

Temperature Dependence of the Mitochondrial Inner Membrane Anion Channel

THE RELATIONSHIP BETWEEN TEMPERATURE AND INHIBITION BY MAGNESIUM*

Received for publication, September 22, 2003

Published, JBC Papers in Press, November 13, 2003, DOI 10.1074/jbc.M310475200

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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, thus allowing the respiratory chain proton pumps to drive salt efflux. How it is regulated is uncertain; however, it is inhibited by matrix Mg^{2+} and matrix protons. Previously determined values for the IC_{50} 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. (1996) *J. Biol. Chem.* 271, 19717–19723), it was demonstrated that the channel is highly temperature-dependent, and that a large component of this sensitivity resulted from an effect on the pIC_{50} for protons. We have now investigated the effect of temperature on the inhibition by Mg^{2+} and have found that it too is temperature-dependent. When the temperature is raised from 20 °C to 45 °C, the IC_{50} increases from 22 to 350 μM at pH 7.4 and from 80 to 1.5 mM at pH 8.4, respectively. The Arrhenius plot for the IC_{50} is linear with a slope = -80 kJ/mol. The IC_{50} 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, we conclude that inhibition by matrix Mg^{2+} and protons is necessary to limit its activity under physiological conditions. We conclude that the primary role of Mg^{2+} is to ensure IMAC is 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 Mg^{2+} IC_{50} . 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.

The mitochondrial inner membrane anion channel (IMAC)¹ is a non-selective anion channel that carries a wide variety of anions ranging from small, singly charged ions, such as Cl^- and HCO_3^- , to multicharged anions, such as citrate, ferrocya-

nide, 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 180 mV negative on the inside, we have hypothesized that IMAC, in conjunction with the K^+/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 and coworkers (3, 4) have 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 after the addition of the potassium ionophore valinomycin to mitochondria suspended in potassium salts of the test anion (1). By using this assay, it has been shown that potential physiological regulators of IMAC include matrix 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 *N,N'*-dicyclohexylcarbodiimide, 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 to 40 °C. A large part of this stimulation seems 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 what has been predicted from previous studies that were carried out at 25 °C. The actual activity under physiological conditions cannot be predicted, however, because 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.

* This work was supported in part by the American Heart Association, Ohio Affiliate, Columbus, Ohio. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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

The data presented show that the IC_{50} for Mg^{2+} is strongly dependent upon temperature increasing from about $30 \mu M$ at $25^\circ C$ to about $100 \mu M$ at $37^\circ C$; moreover, at $37^\circ C$ 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 $37^\circ C$ 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 $400 nmol/min \cdot mg$.

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, 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 ; its rate of change 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, to investigate inhibition by Mg^{2+} , we chose to use Cl^- , because the association between Mg^{2+} and Cl^- is much weaker than between Mg^{2+} and malonate, and much less Mg^{2+} must be added to obtain a given concentration of free Mg^{2+} .

Pretreatment of Mitochondria with A23187—To deplete the mitochondria of endogenous Mg^{2+} , the normal stock suspension was pretreated as described in Ref. 7, except that the amount of A23187 was increased to $10 nmol/mg$ mitochondrial protein to allow rapid equilibration of Mg^{2+} across the inner membrane after transfer to the assay medium. In brief, the mitochondrial stock suspension ($50 mg/ml$) was diluted 1:5 into a medium containing potassium salts of MOPS ($25 mM$) and EDTA ($5 mM$), adjusted to pH 7.4 at $25^\circ C$, and maintained at $0^\circ C$. A23187 ($10 nmol/mg$), nigericin ($0.5 nmol/mg$), and rotenone ($0.5 \mu g/mg$) were added, and at least 10 min was 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 the addition of the pretreated mitochondria, the final assay medium also contained EDTA ($50 \mu M$) and sucrose ($0.5 mM$). All media were 110 milliosmolar. For experiments in which the temperature was to be varied, the pH of the medium was adjusted at $25^\circ C$ to a value, calculated on the basis of -0.0095 for $\Delta pK^\circ/C$ for MOPS and -0.021 for $\Delta pK^\circ/C$ 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 $\Delta pK^\circ/C$ 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 Mg^{2+} was calculated for each temperature and pH value using the program WinMaxC (courtesy of C. Patton, Stanford University). The same program was used to calculate the total Mg^{2+} 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 dimethyl sulfoxide. Rat liver mitochondria were prepared from 30 to 35-day-old Sprague-Dawley rats as described previously (9), except that the first trace-spin pellet was discarded and not resuspended, and the rats were not starved.

