Temperature Dependence of the Mitochondrial Inner Membrane
Anion Channel: The relationship between temperature and inhibition by magnesium

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SUMMARY

The mitochondrial inner membrane anion channel (IMAC) carries a wide variety of anions and is postulated to be involved in mitochondrial volume homeostasis in conjunction with the K⁺/H⁺ antiporter allowing the respiratory chain proton pumps to drive salt efflux. How it is regulated is uncertain; however, it is inhibited by matrix Mg²⁺ and matrix protons. Previously determined values for the IC₅₀ suggested that the channel would be closed under physiological conditions. In a previous study (Liu, G., Hinch, B., Davatol-Hag, H., Lu, Y., Powers, M., and Beavis, A. D., J. Biol. Chem. 271, 19717-19723, 1996), it was demonstrated that the channel is highly temperature dependent and that a large component of this sensitivity resulted from an effect on the pIC₅₀ for protons. We have now investigated the effect of temperature on the inhibition by Mg²⁺ and have found that it too is temperature dependent. When the temperature is raised from 20°C to 45°C, the IC₅₀ increases from 22 µM to 350 µM at pH 7.4 and from 80 µM to 1.5 mM at pH 8.4 respectively. The Arrhenius plot for the IC₅₀ is linear with a slope = -80 kJ/mol. The IC₅₀ is also strongly pH dependent and at 37°C increases from 90 µM at pH 7.4 to 1230 µM at pH 8.4. In view of the extremely rapid fluxes that IMAC is capable of conducting at 37°C, it is concluded that inhibition by matrix Mg²⁺ and protons is necessary to limit its activity under physiological conditions. It is concluded that primary role of Mg²⁺ is to ensure IMAC poised to allow regulation by small changes in pH in the physiological range. This control is mediated by a direct effect of H⁺ on the activity in addition to an indirect effect mediated by a change in the Mg²⁺ IC₅₀. The question that remains is not whether IMAC can be active at physiological concentrations of Mg²⁺ and H⁺, but what other factors might increase its sensitivity to changes in mitochondrial volume.
INTRODUCTION

The mitochondrial inner membrane anion channel (IMAC) is a non-selective anion channel that carries a wide variety of anions ranging from small singly charged ions, such as $\text{Cl}^-$ and $\text{HCO}_3^-$, to multicharged anions, such as citrate, ferrocyanide and even ATP (reviewed in ref. 1). In view of the variety of anions transported and the fact that under physiological conditions mitochondria generate a membrane potential that is about 180mV negative on the inside, we have hypothesized that IMAC in conjunction with the $\text{K}^+/\text{H}^+$ antiporter is involved in mitochondrial volume homeostasis (1). The combined action of these transporters coupled to the proton pumps of the respiratory chain provides a mechanism for respiratory energy to drive salt efflux. In recent years, other roles have been proposed for IMAC. Vanden Hoek, et al (2) have proposed that IMAC may also be involved in the efflux of the superoxide anion from mitochondria during ischemic preconditioning, and O’Rourke’s group (3, 4) has provided evidence suggesting IMAC is involved in synchronized oscillations of mitochondrial membrane potential in isolated cardiac myocytes.

Although, in energized mitochondria, the anion flux through IMAC is expected to be in the outward direction, IMAC is most easily assayed in de-energized mitochondria by monitoring the rate of passive mitochondrial swelling that occurs following the addition of the potassium ionophore valinomycin to mitochondria suspended in potassium salts of the test anion (1). Using this assay, it has been shown that potential physiological regulators of IMAC include matrix $\text{Mg}^{2+}$ and matrix protons (5, 6). A number of nonphysiological inhibitors have also been identified. These include amphiphilic amines such as propranolol, the irreversible inhibitor $\text{N,N’-dicyclohexylcarbodiimide}$ (DCCD), and tributyltin, which is probably the most potent inhibitor of IMAC identified to date (see ref. (1) for review of properties).
More recently, we demonstrated that IMAC is extremely sensitive to temperature, in a manner that suggests the open probability is temperature dependent (7). For example, using malonate as the substrate anion, the flux increased about 1000-fold when the temperature was raised from 5°C to 40°C. A large part of this stimulation appears to result from a decrease in the pIC$_{50}$ of one of the inhibitory protonation sites. The most significant aspect of these findings is that they suggest the activity of IMAC might be significantly higher at physiological pH values and temperatures, than predicted from previous studies, which were carried out at 25°C. The actual activity under physiological conditions cannot be predicted, however, since the effects of temperature were determined in Mg$^{2+}$-depleted mitochondria and the effect of temperature on the inhibition by matrix Mg$^{2+}$ is unknown. On the basis of published values for the IC$_{50}$ for Mg$^{2+}$ determined at 25°C (6), Jung and Brierley (8) have pointed out that activation of IMAC is unlikely at physiological concentrations of Mg$^{2+}$ and, for similar reasons, O’Rourke (3) has stated that the physiological role of IMAC is unclear. Thus, the goal of the present work was to determine the effect of temperature on the inhibition of IMAC by Mg$^{2+}$ and thus shed light on its physiological significance.

