Evidence for the Allosteric Regulation of the Mitochondrial K⁺/H⁺ Antiporter by Matrix Protons*

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It is well accepted that the mitochondrial K⁺/H⁺ antiporter is regulated by matrix Mg²⁺; however, this is not the only factor controlling its activity. The precise conditions used to deplete divalent cations have profound effects on the subsequent activity of the antiporter in a KOAc assay medium. Examination of the proton fluxes during both pretreatment and subsequent assay of K⁺/H⁺ antiport reveals that differences in K⁺/H⁺ antiporter activity correlate very well with differences in matrix pH. Thus, inhibition of the K⁺/H⁺ antiporter following depletion of Mg²⁺ appears to result from inhibition by matrix protons. To test this hypothesis, we have examined the effect of modulating matrix pH in three different ways on the activity of the K⁺/H⁺ antiporter: 1) lowering the pH of the K⁺ pretreatment medium to 6.7 leads to inactivation of the K⁺/H⁺ antiporter; 2) adding NH₄⁺ to the assay medium eliminates the lag in activity induced by depleting Mg²⁺ in a pretreatment medium containing NH₄⁺; 3) permitting mitochondria to respire in a tetraethylammonium⁺-containing pretreatment medium activates the K⁺/H⁺ antiporter. Each one of these procedures leads to a change in matrix pH and an effect on K⁺/H⁺ antiport which appears to require regulation of the K⁺/H⁺ antiporter by matrix protons. This finding is not only physiologically significant but also provides a useful definition of conditions required for unmasking the K⁺/H⁺ antiporter in a reproducible manner.

Consistent with its proposed role in volume homeostasis of the mitochondrial matrix (reviewed in Ref. 1), the mitochondrial K⁺/H⁺ antiporter is found to be regulated. This regulation is thought to be exerted primarily via changes in the activity of matrix Mg²⁺ (1-4); however, regulation of the antiporter is probably more complex. For example, Brierley et al. (5) have pointed out that rates of swelling in K⁺ salts are strongly dependent on the conditions used to remove the divalent cations and conclude that factors other than simply the divalent cation content of the mitochondria control K⁺/H⁺ antiport. In addition, Bernardi and Azzzone (6) have suggested that the activity is also controlled by "membrane stretching" and by the membrane potential. These findings implicate a second physiological regulator of the K⁺/H⁺ antiporter in Mg²⁺-depleted mitochondria.

The most likely candidate for this role is the proton, since both mitochondrial swelling in KOAc (7) and respiration-driven loss of mitochondrial K⁺ (5, 6, 8) are strongly pH-dependent. The inhibitory effect of protons on K⁺/H⁺ exchange could simply reflect "product inhibition" or competition between K⁺ and H⁺ for binding at the cation-binding site. We have suggested (1, 3, 9), however, that the inhibition is allosteric and results from protonation of the vacant Mg²⁺-binding site in the matrix. This hypothesis is based on the following lines of evidence: (a) When matrix proton compensation depends on respiration-driven TEA⁺ uptake, the rate of K⁺ efflux on the antiporter depends on medium TEA⁺ concentration (3). (b) The substrate, K⁺, does not protect the K⁺/H⁺ antiporter against irreversible inhibition by dicyclohexylcarbodiimide (9). On the other hand, "allosteric" inhibitors, such as Mg²⁺, quinine, and "a contracted matrix," do protect (9, 10). Similarly, protons protect against inhibition and labeling by DCCD (9, 10), placing protons in a class with other allosteric inhibitors. Thus, it seems likely that both the inhibitory effect and protective effect of protons are exerted via an allosteric site (c) Whether K⁺/H⁺ antiport induced by A23187 is operating in the direction of K⁺ efflux (3) or K⁺ influx (9), maximal activity is only observed after a lag. Since A23187 catalyzes Mg²⁺/Zn⁺ exchange this lag could reflect inhibition of K⁺/H⁺ antiport by matrix H⁺. Until now, more direct evidence for allosteric inhibition by protons has been lacking. In fact, Jung and Brierley (11) have failed to detect a significant pH gradient in Mg²⁺-depleted heart mitochondria, and Bernardi and Azzzone (6) have concluded that changes in the pH gradient have no regulatory effect on the K⁺/H⁺ antiporter in liver mitochondria.