Analysis of Data—All figures were prepared and nonlinear regression was accomplished by using the program GraphPad Prism version 3.03 (GraphPad, San Diego, CA).

RESULTS

Effect of Temperature on Inhibition of IMAC by Mg^{2+} —In a previous paper (6), we showed that when the mitochondrial IMAC is assayed at $25^\circ C$, it is inhibited by matrix Mg^{2+} , and that the IC_{50} rises from $38 \mu M$ at pH 7.4 to $250 \mu M$ at pH 8.4. The values obtained were not affected by the method used to remove endogenous Mg^{2+} nor by the time of addition of Mg^{2+} to the assay medium. In the present study, because the rate of swelling is very rapid at high temperature, 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

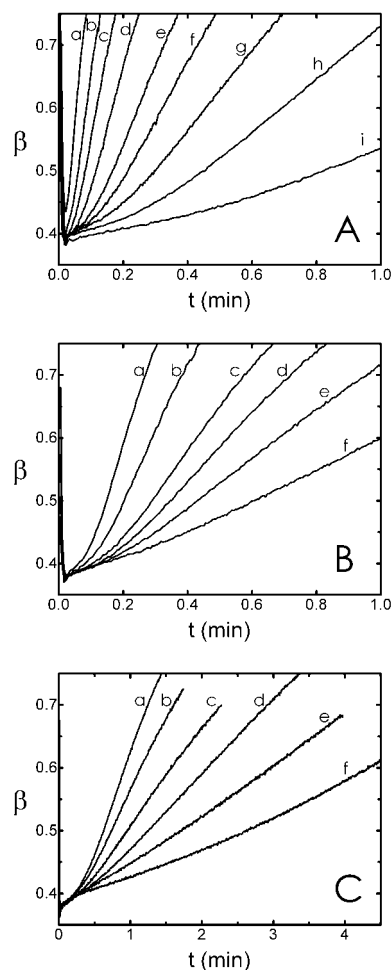


FIG. 1. Effect of temperature on inhibition of IMAC by magnesium. Light-scattering kinetics of mitochondrial ($0.11 mg/ml$) swelling in potassium chloride assay medium at pH 7.4 containing valinomycin ($0.5 nmol/mg$), A23187 ($10 nmol/mg$), and various concentrations of Mg^{2+} are shown. *A*, temperature of $37^\circ C$ 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. *B*, temperature of $25^\circ C$ with free Mg^{2+} (μM): trace *a*, 0; *b*, 16; *c*, 54; *d*, 74; *e*, 103; *f*, 153. *C*, temperature of $15^\circ C$ 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 ($0.5 \mu g/mg$), as described under "Experimental Procedures." The assay media and calculation of free Mg^{2+} are described under "Experimental Procedures."

at three temperatures: $25^\circ C$ for comparison with previous data, $37^\circ C$ to determine the flux at physiological temperatures, and $15^\circ C$ 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 $114 \mu M$, $37 \mu M$, and $17 \mu M$ at $37^\circ C$, $25^\circ C$, and $15^\circ C$, 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 $0.23 \mu mol Cl^-/min \cdot mg$ at $37^\circ C$, $25^\circ C$, and $15^\circ C$, respectively.

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 to $45^\circ C$ and at two pH values, pH 7.4 and 8.1. For each temperature, the pH of the assay medium was adjusted

