix $[Mg^{2+}]$ increases from about 0.55 mM to 1.15 mM in the presence of P_i and to 1.0 mM in its absence in 13 min in the above experiment. Matrix $[Mg^{2+}]$ remains relatively constant in the absence of respiration or P_i in this protocol (not shown).

3.3. Relationship of $[Mg^{2+}]_m$ to total mitochondrial Mg^{2+}

A plot of the data from 3 experiments in which Mg^{2+} efflux was measured in the presence of P_i (conditions of Fig. 1) shows that $[Mg^{2+}]_m$ is a linear function of total Mg^{2+} in the range from about 8 to 25 nmol/mg total Mg^{2+} (Fig. 2A, triangles). About 8 nmol total Mg^{2+}/mg remain when $[Mg^{2+}]_m$ approaches zero (Fig. 2A). A plot for 2 replications of the respiration-dependent uptake of Mg^{2+} shows that $[Mg^{2+}]_m$ increases with total Mg^{2+} accumulation (Fig. 2A, squares). The plot is roughly linear in the range from about 30 to 70 nmol Mg^{2+}/mg and shows that $[Mg^{2+}]_m$ increases by only about 0.006 mM for each nmol/mg increment in total Mg^{2+} gained in this region (Fig. 2A). It appears that the simultaneous uptake of P_i with Mg^{2+} under these conditions provides additional Mg ligands and limits the increase in $[Mg^{2+}]_m$.

Determinations of $[Mg^{2+}]_m$ and total Mg^{2+} for three different experiments in which respiring mitochondria were exposed to 2 mM $[Mg^{2+}]$ in the absence of P_i are plotted in Fig. 2B (closed circles). Initial values of $[Mg^{2+}]_m$ and total Mg^{2+} for 15 preparations of heart mitochondria determined in the absence of P_i are also plotted in Fig. 2B (open triangles). The total Mg^{2+} values range from 15 to 26 nmol/mg with a mean ($\pm$SD) of 21.1 ± 4.3 whereas $[Mg^{2+}]_m$ ranges from 0.33 to 1.25 mM with a mean of 0.67 ± 0.21 mM. The open circles in Fig. 2B compare $[Mg^{2+}]_m$ and total Mg^{2+} values obtained when mitochondria respiring under the conditions of Fig. 1 are treated with a low concentration of BrA23187. This produces a slow decrease in both

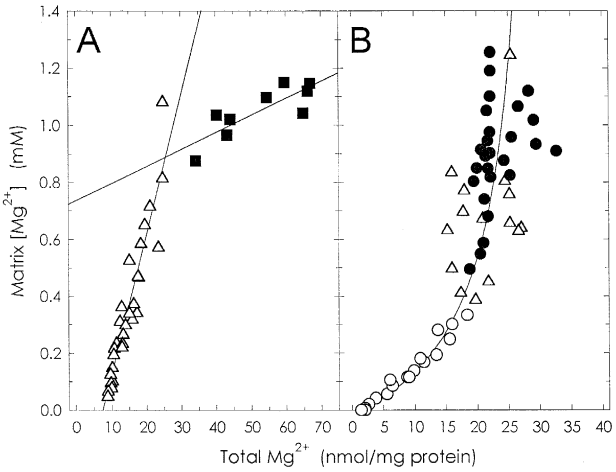

Fig. 2. Plots of $[Mg^{2+}]_m$ vs total Mg^{2+} for heart mitochondria respiring in the presence (A) or absence (B) of P_i. In A, the open triangles are the data from 3 replications of the respiration-dependent extrusion of total Mg^{2+} and decrease in $[Mg^{2+}]_m$ seen in Fig. 1 in the presence of 3.3 mM P_i. The regression line through these points has a slope of 0.05 and an intercept on the *X*-axis of 7.5 nmol/mg ($r = 0.940$). The solid squares are from two replications of the respiration-dependent accumulation of Mg^{2+} seen when mitochondria are suspended in the medium of Fig. 1 containing P_i and 2 mM buffered Mg^{2+}. The regression line for these points has a slope of 0.006 ($r = 0.828$). In B, the open triangles are the initial values of total Mg^{2+} and $[Mg^{2+}]_m$ for the 15 preparations evaluated in this study (see Section 2). The closed circles are data from three experiments in which Mg^{2+} was taken up by mitochondria respiring in the presence of 2 mM $[Mg^{2+}]_m$, but in the absence of P_i. The open circles show the decrease in these components in three experiments in which mitochondria were suspended in the medium of Fig. 1 in the absence of P_i and treated with a low level of BrA23187 (0.1 μM). The solid line is a hyperbolic curve-fit for all the data points and shows a maximum total Mg^{2+} of 31.6 nmol/mg and a K_d near 0.34 mM for Mg-binding sites under these conditions.

parameters as a function of time (not shown). All of the data in Fig. 2B are for mitochondria respiring with succinate in the absence of P_i. It is clear that when external Mg^{2+} is present, $[Mg^{2+}]_m$ can be increased with only minimal change in total Mg^{2+} and that total Mg^{2+} does not exceed a limit of about 30 nmol/mg. The ionophore depletes total Mg^{2+} to about 2–3 nmol/mg. The data of Fig. 2B can be fit to a rectangular hyperbola with a maximum at about 32 nmol/mg (solid line). The apparent K_d for mitochondrial binding sites in the absence of P_i is 0.34 mM.