The data presented show that the IC$_{50}$ for Mg$^{2+}$ is strongly dependent on temperature increasing from about 30 µM at 25°C to about 100 µM at 37°C; moreover, at 37°C it increases further to 1.2 mM when the pH is raised to 8.4. From these results, we estimate that in the presence of 0.5 mM Mg$^{2+}$ at 37°C and pH 7.4, IMAC may be about 7% active. In view of the very high intrinsic activity of this channel, this represents a very significant flux of 400 nmol/min.mg.
EXPERIMENTAL PROCEDURES

Assay of anion transport

Anion transport was assayed by following the swelling that accompanies net salt transport, using the light scattering technique as described in detail elsewhere (9-11). In brief, reciprocal absorbance, which as a function of mitochondrial volume, is monitored and normalized for mitochondrial protein concentration in the assay to yield a parameter referred to as β, the rate of change of which can be converted to a rate of ion transport in nmol/min per mg mitochondrial protein. In a previous study of the effect of temperature on IMAC (7), we used malonate as the substrate anion; however, in order to investigate inhibition by Mg²⁺ we chose to use Cl⁻, since the association between Mg²⁺ and Cl⁻ is much weaker than between Mg²⁺ and malonate and much less Mg²⁺ must be added to obtain a given concentration of free Mg²⁺.

Pretreatment of Mitochondria with A23187

In order to deplete the mitochondria of endogenous Mg²⁺, the normal stock suspension was pretreated as described in ref. 7, except the amount of A23187 was increased to 10nmol/mg mitochondrial protein to allow rapid equilibration of Mg²⁺ across the inner membrane following transfer to the assay medium. In brief, the mitochondrial stock suspension (50µg/ml) was diluted 1:5 into a medium containing potassium salts of MOPS (25 mM) and EDTA (5 mM) adjusted to pH 7.4 at 25°C and maintained at 0°C. A23187 (10 nmol/mg), nigericin (0.5 nmol/mg) and rotenone (0.5 µg /mg) were added and at least 10 minutes allowed to elapse before transfer of aliquots to the assay medium.
Assay Media for Anion Transport

The potassium chloride medium for assay of IMAC contained the potassium salts of Cl- (55 mM), EGTA (0.1 mM) and MOPS (5 mM for assays at pH 7.4) or TAPS (5 mM for assays at pH 8.4). After addition of the pretreated mitochondria, the final assay medium also contained EDTA (50 µM) sucrose (0.5 mM). All media were 110 mOsm. For experiments in which the temperature was to be varied, the pH of the medium was adjusted at 25°C to a value, calculated on the basis of –0.0095 for ΔpK/°C for MOPS and –0.021 for ΔpK/°C for TAPS, that would yield the desired pH at the assay temperature. Thus, separate assay media were prepared for each temperature to be studied. The value of ΔpK/°C was determined experimentally in the assay media described above. The temperature of the medium in the assay tube was measured during the experiment to ensure that a steady value had been achieved. The free concentration of Mg²⁺ was calculated for each temperature and pH value using the program WinMaxC, available from C. Patton, Ph.D., Stanford University, Hopkins Marine Station, CA. The same program was used to calculate the total Mg²⁺ to be added to obtain a specific free concentration.