We now present the results of an investigation into the effects of the conditions of Mg²⁺ depletion on matrix pH and subsequent K⁺/H⁺ antiport activity. Our results provide strong evidence that K⁺/H⁺ antiporter is regulated allosterically by matrix protons. This study also establishes the experimental conditions under which Mg²⁺ depletion leads to maximum, reproducible activity of the K⁺/H⁺ antiporter during the light scattering technique.

**EXPERIMENTAL PROCEDURES**

Pretreatment Procedures for Mg²⁺ Depletion—To deplete matrix Mg²⁺, mitochondria were pretreated with A23187 (1 nmol/mg) and EDTA. To prevent swelling during pretreatment, osmotic support was supplied by salts of impermeant cations (e.g. TEA⁺) or impermeant anions (e.g. TES, glucuronate) or impermeant nonelectrolytes.

1 The abbreviations used are: TEA, tetraethylammonium; DCCD, dicyclohexylcarbodiimide; EDTA, [ethylenebis(oxyethylenenitrilo)tetraacetic acid; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; L.S. light scattering.
(e.g. sucrose). The osmolality, inclusive of sucrose added with the mitochondria, was adjusted to 110 mosm to equal that of the assay medium.

Pretreatment Media Used for Light Scattering Studies—The final composition of the pH 7.4 pretreatment media, including sucrose added with the mitochondria were as follows: "K" medium, "NH -medium," and "TEA" medium contained sucrose (52.6 mM) and the designated salts of EDTA (4.4 mM) and TES (35.3 mM). Total salt concentrations were 20 mM. "TEA OAC medium" contained sucrose (52.6 mM) TEA OAc (18.4 mM) and TEA -salts of EDTA (4.4 mM) and TES (4.4 mM); "sucrose medium" contained sucrose (80.3 mM) and TEA -salts of EDTA (4.4 mM) and TES (4.4 mM). K -medium at pH 6.7 contained sucrose (52.6 mM) and K- salts of EDTA (4.4 mM) and TES (4.4 mM) and K- medium at pH 7.8 contained sucrose (52.6 mM) and K- salts of EDTA (4.4 mM) and TES (30.5 mM).

Pretreatments were carried out at 25 °C, and rotenone (0.5 μg/ml) was added just prior to the addition of the mitochondria (50 mg/ml stock) unless indicated otherwise. After 1 min, to permit temperature equilibration, 123187 (1 nmol/mg) was added. Unless indicated otherwise, after a further 2.5 min the suspension was placed on ice and 30 μl samples transferred to 3 ml of the assay medium.

To follow volume changes during pretreatment, mitochondria were added at 0.1 mg/ml to a dummy pretreatment medium. The media were made identical to those described above by the addition of an appropriate volume of 0.25 M sucrose to compensate for the smaller volume of mitochondrial suspension added. Rotenone was added to 2 μg/ml just prior to the addition of mitochondria.

Pretreatment Media Used for Simultaneous pH and L.S. Measurements—Modified pretreatment media were used for experiments in which both L.S. and pH changes were to be followed in the KOAc assay medium. These media contained the TEA - or K- salts of glucuronate (21 mM), TES (1.2 mM), and EDTA (4.4 mM) plus sucrose (52.6 mM) derived from the mitochondrial stock suspension. Since in these combined L.S. and pH studies we wished to maximize mitochondrial concentration, we pretreated the mitochondria at 20 mg/ml. The sucrose concentration was maintained the same as in our standard pretreatment by adjusting the stock mitochondrial concentration to 100 mg/ml. This procedure allowed the pretreated mitochondria to be diluted 10-fold into the KOAc assay medium yielding a final mitochondrial concentration of 2 mg/ml. Subsequent L.S. changes could be followed simultaneously with the pH changes, using a colorimetric probe with a short path length (0.5 cm total light path).

For studies in which L.S. and pH were to be followed in the pretreatment itself, mitochondria were added to give a concentration of 2 mg/ml. Again, the medium was supplemented with sucrose to compensate for the smaller volume of mitochondrial stock suspension added.