3.4. Changes in $[Mg^{2+}]_m$ and total Mg^{2+} as a function of extramitochondrial Mg^{2+}

As shown in Fig. 1 mitochondria respiring in the presence of P_i, but no Mg^{2+}, lose both total Mg^{2+} and $[Mg^{2+}]_m$. When this protocol is repeated with increasing concentrations of buffered external $[Mg^{2+}]$, the decrease in $[Mg^{2+}]_m$ is considerably less at 0.1 mM than at zero external $[Mg^{2+}]$ (Fig. 3A, squares) and an increase in $[Mg^{2+}]_m$ is seen at and above 0.2 mM external $[Mg^{2+}]$. The loss of total Mg^{2+} is also less at 0.1 mM than at zero external $[Mg^{2+}]$ and at 0.2 mM there is a marked increase in this component (Fig. 3B). As external $[Mg^{2+}]$ is increased to 1.0 mM, $[Mg^{2+}]_m$ continues to increase toward a limit of about a 0.5 mM increase over the initial value (Fig. 3A). In contrast, the increase in total Mg^{2+} reaches a maximum at much lower external $[Mg^{2+}]_m$ (Fig. 3B).

In the presence of 10 mM acetate, total Mg^{2+} shows only a small increase as external $[Mg^{2+}]$ is increased (Fig. 3B, triangles). Acetate, like P_i, causes an increase in $\Delta\Psi$ that should promote cation uptake and the increase in $[Mg^{2+}]_m$ is greater as external $[Mg^{2+}]$ increases in the acetate medium (Fig. 3A). Acetate does not provide an effective Mg^{2+} ligand, so net accumulation is minimal. It is of interest that acetate does not support the respiration-dependent efflux of total Mg^{2+} and decrease in $[Mg^{2+}]_m$ seen with P_i (Fig. 3). In the absence of either P_i or acetate, $[Mg^{2+}]_m$ increases with increasing external $[Mg^{2+}]$ with only slight change in total Mg^{2+} (Fig. 3, circles). Changes in $[Mg^{2+}]_m$ that do not reflect changes in total Mg^{2+} may result from a redistribution of matrix anions or K^+ that alter matrix Mg^{2+} buffering.

3.5. Nigericin induces respiration-dependent uptake of Mg^{2+} and increased matrix $[Mg^{2+}]$

Nigericin promotes an extensive loss of endogenous K^+, equilibration of the $[K^+]$ gradient with the $[H^+]$ gradient and an increase in $\Delta\Psi$ at the expense of ΔpH when added to mitochondria respiring in the sucrose medium of Fig. 1. When the medium contains $[Mg^{2+}]$ buffered at 1 mM, but no P_i, the addition of nigericin results in a rapid increase in mitochondrial Mg^{2+}. Mitochondrial preparations (not equilibrated with furaptra) that averaged 30.5 ± 1.2 nmol Mg^{2+}/mg protein ($n = 7$) increased total Mg^{2+} to 55.1 ± 3.7 nmol/mg in 3 min when challenged with 1 μM nigericin. There was no change in the absence of nigericin under these conditions. There is a net loss of 50–60 nmol K^+/mg on addition of nigericin and a net increase of about 25 nmol Mg^{2+}/mg, so lost K^+ is essentially replaced by Mg^{2+} in this protocol.