Drugs, Reagents and Mitochondria

Most drugs were obtained from Sigma. Valinomycin, nigericin and rotenone were dissolved in ethanol and A23187 (20 mM stock) was dissolved in dimethylsulfoxide. Rat liver mitochondria were prepared from 30-35 day old Sprague-Dawley rats as described previously (9), except that the first slow-spin pellet was not resuspended, but discarded and the rats were not starved.
Analysis of data

All figures were prepared and nonlinear regression accomplished using the program GraphPad Prism version 3.03 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

RESULTS

Effect of Temperature on Inhibition of IMAC by Mg$^{2+}$

In a previous paper (6), we showed that when the mitochondrial inner membrane anion channel (IMAC) is assayed at 25°C, it is inhibited by matrix Mg$^{2+}$ and that the IC$_{50}$ rises from 38µM at pH 7.4 to 250µM at pH 8.4. The values obtained were not affected by the method used to remove endogenous Mg$^{2+}$ nor the time of addition of Mg$^{2+}$ to the assay medium. In the present study, because at high temperature the rate of swelling is very rapid, the mitochondria were pretreated with A23187 and EDTA to ensure complete depletion of endogenous Mg$^{2+}$, and Mg$^{2+}$ and valinomycin were added to the assay at zero time, i.e. before the mitochondria. The data contained in Fig. 1 show typical traces obtained at 3 temperatures, 25°C for comparison with previous data, 37°C to determine the flux at physiological temperatures and 15°C to illustrate behavior at low temperatures. In each case, there is a short acceleration phase before maximum swelling rates are observed. Mg$^{2+}$ inhibited the fluxes at all three temperatures and the IC$_{50}$ values obtained from the dose response curves shown in Fig. 2 are 114 µM, 37µM and 17µM at 37°C, 25°C and 15°C respectively. Note, however, that the Hill coefficients tend to increase with the temperature. Consistent with our previous studies (7), the control rates are strongly temperature dependent with values equal to 4.0, 1.2 and 0.23 µmol Cl/ min.mg at 37°C, 25°C and 15°C respectively.
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Relationship Between Mg$^{2+}$ IC$_{50}$ and Temperature

To examine more closely the relationship between the Mg$^{2+}$ IC$_{50}$ and temperature and how this might be affected by pH, we carried out dose response studies at a series of temperatures ranging from 5°C to 45°C and at two pH values pH7.4 and 8.1. For each temperature, the pH of the assay medium was adjusted separately to ensure that the assay pH did not vary with temperature.

The data contained in Fig. 3 show Arrhenius plots of the control rates of Cl$^-$ transport in the absence of Mg$^{2+}$ at the two pH values. Non-linear relationships are observed, consistent with the data obtained previously using malonate as the substrate for IMAC (7). Note that there is a 275-fold increase in rate when the temperature is raised from 10°C to 45°C at pH 7.4, and at 10°C, there is a 15-fold increase in rate when the pH is raised from 7.4 to 8.1.

The curves are drawn using equation 1 (Eq. 9 of ref. 7), which was derived on the basis of a model in which the open probability of the channel is temperature dependent.

$$\ln J = A + \ln T - \frac{\Delta H_{flux}}{RT} + \frac{\Delta S_{flux}}{R} - \ln \left( 1 + \exp \left( \frac{\Delta H_{open}}{R} \left( \frac{1}{T} - \frac{1}{T_{50}} \right) \right) \right)$$

where $\Delta H_{flux}$ and $\Delta S_{flux}$ are the activation enthalpy and entropy of the transport process and $T_{50}$ is the temperature at which 50% of the channels are open, which is given by $T_{50} = \frac{\Delta H_{open}}{\Delta S_{open}}$ where $\Delta H_{open}$ and $\Delta S_{open}$ are the enthalpy and entropy of channel opening respectively.

Like the transport rate, the Mg$^{2+}$ IC$_{50}$ is strongly dependent on the temperature increasing exponentially as the temperature is raised (Fig. 4A). In contrast to the Arrhenius plots for the transport rates, the Arrhenius plots of the IC$_{50}$ values are essentially linear. Using values interpolated from the curves fitted to the data, the ratio of IC$_{50}$ values range from about 4.6 at 45°C to 5.3 at 20°C. The narrow temperature range and scatter in the data do not allow one to
determine to what extent the effect of pH results from a change in the slope or intercept.