Assay of K+/H+ Antiport Activity—Assays of K+/H+ antiport were carried out at pH 7.4 and 25 °C in KOAc assay medium which contained the K+ salts of acetate (55 mM), TES (5 mM), EDTA (0.1 mM), and EGTA (0.1 mM). K+/H+ antiport was assayed by following swelling, which accompanies net salt transport, using the light scattering technique as described in detail elsewhere (12, 13). Using this technique we generate a light scattering variable, β, which normalizes reciprocal absorbance for mitochondrial protein concentration, P (milligrams/ml), according to the formula

$$\beta = \frac{P}{P_0} (A - a)$$

where a is a machine constant and $P_0$ (equals 1 mg/ml) is a constant introduced to make β dimensionless.

The rate of salt transport is calculated from the rate of change of β according to the formula (13)

$$J_s = \frac{aS_n}{n} \frac{d\beta}{dt}$$

where φ is the medium osmolality (110 milliosmol in most studies reported here), $S_n$ the solute content of the stock preparation of mitochondria (190 nmol/mg), b (15 mmol H+/mol) is the slope of the equilibrium absorbance osmotic curve (12), and n is the number of moles of osmotically active particles which make up 1 mol of the transported salt. At φ = 110 milliosmol $aS_n/b$ is about 1400 nmol/mg.

To determine rates of solute transport, we use a Brinkmann Probe Colorimeter (model PC700) with a 1-cm probe (2 cm light path). For optimum sensitivity we normally use mitochondrial concentrations between 0.1 and 0.2 mg/ml. However, for studies in which H+ fluxes are examined, it is necessary to use mitochondria at about 2 mg/ml. To follow L.S. changes in these suspensions we employ a probe with a variable path length and adjust the gap to about 2.5 mm. It should be noted, however, that these data cannot be compared quantitatively with those obtained with the 1 cm probe. Although b is normalized for mitochondrial concentration, it remains dependent on the length of the light path and the system used to determine light scattering changes. Thus, to determine mitochondrial volume and solute fluxes, the absorbance osmotic curve must be determined with the same apparatus. For this reason in this study the L.S. data obtained with the variable path length probe are simply presented in terms of $A^{-1}$.

Determination of Mg2+ Fluxes—Mg2+ fluxes were determined by centrifugation of the mitochondrial suspension in an Eppendorf microcentrifuge and analysis of extracts of both supernatant and pellets by atomic absorption spectroscopy.

Drugs and Reagents—Most drugs were obtained from Sigma. Rat liver mitochondria were prepared as described previously (12).

RESULTS

Dose Dependence of A23187-induced K+/H+ Antiport—Freshly isolated mitochondria exhibit very low K+/H+ antiport activity. Thus, as illustrated by curve a of Fig. 1, mitochondria swell very slowly when suspended in KOAc medium. Addition of an exogenous K+/H+ antiporter, nigericin, induces immediate swelling due to a net influx of KOAc and water (trace b, Fig. 1). The rate is dose-dependent (14), and swelling begins immediately after the ionophore is added. Swelling is also induced by addition of A23187 (trace c, Fig. 1); however, in this case, the maximum rate is independent of the dose at or above 2 nmol of A23187/mg (0.2 μM), and furthermore, there is a dose-dependent lag (see Fig. 2). These findings are consistent with the interpretation that, unlike nigericin, A23187 does not catalyze K+/H+ antiport but leads to activation of the endogenous K+/H+ antiporter secondary to depletion of endogenous matrix divalent cations (3, 4).
The data in Fig. 2 indicate that to minimize the lag at least 5 nmol of A23187/mg protein (0.5 μM) should be added to the assay medium. The data in Fig. 2 also suggest that there is a residual lag of about 0.3 min which cannot be eliminated by raising the concentration of A23187 further. The existence of a lag in K+/H+ antiport activity became more obvious when we varied the pretreatment conditions for depleting endogenous divalent cations prior to adding the mitochondria to the KOAc assay medium.