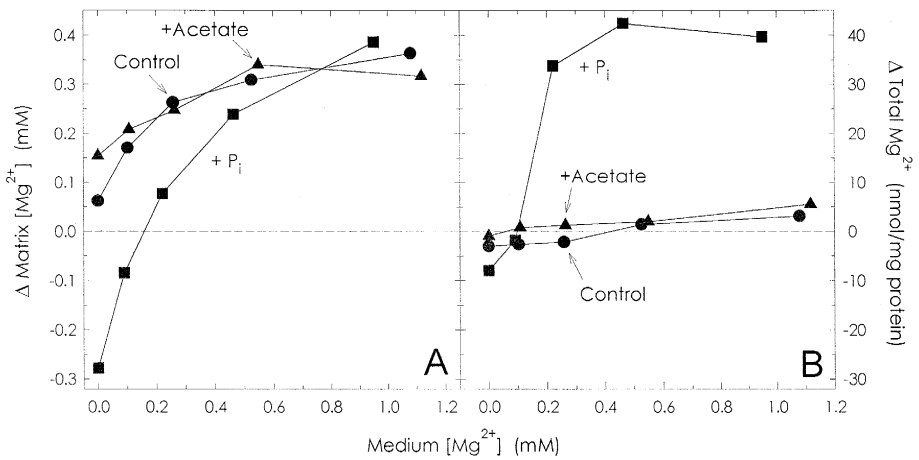

Fig. 3. Changes in $[Mg^{2+}]_m$ (A) and total Mg^{2+} (B) as a function of extramitochondrial $[Mg^{2+}]$ in respiring heart mitochondria. Furaptra-loaded mitochondria were suspended at 0.5 mg/ml in the medium of Fig. 1 containing either 3.3 mM Tris P_i (squares), 10 mM TEA acetate (triangles) or no further addition (circles). The free $[Mg^{2+}]$ of the medium was established using Mg/EGTA buffers and the mM value for each of the media was computed [14]. The change in $[Mg^{2+}]_m$ in 8 min at 25°C was measured using furaptra fluorescence and shown in A; the change in total Mg^{2+} shown in B was obtained from parallel incubations centrifuged at zero and 8 min of incubation as described in Fig. 2.

A marked increase in $[Mg^{2+}]_m$ accompanies the uptake of total Mg^{2+} induced by nigericin and is readily followed by furaptra fluorescence (Fig. 4). In this protocol $[Mg^{2+}]_m$ doubles in the first 60 sec after the addition of nigericin and reaches about 2.0 mM after 3 min, the time at which total Mg^{2+} has increased by 25 nmol/mg protein. The increase in $[Mg^{2+}]_m$ is roughly 5% of the increase in total Mg^{2+} under these conditions.

The nigericin-induced increase in total Mg^{2+} and $[Mg^{2+}]_m$ are both completely inhibited by uncouplers of oxidative phosphorylation or inhibitors of respiration (not shown). The nigericin-induced uptake of Mg^{2+} is only partially inhibited by ruthenium red (1 μM inhibits $35 \pm 4\%$; $n = 3$), by quinine (500 μM inhibits 28%) and by spermine (90 μM inhibits 50%). No completely specific inhibitor of Mg^{2+} uptake has been identified. The partial inhibition by ruthenium red suggests that a portion of the uptake may occur via the Ca-uniport. Nigericin-induced accumulation of Mg^{2+}, as well as increased $[Mg^{2+}]_m$, is abolished when an ionic medium, such as 100 mM KCl, NaCl or tetraethylammonium chloride, is used in place of sucrose. The increase in both components is also inhibited by lower concentrations of K^+ with 30 mM KCl inhibiting the rate of increase in $[Mg^{2+}]_m$ by 50% or more (Fig. 4). Increasing $[K^+]$ in the medium results in less loss of endogenous K^+ on nigericin addition and somewhat lower $\Delta\Psi$ in this protocol, so some of the inhibition could result from a decrease in driving force for Mg^{2+} uptake. However, 30 mM tetraethylammonium chloride has no effect on the driving force and still inhibits the accumulation of Mg^{2+} by 30% under these conditions (not shown). This indicates that the Mg^{2+} uptake induced by nigericin is effectively antagonized by the presence of more permeant cations in the medium, as we have noted for Mg^{2+} uptake under other conditions [9,12].

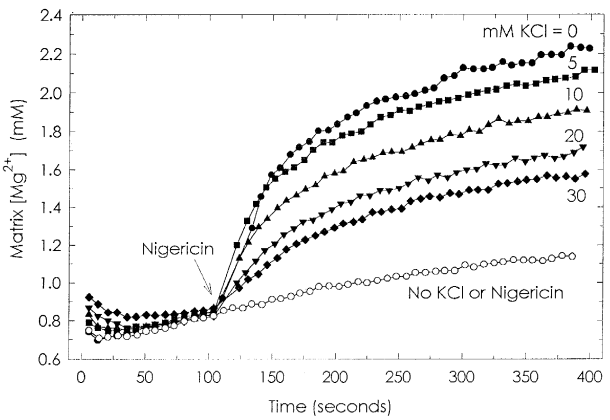

Fig. 4. Respiration-dependent increase in $[Mg^{2+}]_m$ in heart mitochondria induced by nigericin. Mitochondria were equilibrated with furaptra and suspended at 0.5 mg/ml and 25°C in a medium identical to that of Fig. 1 except that Tris succinate was 5 mM. $MgCl_2$ (1.5 mM) and Tris EGTA (2.3 mM) were added to buffer external $[Mg^{2+}]$ at 1 mM as well as the indicated concentrations of KCl. $[Mg^{2+}]_m$ was estimated from furaptra fluorescence [6]. Except where indicated, nigericin (1 μM) was added at 100 s to each incubation.