However, whatever the cause of the shift, there is about a five-fold increase in Mg\(^{2+}\) IC\(_{50}\) as the pH is raised from 7.4 to 8.1 over the temperature range examined.

**Influence of Temperature on the Relationship between Mg\(^{2+}\) IC\(_{50}\) and pH**

To examine further the effect of temperature on the relationship between the IC\(_{50}\) for Mg\(^{2+}\) and pH, the IC\(_{50}\) was determined at a series of pH values ranging from 7.35 to pH 8.35 at 25°C and 37°C. The results of two independent experiments contained in Fig. 5 reveal that the IC\(_{50}\) increases 10-fold and 16-fold at 25°C and 37°C respectively. The curves drawn were fitted to the data using a model, previously shown to describe the relationship between the Mg\(^{2+}\) IC\(_{50}\) and pH at 25°C (6), in which

\[
IC_{50} = K_{Mg}(1 + (K_H/[H^+])^n)
\]  

(2)

Because the intercept on the ordinate is so close to the origin, the data do not allow one to determine whether the effect of temperature is mediated via an effect on \(K_{Mg}\) or the pK for the protonation site. The slopes, however, which are determined by the product \(K_{Mg} K_H\) and \(n\) differ by a factor of 7. Using the constants provided in the legend, values of IC\(_{50}\) can be calculated. Thus, at 37°C, the IC\(_{50}\) rises from 90 µM at pH 7.4 (cf. 114 µM in Fig. 1) to 250 µM at pH 7.8. This latter value is considerably closer to published estimates of the physiological free matrix Mg\(^{2+}\) concentration (8).

**Estimate of IMAC Activity at Physiological Mg\(^{2+}\) Concentration**

It is now well accepted that the physiological concentration of free Mg\(^{2+}\) in the mitochondrial matrix is close to 0.5 mM (8). It is also likely that dramatic changes in the free concentration do not occur under most physiological conditions. Thus, the temperature
dependence of IMAC in the presence of this fixed concentration of Mg$^{2+}$ is of interest. Since at pH 7.4 rates are extremely low at low temperatures, especially with Mg$^{2+}$ present, we carried out this experiment at both pH 7.4 and pH 8.4. The data presented in Fig 6A show that at pH 8.4, both in the presence and absence of Mg$^{2+}$, the Arrhenius plot is nonlinear. The curves were fitted to the data using equation 1, in which the nonlinearity is attributed to a change in the open probability of the channel. Interestingly, comparison of these data with those in Fig. 3 reveals that the curve in the presence of 0.5 mM Mg$^{2+}$ at pH 8.4 is very similar to that obtained in the absence of Mg$^{2+}$ at pH 7.4. Thus, it is evident that like H$^+$, Mg$^{2+}$ changes the temperature dependence of the channel. This effect is most conveniently expressed as a change in T$_{50}$, brought about by the preferential binding of H$^+$ and Mg$^{2+}$ to the closed state of the channel. The curves fitted indicate that the T$_{50}$ increases from about 10° C in the control curve to about 29° C in the presence of 0.5 mM Mg$^{2+}$. The latter curve was fitted using the $\Delta H_{\text{flux}}$ obtained from the control curve. The narrow temperature range and scatter in the data do not permit one to determine whether this change results from a change in $\Delta H_{\text{open}}$ or $\Delta S_{\text{open}}$. Due to the fact that it is difficult to measure accurately the very low rates observed when both pH and temperature are low, only rates measured between 45° C and 30° C are shown for pH 7.4. To allow comparison of the T$_{50}$ with the other values, the curve was fitted assuming that $\Delta H_{\text{open}}$ and $\Delta H_{\text{flux}}$ were the same as at pH 8.4 yielding a value of T$_{50}$ = 49° C.

To better illustrate the effect of Mg$^{2+}$ and temperature on the activity of IMAC, we have plotted the data as percent inhibition of the rate at 45° C in the absence of Mg$^{2+}$ (Fig. 6B). The control curve (pH 8.4, no Mg$^{2+}$, solid circles) shows the “inhibitory” effect of lowering temperature. Between 45° C and 25° C, IMAC is inhibited by about 75% with a Q$_{10}$ = 2. The open circles and open squares show the inhibition of the flux by temperature plus 0.5 mM Mg$^{2+}$. 