Effect of Pretreatment Conditions for Mg2+ Depletion on Swelling in KOAc—The minimum requirements for a pretreatment medium designed to deplete endogenous divalent cations are that it contains EDTA, to chelate the divalent cations, and impermeant salts or non-electrolytes, to prevent swelling during pretreatment (see "Experimental Procedures"). The nature of the major cation in the pretreatment medium was found to have a dramatic effect on the subsequent K+/H+ antiport activity. When the pretreatment medium contained K+ and was adjusted to pH 7.4 or higher, the mitochondria began swelling rapidly, immediately they were transferred to the assay medium (see trace a of Fig. 3). In contrast, when K+ salt was replaced by TEA+ salt or sucrose, rapid swelling was only observed after a lag of about 1.2 min (see trace b of Fig. 3). A much longer lag was observed when K+ was replaced by NH4+. In this case, about 5 min elapsed before the swelling rate became comparable with that observed with the other media (see trace c of Fig. 3). All three of the traces shown in Fig. 3 were obtained following a pretreatment period of about 2 min; however, extension of the pretreatment period to 10 min had no effect on the character of curves obtained. Direct measurement of mitochondrial Mg2+ under each of these conditions revealed that depletion was essentially complete (see Table I). Thus, incomplete equilibration of Mg2+ does not appear to be responsible for the lag in swelling.

The different lags observed following these preincubations could either result from a difference in K+/H+ antiporter activity or from a change in the gradient driving salt transport. To distinguish between these possibilities, we examined the swelling induced by nigericin with mitochondria from the same preincubations. As shown by the results contained in Fig. 3B, rapid swelling was observed in all cases with no lag. These data, therefore, suggest that the lag in swelling observed in the absence of nigericin is a consequence of inhibition of the antiporter by something other than Mg2+.

Effects of Pretreatment on Mitochondria—To determine why the pretreatment conditions have such a profound effect on subsequent swelling, we examined the changes in mitochondrial volume (Fig. 4) and ion content (Table I) which take place during pretreatment. Upon addition of A23187 small volume changes can result from efflux of Mg2+ and, after activation of the K+/H+ antiporter (3, 7, 15) and the anion uniporter (16-18), from movements of K+ and endogenous anions. Nigericin was used to establish the direction and extent of volume changes secondary to equilibration of K+/H+ antiport.

Fig. 4A shows results obtained in the K+ medium, note that the data are plotted on an expanded scale (of Fig. 3). Addition of A23187 induces a short period of swelling (trace a) and the subsequent addition of nigericin causes no further change in volume. In contrast, addition of nigericin prior to A23187 causes rapid shrinkage which is reversed by subsequent addition of A23187 (trace b). The rapid increase in volume observed upon addition of A23187 is most simply explained by the net exchange of osmotically inactive matrix Mg2+ for osmotically active K+. As shown in Table I, under these conditions A23187 induces the efflux of about 28 nmol Mg2+.

If the pH of the pretreatment is raised to 7.8 there is a more rapid increase in volume subsequent to the addition of A23187; however spontaneous swelling is then observed (see trace a, Fig. 4B). This shrinkage can be blocked by pretreatment of the mitochondria with DCCD under conditions where the anion uniporter, but not the K+/H+ antiporter, is blocked (19) (trace b, Fig. 4B). We conclude, therefore, that the
shrinkage represents loss of endogenous anions via the unipporter.

The results presented in Fig. 4, A and B, suggest that loss of matrix Mg^{2+}, which occurs via Mg^{2+}/2H^{+} exchange (20), reverses the gradient for K^{+}/H^{+} exchange (20) and drives K^{+} uptake. This occurs very rapidly in the presence of nigericin (Fig. 4A, trace b) or slowly via the K^{+}/H^{+} antiporter (Fig. 4A, trace a) and is more extensive when the pH of the medium is raised.

Different results are obtained when NH_{4}^{+} is substituted for K^{+} (Fig. 4C). Addition of A23187 induces a small but rapid increase in volume (trace a) and depletes matrix Mg^{2+} (see Table I). This is followed by a slower contraction, which probably reflects loss of K^{+} via the K^{+}/H^{+} antiporter, since subsequent addition of nigericin causes no further change and direct measurements reveal that matrix K^{+} declines to 7 nmol of K^{+}/mg (see Table I). Addition of nigericin prior to A23187 causes loss of matrix K^{+} (see Table I) and a rapid shrinkage (trace b, Fig. 4C). Again, addition of A23187 depletes Mg^{2+} to very low levels (<10.5 nmol/mg; Table I) and induces very rapid swelling back to the original volume. Since matrix K^{+} remains at a very low level (see Table I), this increase in volume is attributable to net Mg^{2+}/2H^{+} exchange.