3.6. Mersalyl increases $[Mg^{2+}]_m$ in respiring mitochondria

Mersalyl has been shown to increase the rate and extent of Mg^{2+} accumulation in respiring heart mitochondria [12]. An uptake of more than 30 nmol/mg was seen in 4 min at 30°C in mitochondria respiring with succinate in a medium containing Mg^{2+} but no P_i [12]. The increase in total Mg^{2+} is accompanied by a marked increase in $[Mg^{2+}]_m$ when mersalyl (50 μM) is added to furaptra-loaded mitochondria under these conditions (Fig. 5). The excitation spectra show a large decrease in fluorescence intensity at 380 nm_{ex} as a function of time after mersalyl addition (Fig. 5) that is consistent with increasing $[Mg^{2+}]_m$. Fluorescence intensity in the 330–340 nm_{ex} region increases as $[Mg^{2+}]_m$ increases (Fig. 5), but as noted in a previous study [4], fluorescence of the matrix-sequestered probe is strongly attenuated in this region. Addition of Ca^{2+} increases fluorescence intensity further at 340 nm_{ex} (Fig. 5), but the peak intensity is attenuated relative to the Ca^{2+} spectrum seen in the absence of Mg^{2+} accumulation (see Fig. 5 in [4]).

$[Mg^{2+}]_m$ calculated from the change in fluorescence intensity as described in [4] increases from about 1 mM to over 4 mM in 9 min (Fig. 5 inset). In a comparable experiment in the absence of mersalyl, $[Mg^{2+}]_m$ increased by less than 0.5 mM in 9 min (not shown).

3.7. Changes in matrix $[Mg^{2+}]$ with changes in mitochondrial volume

When mitochondria respire in a KCl medium containing no added Mg^{2+}, addition of P_i results in

osmotic swelling due to K^+ phosphate accumulation (see [15] for a review). The extent of this swelling increases with increasing phosphate concentration (Fig. 6A). A parallel incubation monitoring furaptra fluorescence (Fig. 6B) shows that $[Mg^{2+}]_m$ decreases to a minimum that is about 60% of the initial value as the uptake of salt and osmotic swelling increase. A portion of this decline in $[Mg^{2+}]_m$ coincides with a small and somewhat variable loss of total Mg^{2+} under these conditions (not shown), but the increased availability of P_i as a Mg-ligand in the matrix undoubtedly contributes to the observed decrease (see [6]).

Mitochondria challenged with acetate under these conditions accumulate K^+ acetate and swell to the same extent as those accumulating the phosphate salt (Fig. 6A). However, there is a small increase in $[Mg^{2+}]_m$ with swelling in acetate rather than the decrease seen with P_i (Fig. 6B). It appears that, as K^+ acetate accumulates, sufficient Mg^{2+} is released from matrix binding sites to prevent a decrease in $[Mg^{2+}]_m$ as the matrix volume increases. These results suggest that $[Mg^{2+}]_m$ is buffered in such a way that it changes very little during osmotic swelling unless there is a net loss of total Mg^{2+} or an increase in ligand availability.

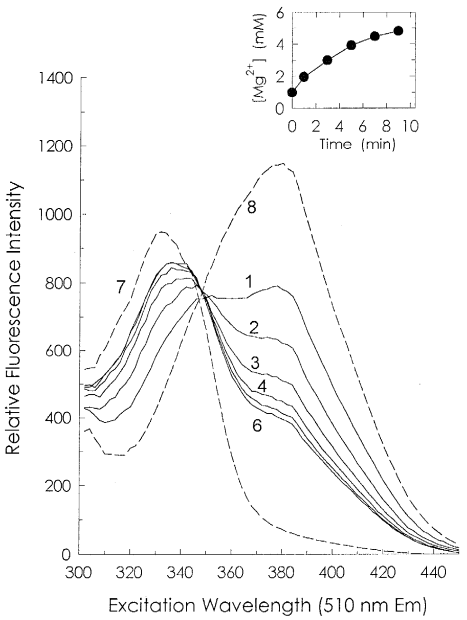

Fig. 5. Respiration-dependent increase in $[Mg^{2+}]_m$ induced by mersalyl. Heart mitochondria were equilibrated with furaptra and suspended at 0.125 mg/ml in a medium containing sucrose (0.2 M), TEA-HEPES (15 mM, pH 7.2), TEA-EGTA (30 μM), TEA-succinate (5 mM), $MgCl_2$ (l.5 mM), rotenone (1 μg/ml), oligomycin (1.5 μg/ml) and cyclosporin A (1 μM). The excitation spectrum was recorded at 25°C in a SLM 8100 fluorimeter with emission (Em) at 510 nm. Scan 1 had no further additions; scans 2–6 were recorded 1, 3, 5, 7 and 9 min after addition of mersalyl (50 μM). Scan 7 was recorded 3 min after further addition of $CaCl_2$ (2 mM). Scan 8 was recorded after a fresh cuvette was treated with BrA23187 (2 μM). The latter two scans were used to establish max and min fluorescence [4,6]. The inset shows the increase in $[Mg^{2+}]_m$ calculated from fluorescence intensity at 380 nm_{ex} as described in [4].