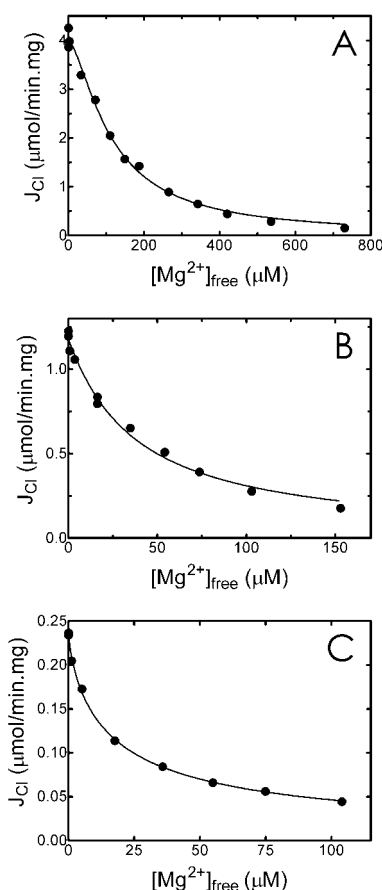


FIG. 2. Effect of temperature on dose-response curves for inhibition of IMAC by magnesium. Dose-response curves for inhibition of Cl^- fluxes determined from the traces contained in Fig. 1 are shown. Rates were determined from the traces by applying linear regression to the data points collected between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

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 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 (equation 9 of Ref. 7), which was derived on the basis of a model in which the open probability of the channel is temperature-dependent.

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

where ΔH_{flux} and ΔS_{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 $T_{50} = \Delta H_{\text{open}} / \Delta S_{\text{open}}$ where ΔH_{open} and ΔS_{open} are the enthalpy and entropy of channel opening, respectively.

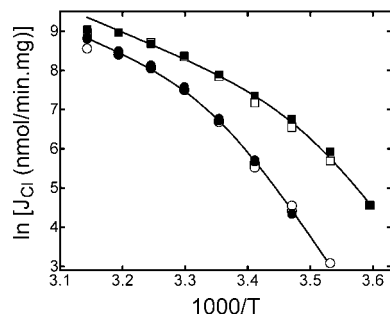


FIG. 3. Effect of temperature on Cl^- flux in the absence of Mg^{2+} . Arrhenius plots are shown for Cl^- fluxes measured at temperatures ranging from 5 to 45 °C at pH 7.4 (circles) and 8.1 (squares). Rates of Cl^- transport were determined as described in Figs. 1 and 2. The curves were fitted by using nonlinear regression, as described in the text. The black and white 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.

Like the transport rate, the Mg^{2+} IC_{50} is strongly dependent upon 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 5-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

$$\text{IC}_{50} = K_{\text{Mg}} (1 + (K_{\text{H}} / [\text{H}^+])^n) \quad (\text{Eq. 2})$$

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 by means of an effect on K_{Mg} or the pK for the protonation site. The slopes, however, which are determined by the product $K_{\text{Mg}} \cdot K_{\text{H}}$ 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 (compare 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. Because 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

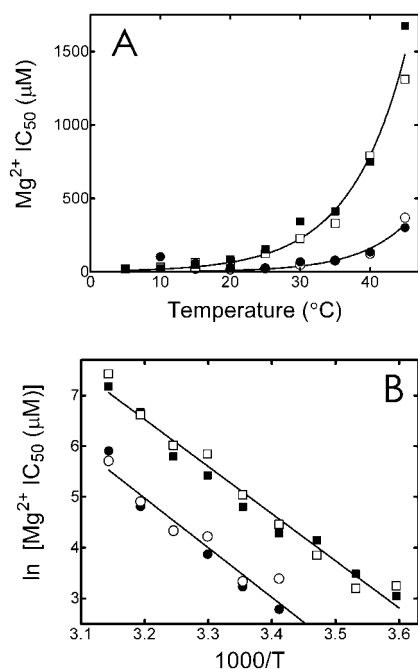


FIG. 4. **Effect of temperature on the IC_{50} for Mg^{2+} .** The Mg^{2+} IC_{50} was determined at various temperatures ranging from 5 to 45 °C, as described in Figs. 1 and 2, in KCl assay media at pH 7.4 (circles) and 8.1 (squares). A, exponential increase in IC_{50} as the temperature is raised. B, Arrhenius plots of the data. White and black symbols represent data from two independent experiments using different mitochondrial preparations. See "Experimental Procedures" and text for further details.