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At pH 8.4, the flux increases dramatically between 35°C and 45°C, while at pH 7.4 the channel is almost completely inhibited at 40°C and below. The open triangles show the percent inhibition, relative to the individual control rates at each temperature, produced by 0.5 mM Mg²⁺ at pH 8.4. Note that at 37°C there is 34% inhibition. These data suggest that Mg²⁺ poises the channel to be very sensitive to changes in mitochondrial matrix pH at physiological temperatures.

**DISCUSSION**

In this paper, we have examined the temperature dependence of the IC₅₀ for matrix Mg²⁺ for inhibition of the mitochondrial inner membrane anion channel. In view of the combined effects of pH and temperature on the Mg²⁺ IC₅₀ and their direct effects on the activity of IMAC, the data presented provide strong evidence that IMAC may have considerable activity under physiological conditions.

The data presented demonstrate that Mg²⁺ is an efficacious inhibitor over a temperature range that is associated with a 350-fold change in anion flux. We have observed, however, that the Hill coefficient for the inhibition tends to increase with temperature. It is quite likely that this reflects the involvement of more than one inhibitory Mg²⁺ binding site at high temperatures. A similar but more pronounced phenomenon was observed for inhibition by protons (7), which was explained by the presence of a second inhibitory site with a lower temperature dependence. The Hill coefficient of less than unity seen at 15°C may reflect the presence of a small uninhibitable component, which only becomes significant at the very low fluxes observed at the low temperatures. For consistency, however, all IC₅₀ values were determined on the basis of the assumption that the flux could be completely inhibited.

The values for Mg²⁺ IC₅₀ increase quite dramatically as the temperature is raised. At 45°C, values of 1.5 mM and 0.35 mM were obtained at pH 8.4 and pH 7.4 respectively. It is also
evident that the IC50 remains pH dependent over the whole temperature range. We have previously reported (6) that the IC50 may be expressed as a function of the binding constants for a magnesium binding site (K_{Mg}) and a proton binding site (K_H) (see equation 2). Consequently, the slope of the Arrhenius plot for the IC50, which is linear and equal to -77-81 kJ/mol, is dependent on the enthalpies of binding for both Mg^{2+} and H^+ (\Delta H_{Mg} and \Delta H_{H}). From these data, the interpolated values for the Mg^{2+} IC50 at 37°C are 114 \mu M and 550 \mu M at pH 7.4 and 8.1 respectively.

The dependence of the Mg^{2+} IC50 on pH (Fig. 5) is consistent with the model previously proposed (6), although the Hill coefficient appears to increase slightly with temperature so that the increase in IC50 with pH is greater at 37°C than it is at 25°C. Because both the curves extrapolate to values of IC50 very close to the origin, it is not possible to determine whether K_{Mg} is affected by temperature. Both curves shown were fitted by setting K_{Mg} = 8 \mu M and allowing pK_H and the Hill coefficients (n) to vary. Using this approach, the effect of raising the temperature from 25°C to 37°C is explained by a decrease in the value of pK_H from 6.9 to 6.5, and an increase in n from 0.89 to 1.17. Curves can be fit equally well, however, if it is assumed that pK_H = 6.7 at both temperatures and K_{Mg} and n are allowed to vary. In this case, the increase in IC50 can be explained by an increase in K_{Mg} from 6.3 to 13 \mu M together with the above-mentioned increase in n. Both models allow the Mg^{2+} IC50 to be calculated. For example, at 37°C interpolated values of the IC50 are 90, 250 and 1230 \mu M free Mg^{2+} at the pH values of 7.4, 7.8 and 8.4 respectively.