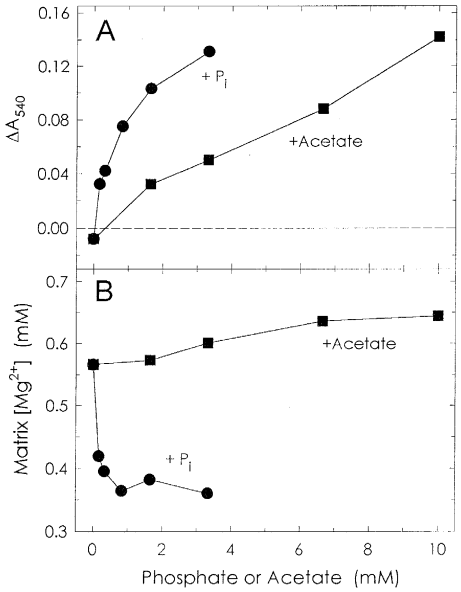

Fig. 6. Changes in $[Mg^{2+}]_m$ with osmotic swelling in K^+ phosphate and acetate. A: mitochondrial swelling as a function of P_i or acetate concentration. Mitochondria were suspended at 0.5 mg/ml and 25°C in a medium of KCl (0.1 M), K^+ HEPES (10 mM, pH 7.2), K^+ succinate (3 mM), Tris EGTA (2 μM), rotenone (1 μg/ml) and the indicated amount of K^+ P_i or acetate was added after 60 s. Swelling was recorded as the change in absorbance at 520 nm in 5 min. B: $[Mg^{2+}]_m$ calculated from furaptra fluorescence after 5 min under the conditions of A.

4. Discussion

These studies confirm and extend earlier indications that $[Mg^{2+}]_m$ increases and decreases with changing total Mg^{2+} in heart mitochondria [6,7,17,18]. $[Mg^{2+}]_m$ decreases to a very low value (less than 0.1 mM) as total Mg^{2+} is lost to a Mg-free

medium in a respiration-dependent reaction (Fig. 2A). When Mg^{2+} and P_i are accumulated, $[Mg^{2+}]_m$ increases with total Mg^{2+} (Fig. 2A), but a smaller percentage of the total Mg^{2+} is present as the free cation. $[Mg^{2+}]_m$ increases when total Mg^{2+} uptake is stimulated by increased $\Delta\Psi$, as seen with nigericin (Fig. 4), or when permeability to cations is increased by addition of mersalyl (Fig. 5). Corkey et al. [17] have pointed out that the high concentration and low affinity of mitochondrial binding sites will result in large changes in $[Mg^{2+}]_m$ with small variations in total Mg^{2+}.

The beef heart mitochondria used in the present work contain about 30 nmol total Mg^{2+}/mg protein as isolated. After incubation to load the mitochondria with furaptra this decreases to 21.1 ± 4.5 nmol/mg, a value that agrees well with the 20.6 ± 1.6 nmol/mg reported previously by this laboratory [6]. The water content of the matrix averages about 1 μl/mg protein in these mitochondria [12], so this value is equivalent to a total Mg^{2+} concentration of about 21 mM. The loading procedure decreases endogenous Ca^{2+} to 1.0 nmol/mg or less [6], but decreases total Mg^{2+} to some extent as well. The mean initial value for $[Mg^{2+}]_m$ found in the present study (0.67 ± 0.21 mM) is also very close to the 0.74 ± 0.10 mM found when the values reported in [6] are recalculated using the more appropriate K_d of 2.1 mM [4]. On the average about 3 to 4% of the total Mg^{2+} is present as $[Mg^{2+}]_m$ in these preparations.

4.1. Relationship of $[Mg^{2+}]_m$ to total mitochondrial Mg^{2+}

It is clear that $[Mg^{2+}]_m$ reflects the anion composition of the matrix as well as the total Mg^{2+} available. Previous studies have shown that $[Mg^{2+}]_m$ declines in heart mitochondria when P_i increases [6] and when liver mitochondria accumulate citrate [17]. Changes in mitochondrial $[Ca^{2+}]$ also affect $[Mg^{2+}]_m$ [17], but because the mitochondria used in the present study are Ca-depleted and the medium contains EGTA, such effects should be negligible.

When P_i is present in the suspending medium the uptake of P_i on the phosphate transporter converts ΔpH to $\Delta\Psi$ in respiring mitochondria and keeps matrix phosphate levels high. $[Mg^{2+}]_m$ reacts with the elevated P_i to produce adducts such as $MgHPO_4$ [6]. If external Mg^{2+} is present in a non-polar medium, the cation will accumulate in response to the elevated $\Delta\Psi$ [1,12] and large increases in both Mg and P_i will result (Fig. 2A and Fig. 3). When external $[Mg^{2+}]$ is high, deposition of Mg salts can drive the accumulation to very high levels [9]. Because P_i provides a strong Mg-ligand, the increase in $[Mg^{2+}]_m$ will be low under these conditions (less than 1% of the increase in total Mg^{2+}, Fig. 2A).