Fig. 4D shows results obtained with TEA^{+} pretreatment medium. Addition of A23187 induces loss of matrix Mg^{2+} (Table I) and slow shrinkage which is accelerated by the addition of nigericin (trace a, Fig. 4D). The final volume is independent of the time at which nigericin is added, and mitochondrial K^{+} content is depleted to 30 nmol/mg after about 7 min in the presence or absence of nigericin (Table I). Thus, we conclude that the slow phase of shrinkage represents K^{+} loss on the antiporter.

The results of Fig. 4 and Table I suggest that inhibition of the antiporter by matrix Mg^{2+} is an unlikely cause of the lag since Mg^{2+} depletion appears to be equal in all three pretreatment media. K^{+} content differs, however. In the K^{+} medium it probably increases, whereas in TEA^{+} and NH_{4}^{+} medium it is depleted to 37.7 and 9 nmol/mg, respectively. Since these K^{+} fluxes are mediated via K^{+}/H^{+} exchange it follows that the matrix pH should also be different following the three types of pretreatment. This suggests that the lag in K^{+}/H^{+} antiporter activity may result from inhibition of the antiporter by matrix protons. The following experiments were designed to test this hypothesis.

**Examination of Proton Fluxes during Pretreatment and KOAc Transport**—To follow the transmembrane proton fluxes which take place during pretreatment and swelling, we have used a pH electrode to continuously monitor the medium pH. In order to observe the small fluxes which were anticipated, we decreased the concentration of buffer in the system, replacing it with the impermeant glucuronate anion (16), and raised the concentration of mitochondria to 2 mg/ml. Under these conditions, simultaneous L.S. measurements were possible using a colorimeter probe with a short path length (see "Experimental Procedures").

Fig. 5 compares the proton fluxes which take place during pretreatment in K^{+} and TEA^{+} media. The traces are plotted so that an increase in the signal represents an influx of protons and have been normalized as described under "Experimental Procedures." In the K^{+} medium, addition of A23187 induces the disappearance of about 17 nmol H^{+}/mg from the medium (trace a). Since the chelation of Mg^{2+} by EDTA releases 1.0 H^{+}/Mg^{2+} (21) and 28 nmol of Mg^{2+}/mg are released upon addition of A23187 (Table I), this represents a net influx of 45 nmol of H^{+}/mg. Following this rapid influx, there is a slow efflux of 20.7 nmol/mg at a rate of 15 nmol/min·mg. Addition of nigericin during the efflux phase causes a rapid efflux (trace b, Fig. 5); however, addition of nigericin after the trace has become stable has almost no effect (trace c, Fig. 5). Thus, this efflux of protons reflects equilibration of K^{+}/H^{+} antiport.

In the TEA^{+} pretreatment medium (trace c, Fig. 5), the signal starts about 6 nmol of H^{+}/mg higher (matrix more acid) than in the K^{+} medium consistent with some loss of K^{+} by K^{+}/H^{+} antiport during the stabilization phase. As in the K^{+} medium addition of A23187 induces a rapid influx of protons; however, in this case there is no subsequent efflux. Furthermore, addition of nigericin causes a further rapid uptake of about 31 nmol of H^{+}/mg indicating that K^{+}/H^{+} antiport has not reached equilibrium even after 4 min. The difference between the matrix proton content of mitochondria suspended in K^{+} and TEAs pretreatment media is about 36 nmol of H^{+}/mg. Since the buffering power of the matrix lies between 18 and 36 nmol of H^{+}/pH unit·mg (21), this represents a substantial difference in matrix pH.