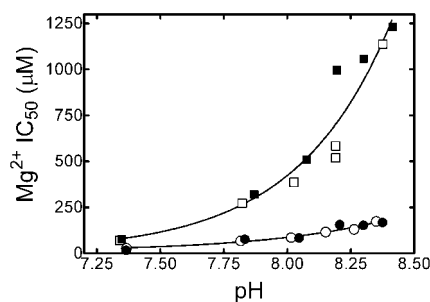


FIG. 5. **Effect of temperature on the relationship between the IC_{50} for Mg^{2+} and pH.** The Mg^{2+} IC_{50} was determined in KCl assay media adjusted to pH values ranging from pH 7.3 to 8.3 at 25 (circles) and 37 °C (squares), as described in Figs. 1 and 2. White and black symbols represent data from two independent experiments using different mitochondrial preparations. Both curves were fitted to the data using nonlinear regression with the equation: $IC_{50} = K_{Mg} \cdot (1 + (K_H/[H^+])^n)$ and setting $K_{Mg} = 8 \mu M$, which was derived from analysis of the data obtained at 37 °C. At 25 °C, $pK_H = 6.88$, and $n = 0.89$, whereas at 37 °C, $pK_H = 6.53$, and $n = 1.17$. See "Experimental Procedures" and text for further details.

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

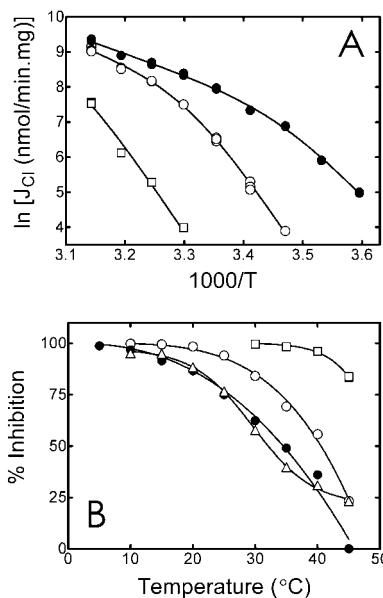


FIG. 6. **Effect of Mg^{2+} and pH on the temperature dependence of IMAC.** Chloride flux through IMAC is shown at pH 8.4 (circles) and pH 7.4 (squares) as a function of temperature in KCl assay medium; in the presence (white symbol) or absence (black symbol) of 0.5 mM free Mg^{2+} . A, Arrhenius plots are shown. 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 (white 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.

29 °C in the presence of 0.5 mM Mg^{2+} . The latter curve was fitted using the ΔH_{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 ΔH_{open} or ΔS_{open} . Because of 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 and 30 °C are shown for pH 7.4. To allow comparison of the T_{50} with the other values, the curve was fitted by assuming that ΔH_{open} and ΔH_{flux} were the same as at pH 8.4, thus 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+} ; Fig. 6B, black circles) shows the "inhibitory" effect of lowering temperature. Between 45 and 25 °C, IMAC is inhibited by about 75%, with a $Q_{10} = 2$. In Fig. 6B, the white circles and white squares show the inhibition of the flux by temperature plus 0.5 mM Mg^{2+} . At pH 8.4, the flux increases dramatically between 35 °C and 45 °C, whereas at pH 7.4, the channel is almost completely inhibited at 40 °C and below. The white triangles show the percent inhibition, relative to the individual control rates at each temperature, produced by 0.5 mM Mg^{2+} at pH 8.4. Note that, at 37 °C, there is 34% inhibition. These data suggest that Mg^{2+} predisposes 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 IC_{50} for matrix Mg^{2+} for inhibition of the mitochondrial inner membrane anion channel. In view of the combined

effects of pH and temperature on the Mg^{2+} IC_{50} 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 Mg^{2+} 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 Mg^{2+} -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 IC_{50} values were determined on the basis of the assumption that the flux could be completely inhibited.

The values for Mg^{2+} IC_{50} increase quite dramatically as the temperature is raised. At 45 °C, values of 1.5 and 0.35 mM were obtained at pH 8.4 and 7.4, respectively. It is also evident that the IC_{50} remains pH-dependent over the whole temperature range. We have previously reported (6) that the IC_{50} 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 IC_{50} , which is linear and equal to -77 to -81 kJ/mol, is dependent upon the enthalpies of binding for both Mg^{2+} and H^+ (ΔH_{Mg} and ΔH_H). From these data, the interpolated values for the Mg^{2+} IC_{50} at 37 °C are 114 and 550 μ M at pH 7.4 and 8.1, respectively.