If P_i but no extracellular $[Mg^{2+}]$ is present, a respiration-dependent loss of total Mg^{2+} and $[Mg^{2+}]_m$ is seen (Fig. 1). Under these conditions $[Mg^{2+}]_m$ shows a good linear relationship to total Mg^{2+} (Fig. 2A, triangles) in the range from about 25 to 8 nmol/mg total Mg^{2+}. The slope of this plot shows that the decrease in $[Mg^{2+}]_m$ is about 5% of the total Mg^{2+} lost in this range. Extrapolation of this plot to zero $[Mg^{2+}]_m$ suggests that 7–8 nmol/mg Mg^{2+} is bound so tightly that it does not contribute to $[Mg^{2+}]_m$ under these conditions (Fig. 2A). A comparable component is not seen in the absence of P_i (Fig. 2B) and it seems likely that the high concentration of P_i contributes to this tight binding.

In the absence of P_i or acetate a plot of $[Mg^{2+}]_m$ vs total Mg^{2+} (Fig. 2B) can be fit to a rectangular hyperbola. This plot indicates that, under these conditions, a limit of about 32 nmol/mg Mg-binding sites are available with an apparent K_d of 0.34 mM (Fig. 2B). Corkey et al. [17], using a null-point analysis, estimated $[Mg^{2+}]_m$ to be 0.38 mM in rat liver mitochondria containing 31.7 nmol total Mg^{2+}/mg protein. The liver mitochondria contained about 26 nmol non-diffusible Mg-binding sites per mg with an apparent K_d of 0.22 mM [17]. These values are close to those obtained for heart mitochondria from the plot in Fig. 2B. Corkey et al. [17] estimated that 23% of total Mg^{2+} was present as the ATP complex, 51% was bound to non-diffusable binding sites, and the remainder complexed with ADP, P_i and citrate [17]. These liver mitochondria contained about 10 nmol/mg ATP and 16 nmol/mg total AN [17]. The beef heart mitochondria used in the present study show quite satisfactory morphology, respiratory control and other properties, but contain only low levels of ATP and AN (about 1 and 5 nmol/mg, respectively) [20]. This means that very little of the bound Mg^{2+} in these mitochondria is associated with AN

and that non-diffusible binding sites probably account for the bulk of the bound Mg^{2+}.

The plot in Fig. 2B includes points obtained when respiring mitochondria extrude Mg^{2+} in the presence of a low concentration of BrA23187. This plot reflects the equilibration of bound Mg^{2+} with $[Mg^{2+}]_m$ followed by efflux of free Mg^{2+} by $Mg^{2+}/2H^+$ exchange on the ionophore. Under these conditions, 2–3 nmol/mg total Mg^{2+} is not removed (Fig. 2B) and appears to be very tightly bound. An earlier null-point analysis also identified a tightly bound fraction of total Mg^{2+}, and tight-binding of this cation by mitochondrial components such as the F_0F_1 ATPase and cytochrome oxidase has been reported [21,22]. In addition to this very tightly bound component, about 5 nmol/mg of the total Mg^{2+} is bound tightly enough to be retained during respiration-dependent extrusion in the presence of P_i (Fig. 2A).

The limit of 32 nmol/mg for Mg-binding sites is readily exceeded when the uptake of P_i provides additional Mg-ligands (Fig. 2A and Fig. 3) or when accumulation of Mg acetate produces osmotic swelling [19]. When mitochondrial K^+ is depleted, as in the nigericin protocol in Fig. 4, additional Mg^{2+} binding sites appear to be available and substantial Mg^{2+} uptake occurs.

4.2. Respiration-dependent Mg^{2+} efflux

There is a large decrease in $[Mg^{2+}]_m$ during the respiration-dependent efflux of Mg^{2+} first reported by Crompton et al. [8] and both the loss of total Mg and the decrease in $[Mg^{2+}]_m$ require the presence of P_i (Fig. 1). Mg^{2+} does not come to electrochemical equilibrium across the inner membrane, so an effective Mg^{2+} efflux pathway must be available to mitochondria in situ [1]. It has been suggested that respiration-dependent Mg^{2+} efflux occurs via a $Mg^{2+}/2H^+$ antiport [12,16,23]. However, such a pathway would not be favored by the low ΔpH seen in the presence of P_i. If the antiport were not electroneutral, exchanges such as $Mg^{2+}/3H^+$ could result in Mg^{2+} extrusion driven by $\Delta\Psi$. Such a situation appears to contribute to Ca^{2+} efflux on the Ca^{2+}/nNa^+ antiport of heart mitochondria [13].