These data indicate that at 37°C, as the pH rises from 7.4 to 8.4, the IC50 for Mg^{2+} passes from values that are about 15% of the free Mg^{2+} to values that are at least two-fold higher than the free Mg^{2+}. Thus, as the pH changes over this range, the activity of IMAC not only increases
because of the diminished inhibition by protons, but also due to a decrease in the IC\textsubscript{50} for Mg\textsuperscript{2+}. Needless to say, the flux through a pathway is not simply dependent on the percent inhibition, but also the intrinsic activity of the process. Because IMAC has a very high J\textsubscript{max}, even when Mg\textsuperscript{2+} and H\textsuperscript{+} concentrations are significantly above their IC\textsubscript{50} values, IMAC still has considerable activity. From the data presented here and the pH dependence previously described (7), we estimate that at 37°C the J\textsubscript{max} ([H\textsuperscript{+}] = 0, [Mg\textsuperscript{2+}] = 0) is 6-7 µmol Cl\textsuperscript{-}/min.mg. To place this value in perspective, it should be noted that the maximum rates of respiration on succinate are of the order of 200 nmol O/min.mg. Moreover, since the net H\textsuperscript{+}/O stoichiometry of proton pumping is 6-7 (11), this respiration rate corresponds to a proton flux of about 1400 nmol H\textsuperscript{+}/min.mg, which is less than 25% of the capacity of IMAC. Thus, in order to avoid uncoupling of oxidative phosphorylation, in vivo IMAC must be tightly controlled and could have sufficient activity even at just a few percent of its maximum activity to play an important role in volume homeostasis. In order for the respiratory chain to drive net salt efflux, activity of both IMAC and the K\textsuperscript{+}/H\textsuperscript{+} antiporter is required (1). In a preliminary study, we have found that at 37°C and pH 8.4 the K\textsuperscript{+}/H\textsuperscript{+} antiporter has a J\textsubscript{max} ([Mg\textsuperscript{2+}] = 0) of 1.3 µmol/min.mg and a Mg\textsuperscript{2+} IC\textsubscript{50} of 220 µM. (results not shown). Thus, under physiological conditions, the K\textsuperscript{+}/H\textsuperscript{+} antiporter could also have significant activity. Note that the relatively high activity of IMAC, would ensure that respiration (H\textsuperscript{+} pump) driven anion efflux would elevate matrix pH and further activate both the K\textsuperscript{+}/H\textsuperscript{+} antiporter and IMAC, which are both regulated by matrix protons (1,14). Note also that the very high J\textsubscript{max} for IMAC relative to the rate of mitochondrial respiration is also consistent with the suggestion by Aon \textit{et al} (4) that IMAC is responsible for depolarization of mitochondria in myocytes observed in their studies.
In order to illustrate better the potential roles of H\(^+\) and Mg\(^{2+}\) in the regulation of IMAC, we have plotted the Cl\(^-\) flux calculated on the basis of the Mg\(^{2+}\) dependence presented here and the pH dependence reported previously (1). Fig. 7A shows the predicted flux as a function free Mg\(^{2+}\) at different pH values taking into account direct effects of pH on the flux as well as its effect on the Mg\(^{2+}\) IC\(_{50}\). Given that estimates of physiological free Mg\(^{2+}\) are 0.5-0.6 mM, only as the pH becomes alkaline do small changes in Mg\(^{2+}\) significantly affect the rate. Fig. 7B shows the predicted flux as a function of pH at various free Mg\(^{2+}\) concentrations. Note that the effect of physiological concentrations of Mg\(^{2+}\) is to move the pH dependence to a point that the flux will be very sensitive to pH changes in the 7.2-7.8 range. It should also be noted that the range of fluxes shown have been limited to the maximum that could be sustained by the proton pumps of the respiratory chain. The flux at pH7.4 and 0.5 mM Mg\(^{2+}\) is 400 nmol/min.mg, which is only 6.5% of the J\(_{\text{max}}\) ([H\(^+\])=0, [Mg\(^{2+}\]) = 0), but still high enough that in vivo some other factor probably limits the flux further.

Even though changes in Mg\(^{2+}\) may not be sufficient to regulate IMAC, the major function of Mg\(^{2+}\) may be to ensure that the channel is poised to be responsive to other factors that fine tune its activity and to ensure that it does not have excessive activity under physiological conditions. The large number of inhibitors that have so far been identified (1,12,13) suggests that there is a very delicate balance between the open and closed states of IMAC. Many unidentified factors could be involved in regulation IMAC’s open probability. In addition to pH, these factors could include changes in matrix volume. As also suggested by others (8), volume \textit{per se} could exert effects via cytoskeletal proteins that might be involved in control of mitochondrial shape and membrane folding. In view of the complex convoluted nature of the mitochondrial inner membrane and its cristae, this could provide a much more sensitive sensor of
mitochondrial volume changes. Aon et al (4) have recently proposed that superoxide may also activate IMAC. Thus, the question that remains is not whether IMAC can be active at physiological concentrations of Mg$^{2+}$ and H$^+$, but what other factors might increase its sensitivity to changes in mitochondrial volume.