The dependence of the Mg^{2+} IC_{50} on pH (Fig. 5) is consistent with the model previously proposed (6), although the Hill coefficient seems to increase slightly with temperature, so that the increase in IC_{50} with pH is greater at 37 °C than it is at 25 °C. Because both the curves extrapolate to values of IC_{50} 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 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 IC_{50} can be explained by an increase in K_{Mg} from 6.3 to 13 μ M, together with the above-mentioned increase in n . Both models allow the Mg^{2+} IC_{50} to be calculated. For example, at 37 °C, interpolated values of the IC_{50} are 90, 250, and 1230 μ 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 IC_{50} for Mg^{2+} passes from values that are about 15% of the free Mg^{2+} to values that are at least 2-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 because of a decrease in the IC_{50} for Mg^{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_{max} , even when Mg^{2+} and H^+ concentrations are significantly above their IC_{50} values, IMAC still has considerable activity. From the data presented here and the pH dependence described previously (7), we estimate that at 37 °C, the J_{max} ($[H^+] = 0$, $[Mg^{2+}] = 0$) is 6–7 μ mol Cl^- /min·mg. To place this value in perspective, it should be noted that the maximum rates of

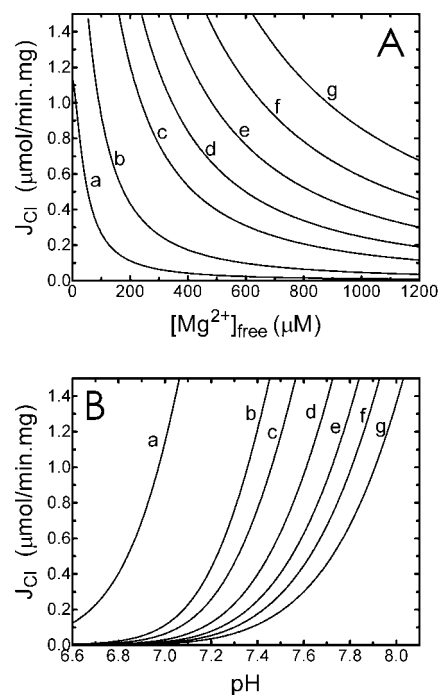


FIG. 7. Effects of Mg^{2+} and pH in regulating IMAC at 37 °C. Theoretical curves are plotted using the equation $J = J_0 / (1 + ([Mg^{2+}]_{free} / Mg^{2+} IC_{50})^n)$ in which the values of Mg^{2+} IC_{50} and n were determined from Figs. 2, 4, and 5, and the values of J_0 for each pH were determined using equation 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 $[Mg^{2+}]$ (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.

respiration on succinate are of the order of 200 nmol O/min·mg. Moreover, because the net H^+ /O stoichiometry of proton pumping is 6–7 (11), this respiration rate corresponds to a proton flux of about 1400 nmol H^+ /min·mg, which is less than 25% of the capacity of IMAC. Thus, 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. For the respiratory chain to drive net salt efflux, activity of both IMAC and the K^+ / H^+ antiporter is required (1). In a preliminary study, we have found that at 37 °C and pH 8.4 the K^+ / H^+ antiporter has a J_{max} ($[Mg^{2+}] = 0$) of 1.3 μ mol/min·mg and a Mg^{2+} IC_{50} of 220 μ M.² Thus, under physiological conditions, the K^+ / H^+ antiporter could also have significant activity. Note that the relatively high activity of IMAC would ensure that respiration-driven (H^+ pump) anion efflux would elevate matrix pH and further activate both the K^+ / H^+ antiporter and IMAC, which are both regulated by matrix protons (1, 14). Note also that the very high J_{max} for IMAC relative to the rate of mitochondrial respiration is also consistent with the suggestion by Aon *et al.* (4) that IMAC is responsible for depolarization of mitochondria in myocytes observed in their studies.

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 pre-

² A. D. Beavis and M. Powers, unpublished data.

dicted flux as a function-free Mg^{2+} at different pH values, taking into account the 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 pH 7.4 and 0.5 mM Mg^{2+} is 400 nmol/min·mg, which is only 6.5% of the J_{max} ($[H^+] = 0$, $[Mg^{2+}] = 0$), but still high enough so 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 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 regulating the open probability of IMAC. In addition to pH, these factors could include changes in matrix volume. As also suggested by others (8), volume *per se* could exert effects via cytoskeletal proteins that might be involved in control of mito-

chondrial 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.

Acknowledgment—We thank Dr. Elizabeth Tietz for the generous donation of rat livers.

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