Acetate produces changes in ΔpH and $\Delta\Psi$ of the same magnitude as P_i, but does not support Mg^{2+} efflux (Fig. 3). This suggests that there is a direct requirement for P_i and that a pathway for the loss of a species, such as $MgHPO_4$, may be available. The transporter described by Aprille [24] might be modified to provide such a pathway, but this component has been reported to be absent in heart mitochondria. Romani et al. [25] have presented evidence that Mg^{2+} is lost in parallel with ATP via a modified adenine nucleotide translocator in liver mitochondria. However, the pool of endogenous AN in beef heart mitochondria is too low to support the level of total Mg loss seen in Fig. 1A by an obligatory Mg-ATP efflux pathway. The extrusion of Mg^{2+} from mitochondria shares many of the features of respiration-dependent K^+ loss (see [1,2] for reviews), but the mechanism for the respiration and P_i-dependent loss of Mg^{2+} seen in Fig. 1 is still obscure.

4.3. Respiration-dependent Mg^{2+} uptake

The question of whether there is a specific transporter for Mg^{2+} uptake is also not resolved. The permeability of the mitochondrion to Mg^{2+} is low as judged from $^{28}Mg^{2+}$ equilibration studies and the uptake of Mg^{2+} by mitochondria is strongly inhibited by both AN and monovalent cations [9,12]. These considerations would keep Mg^{2+} influx into mitochondria in situ very low under normal circumstances. However, two sets of conditions promote the uptake of Mg^{2+} by mitochondria in vitro. The first is an increase in $\Delta\Psi$ as provided by nigericin (Fig. 4) or P_i (Fig. 3) and the second is an increase in permeability to cations, as induced by mersalyl (Fig. 5).

The electrophoretic permeability of the inner membrane of the mitochondrion to H^+ and cations has been shown to increase in a non-ohmic way as $\Delta\Psi$ increases [26]. R. Hafner and M.D. Brand (personal communication) have shown that rat liver mitochondria respiring in a sucrose medium containing 40 mM Mg acetate take up Mg^{2+} and acetate and swell osmotically. This reaction increases dramatically with increasing $\Delta\Psi$ and shows a non-ohmic relationship to the potential. We have confirmed that beef heart mitochondria also respond in this way (not shown). The rapid influx of Mg^{2+} in response to nigericin seen in the present study (Fig. 4) seems best explained by such a diffusive leak in response to a large increase in $\Delta\Psi$. Phosphate also increases $\Delta\Psi$ and

would favor uptake by such a mechanism. Although there is some indirect evidence for the presence of a specific transporter for Mg^{2+} influx [2], the lack of a unique inhibitor profile makes it difficult to ascribe reactions, such as that shown in Fig. 4, to a specific mechanism.

Zn^{2+}, mercurials and other metals that appear to increase membrane permeability also increase respiration-dependent Mg^{2+} accumulation [27]. Elevated levels of mersalyl have been shown to increase passive permeability of the mitochondrion to K^+ [28] and to increase Mg^{2+} uptake in the presence of acetate [19]. The present studies show that mersalyl induces large increases in $[Mg^{2+}]_m$ under conditions that would not increase $\Delta\Psi$ (Fig. 5). Mercurials and similar reagents probably increase permeability by reacting with and modifying transporters that normally have other functions (see [29] or [30]). The present results seem best explained by electrophoretic Mg^{2+} uptake via non-specific leak pathways, but the presence of a low activity uniporter cannot be excluded.

4.4. $[Mg^{2+}]_m$ and matrix volume

Garlid [31,32] has proposed that mitochondrial volume control is maintained by the action of a latent K^+/H^+ antiport and an anion channel that are both regulated by Mg^{2+}. In line with this model, Jung et al. [6], using furaptra fluorescence, showed a marked decrease in $[Mg^{2+}]_m$ when heart mitochondria are swollen hypotonically and a return to the initial value on recontraction. This experiment is consistent with $[Mg^{2+}]_m$ responding as an indicator of matrix volume changes and with an increase in matrix water diluting a constant pool of $[Mg^{2+}]_m$. However, quantitation of the decrease in $[Mg^{2+}]_m$ on hypotonic swelling is undermined by the uncertainty of the value for matrix ionic strength under these conditions and the strong dependence of the K_d for furaptra on this value [4]. In contrast to hypotonic swelling, osmotic swelling under the conditions of Fig. 6 would not be expected to produce large changes in matrix ionic strength [15].

The present work shows that $[Mg^{2+}]_m$ decreases with swelling when a Mg-ligand such as P_i is accumulated, but not when it is due to the uptake of K^+ acetate (Fig. 6). The failure of $[Mg^{2+}]_m$ to decrease during osmotic swelling in K^+ acetate suggests that the buffering power of matrix ligands is sufficient to maintain nearly constant $[Mg^{2+}]_m$ over a wide range of volume changes. If osmotic swelling doubles matrix water to 2 μl/mg protein, for example, this would require release of only 0.6 nmol/mg total Mg^{2+} to maintain matrix $[Mg^{2+}]_m$ at 0.6 mM. The $[K^+]$ of the matrix remains near 0.1 M during osmotic swelling in K^+ acetate [15] and it seems likely that bound Mg^{2+} is replaced by K^+ under these conditions. The decrease in $[Mg^{2+}]_m$ seen with hypotonic swelling [6] may indicate a lack of available matrix cations to displace bound Mg^{2+}. The requirement for a Mg-binding anion to decrease $[Mg^{2+}]_m$ during osmotic swelling gives qualitative support to Garlid's model [31,32] for mitochondrial volume control.