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FOOTNOTES

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1The abbreviations used are: IMAC, inner membrane anion channel; MOPS, 3-(N-morpholino)-propanesulfonic acid; TAPS, N-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid
FIGURE LEGENDS

Fig. 1. **Effect of temperature on inhibition of IMAC by Magnesium.** Light scattering kinetics of mitochondria (0.11 mg/ml) swelling in potassium chloride assay medium at pH 7.4 containing valinomycin (0.5 nmol/mg) and A23187 (10 nmol/mg) and various concentrations of Mg\(^{2+}\) are shown. Panel A: Temperature 37°C with the following concentrations of free Mg\(^{2+}\) (µM); trace a, 0; b, 71; c, 110; d, 187; e, 265; f, 342; g, 420; h, 536; i, 731. Panel B: Temperature 25°C with free Mg\(^{2+}\) (µM); trace a, 0; b, 16; c, 54; d, 74; e, 103; f, 153. Panel C: Temperature 15°C with free Mg\(^{2+}\) (µM); a, 0; b, 5; c, 18; d, 36; e, 75; f, 154. Rates of data collection were 18 points/s for data in panel A and 3.03 points/s in panels B and C. The mitochondria were pretreated with A23187 (10 nmol/mg), nigericin (0.5 nmol/mg), and rotenone (0.5 µg/mg) as described under “Experimental Procedures”. The assay media and calculation of free Mg\(^{2+}\) are described under “Experimental Procedures”.

Fig. 2. **Effect of temperature on dose-response curves for inhibition of IMAC by Magnesium.** Dose-response curves for inhibition of Cl\(^-\) fluxes determined from the traces contained in Fig. 1 are shown. Rates were determined from the traces by applying linear regression to the data points collected between β values of 0.5 and 0.6 as described under “Experimental Procedures”. IC\(_{50}\) values and Hill coefficients (n) were determined using nonlinear regression and the equation \( J = J_0/(1 + ([Mg^{2+}]/IC_{50})^n) \) and assuming complete inhibition at infinite [Mg\(^{2+}\)]. Panel A: Temperature 37°C, IC\(_{50}\) = 114 µM, n = 1.49. Panel B: temperature 25°C, IC\(_{50}\) = 37 µM, n = 1.18. Panel C: Temperature 15°C, IC\(_{50}\) = 117 µM, n = 0.79.

Fig. 3. **Effect of temperature on Cl- flux in the absence of Mg\(^{2+}\).** Arrhenius plots are shown for Cl- fluxes measured at temperatures ranging from 5°C to 45°C at pH 7.4 (circles) and...
pH 8.1 (squares). Rates of Cl⁻ transport were determined as described in Figs. 1 and 2. The curves were fitted using nonlinear regression as described in the text. The open and closed symbols represent data obtained from two independent experiments using different mitochondrial preparations. See “Experimental Procedures” for composition of assay and pretreatment media and other experimental details.

Fig. 4. **Effect of temperature on the IC₅₀ for Mg²⁺.** The Mg²⁺ IC₅₀ was determined at various temperatures ranging from 5°C to 45°C, as described in Figs. 1 and 2, in KCl assay media at pH 7.4 (circles) and pH 8.1 (squares). Panel A shows there is an exponential increase in IC₅₀ as the temperature is raised. Panel B: Arrhenius plots of the data. Open and closed symbols represent data from two independent experiments using different mitochondrial preparations. See “Experimental Procedures” and text for further details.

Fig. 5. **Effect of temperature on the relationship between the IC₅₀ for Mg²⁺ and pH.** The Mg²⁺ IC₅₀ was determined in KCl assay media adjusted to pH values ranging from pH 7.3 to pH 8.3 at 25°C (circles) and 37°C (squares), as described in Figs. 1 and 2. Open and closed symbols represent data from two independent experiments using different mitochondrial preparations. Both curves were fitted to the data using nonlinear regression with the equation: IC₅₀ = K_Mg.(1 + (K_H/[H⁺])ⁿ) and setting K_Mg = 8 µM derived from analysis of the data obtained at 37°C. At 25°C, pK_H = 6.88 and n = 0.89, while at 37°C, pK_H = 6.53 and n = 1.17. See “Experimental Procedures” and text for further details.