It seems clear that $[Mg^{2+}]_m$ can be varied over a considerable range in isolated mitochondria by both transport reactions and changing ligand availability. Even if transport reactions in situ are slow [12], changes in ligand availability with changing P_i, citrate, or adenine nucleotides could well produce significant shifts in $[Mg^{2+}]_m$. It is therefore quite reasonable to visualize changes in this component contributing to the regulation of Mg-sensitive reactions in the mitochondria of functioning heart cells.

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References

[1] D.W. Jung, G.P. Brierley, J. Bioenerg. Biomembr. 26 (1994) 527–535.
[2] J.J. Diwan, Biochim. Biophys. Acta 895 (1988) 155–165.
[3] A. Romani, A. Scarpa, Arch. Biochem. Biophys. 298 (1992) 1–12.
[4] D.W. Jung, C.J. Chapman, K. Baysal, D.R. Pfeiffer, G.P. Brierley, Arch. Biochem. Biophys. 332 (1996) 19–29.
[5] D.W. Jung, G.P. Brierley, Magnesium Trace Elem. 10 (1992) 151–164.

[6] D.W. Jung, L. Apel, G.P. Brierley, Biochemistry 29 (1990) 4121–4128.
[7] G.A. Rutter, N.J. Osbaldson, J.G. McCormack, R.M. Denton, Biochem. J. 271 (1990) 627–634.
[8] M. Crompton, M. Capano, E. Carafoli, Biochem. J. 154 (1976) 735–742.
[9] G.P. Brierley, E. Murer, E. Bachmann, D.E. Green, J. Biol. Chem. 238 (1963) 3482–3489.
[10] G.P. Brierley, M.S. Jurkowitz, T. Farooqui, D.W. Jung, J. Biol. Chem. 259 (1984) 14672–14678.
[11] G. Grynkiewicz, M. Poenie, R.Y. Tsien, J. Biol. Chem. 260 (1985) 3440–3450.
[12] G.P. Brierley, M.H. Davis, D.W. Jung, Arch. Biochem. Biophys. 253 (1987) 322–332.
[13] D.W. Jung, K. Baysal, G.P. Brierley, J. Biol. Chem. 270 (1995) 672–678.
[14] S.P.J. Brooks, K.B. Storey, Anal. Biochem. 201 (1992) 119–126.
[15] G.P. Brierley, in: B.F. Trump and A.V. Arstila (Eds.), Pathophysiology of Cell Membranes, Academic Press, New York, 1983.
[16] G.P. Brierley, M.H. Davis, D.W. Jung, J. Bioenerg. Biomembr. 20 (1988) 229–242.
[17] B.E. Corkey, J. Duszynski, T.L. Rich, B. Matschinsky, J.R. Williamson, J. Biol. Chem. 261 (1986) 2567–2574.
[18] D.W. Jung, G.P. Brierley, J. Biol. Chem. 261 (1986) 6408–6415.
[19] G.P. Brierley, C.T. Settlemire, V.A. Knight, Biochem. Biophys. Res. Commun. 28 (1967) 420–425.
[20] D.W. Jung, G.P. Brierley, J. Biol. Chem. 259 (1984) 6904–6911.
[21] A.E. Senior, J. Biol. Chem. 256 (1981) 4763–4767.
[22] O. Einersdottir, W.S. Caughey, Biochem. Biophys. Res. Commun. 129 (1985) 840–845.
[23] K.E.O. Akerman, J. Bioenerg. Biomembr. 13 (1981) 133–139.
[24] J.R. Aprille, J. Bioenerg. Biomembr. 25 (1993) 473–481.
[25] A. Romani, C. Marfella, A. Scarpa, J. Biol. Chem. 268 (1993) 15489–15495.
[26] G.C. Brown, M.D. Brand, Biochem. J. 234 (1986) 75–81.
[27] G.P. Brierley, G.R. Hunter, W.E. Jacobus, J. Biol. Chem. 242 (1967) 2192–2198.
[28] E. Chavez, D.W. Jung, G.P. Brierley, Arch. Biochem. Biophys. 183 (1977) 460–470.
[29] R. Stappen, R. Kramer, Biochim. Biophys. Acta 1149 (1993) 40–48.
[30] A.D. Beavis, Eur. J. Biochem. 185 (1989) 511–519.
[31] K.D. Garlid, J. Biol. Chem. 255 (1980) 11273–11279.
[32] K.D. Garlid, in: J.J. Lemasters, C.R. Hackenbrock, R.G. Thurman and H.V. Westerhoff (Eds.), Integration of Mitochondrial Function, Plenum Press, New York, 1988, pp. 257–276.