Fig. 6. **Effect of Mg²⁺ and pH on the temperature dependence of IMAC.** Chloride flux through IMAC is shown at pH 8.4 (circles) and pH 7.4 (squares) as a function of temperature in KCl assay medium; in the presence (open symbol) or absence (closed symbol) of 0.5 mM free Mg²⁺. Panel A: Arrhenius plots are shown. Panel B: The percent “inhibition” of Cl⁻ flux by
decrease in temperature, relative to the control rate at pH 8.4 and 45°C, is plotted versus the
temperature. The percent inhibition by Mg\(^{2+}\) relative to the control rate at each temperature is
also shown (open triangles). Fluxes were measured as described in Fig. 1. To maintain a
constant free Mg\(^{2+}\) of 0.5 mM, the amount of Mg\(^{2+}\) added to each assay medium was adjusted to
compensate for the effects of temperature and pH. See “Experimental Procedures” and text for
further details.

Fig. 7. Effects of Mg\(^{2+}\) and pH in regulating IMAC at 37°C. Theoretical curves are
plotted using the equation

\[ J = J_0/(1 + ([\text{Mg}^{2+}]_{\text{free}}/\text{Mg}^{2+}\text{IC}_{50})^n) \]

in which the values of Mg\(^{2+}\) IC\(_{50}\) and \(n\) were determined from Figs. 2, 4 and 5 and values of \(J_0\)
for each pH determined using Eq. 10 of ref. (7) with the value of pK\(_1\) = 7.1 to fit the data
presented in this paper. A. Flux versus free Mg\(^{2+}\) at pH values a, 7.0; b, 7.2; c, 7.4; d, 7.5, e,
7.6; f, 7.7; g, 7.8. B. Flux versus pH at free \([\text{Mg}^{2+}]\) (mM) of a, 0; b, 0.2; c, 0.3; d, 0.5; e, 0.7; f,
0.9; g, 1.2. Note: the range of Y-axis values is limited to focus on the maximum range that could
be coupled to the respiratory chain proton pumps. The maximum value shown represents the
theoretical rate that would be seen if all proton pump activity were coupled to the anion flux
through IMAC.
FIGURES

Figure 1, Beavis and Powers
Figure 2, Beavis and Powers

[A graph showing the relationship between $[\text{Mg}^{2+}]_{\text{free}}$ and $J_{\text{Cl}}$ for three panels: A, B, and C. The graphs illustrate the decrease in $J_{\text{Cl}}$ as $[\text{Mg}^{2+}]_{\text{free}}$ increases.]
Figure 3, Beavis and Powers

\[ \ln(J_{Cl} \text{ (nmol/min.mg)}) = \frac{1000}{T} \]

Graph showing the relationship between \( \ln(J_{Cl}) \) and \( 1000/T \).
Figure 4, Beavis and Powers

A

Mg\(^{2+}\) IC\(_{50}\) (\(\mu\)M)

Temperature (°C)

B

\(\ln [\text{Mg}^{2+} \text{ IC}_{50}\] (\(\mu\)M)]

\(1000/T\)
Figure 5, Beavis and Powers

![Graph showing the relationship between Mg$^{2+}$ IC$_{50}$ (µM) and pH.](http://www.jbc.org/Downloadedfrom)
Figure 6, Beavis and Powers

A

\[ \ln(J_{Cl} \text{ (nmol/min.mg)}) \]

1000/T

\[ 3.1 \ 3.2 \ 3.3 \ 3.4 \ 3.5 \ 3.6 \]

Temperature (°C)

B

% Inhibition

Temperature (°C)
Figure 7, Beavis and Powers

A

\[ J_{Cl} \] (µmol/min.mg)

\[ \left[ Mg^{2+} \right]_{\text{free}} \] (µM)

B

\[ J_{Cl} \] (µmol/min.mg)

pH
Temperature dependence of the mitochondrial inner membrane anion channel: The relationship between temperature and inhibition by magnesium
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