**Table I**

| Effect of pretreatment conditions on mitochondrial Mg^{2+} and K^{+} content |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| K^{+} medium    | S               | M               | T               | S               | M               | T               |
| (pH 7.4)        |                 |                 |                 |                 |                 |                 |
| C               | 7.7             | 26.9            | 34.7            | 37.5            | 0.6             | 38.1            |
| A               | 7.2             | 28.2            | 35.4            | 9.2             | 0.5             | 9.7             |
| N               | 7.2             | 28.2            | 35.4            | 9.2             | 0.5             | 9.7             |
| A+N             | 38.2            | 0.5             | 38.7            | 9.2             | 0.5             | 9.7             |
| TEA^{+} medium  |                 |                 |                 |                 |                 |                 |
| C               | 7.7             | 27.4            | 35.1            | 37.7            | 0.5             | 37.2            |
| A               | 147.2           | 15.4            | 15.4            | 15.4            | 0.8             | 15.2            |
| N               | 138.0           | 7.4             | 145.4           | 7.9             | 27.9            | 35.8            |
| A+N             | 137.4           | 6.0             | 143.4           | 39.3            | 0.20            | 39.55           |
| NH_{4}^{+} medium |                |                 |                 |                 |                 |                 |
| C               | 9.4             | 27.0            | 35.4            | 37.7            | 0.5             | 37.2            |
| A               | 147.2           | 9.4             | 156.4           | 7.9             | 27.9            | 35.8            |
| N               | 138.0           | 7.4             | 145.4           | 7.9             | 27.9            | 35.8            |
| A+N             | 137.4           | 6.0             | 143.4           | 39.3            | 0.20            | 39.55           |
| TEA OAc medium  |                 |                 |                 |                 |                 |                 |
| C               | 7.6             | 27.2            | 34.8            | 37.7            | 0.5             | 37.2            |
| A               | 91.9            | 62.1            | 154             | 36.9            | 0.8             | 37.5            |
| N               | 136.3           | 14.0            | 150.3           | 7.7             | 27.9            | 35.6            |
| A+N             | 111.6           | 29.4            | 141.0           | 37.2            | 2.4             | 39.6            |
| K^{+} medium    | (pH 6.7)        |                 |                 |                 |                 |                 |
| C               | 7.6             | 27.6            | 35.2            | 35.0            | 0.5             | 35.5            |
| A               | 35.0            | 0.5             | 35.5            | 35.0            | 0.5             | 35.5            |
| N               | 8.7             | 28.5            | 37.2            | 35.1            | 0.6             | 35.7            |
| A+N             | 35.1            | 0.6             | 35.7            | 35.0            | 0.5             | 35.5            |

Further reading available upon request.
FIG. 4. Effect of pretreatment conditions on mitochondrial volume. L.S. kinetics of mitochondria (0.1 mg/ml) suspended in dummy pretreatment mixes are shown. A, K' medium, pH 7.4. B, K' medium, pH 7.8. C, NH:- medium. D, TEA+ medium (see "Experimental Procedures" for composition of media). For A-D, trace a shows experiments in which A23187 (A23, 10 nmol/mg) was added at 0.75 min and nigericin (nig, 1 nmol/mg) was added at 3.0 min. In A, C, and D, trace b shows experiments in which nigericin was added at 0.75 min and A23187 was added at 1.5 min. In B, trace b shows the effect of pretreating the stock mitochondrial suspension with dicyclohexylcarbodiimide (50 nmol/mg) for 30 min. Nigericin (1 nmol/mg) was added at 3 min.

FIG. 5. Comparison of proton fluxes during pretreatment in K' and TEA+ media. Proton fluxes measured in modified K' and TEA+ media are compared. The traces represent recordings from a pH electrode immersed in 10 ml of the pretreatment medium containing 20 mg of mitochondrial protein and calibrated by the addition of 500 nmol of H+ pulses at the end of the min (not shown). Recording was begun about 5 min after addition of the mitochondria after the pH gradient had become stable. The scale has been normalized to nmol of H+/mg, and zero has been arbitrarily set equal to the starting point of the traces in the K+ medium (traces a and b). Trace a, modified K+ medium. A23187 (A23, 10 nmol/mg) was added at 0.5 min and nigericin (nig, 1 nmol/mg) at 3.6 min. Trace b, same as a but nigericin was added at 1.4 min. Trace c, modified TEA+ medium. A23187 was added at 0.5 min and nigericin at 3.6 min. See "Experimental Procedures" for composition of the media. of H+/min - mg). This is predicted by the kinetics of KOAc transport if it is limited by the rate of K'/H+ antiport. Under these conditions, HOAc transport should be close to equilibrium, and therefore, the ratio [H+]o/[H+]m should equal the ratio [OAc-]o/[OAc-]m, which will decline as swelling proceeds. For the TEA+-pretreated mitochondria, as predicted from the results of Fig. 5, the matrix starts off more acid (~17 nmol of H+/mg) than the K+-pretreated mitochondria (see trace d). There is a slow efflux of protons (15 nmol/min - mg rising to 25 nmol/min - mg) during the lag in the swelling. The most significant finding is that the lag in the pH trace closely matches the lag in the L.S. trace. This is demonstrated by replottting traces c and d (relabeled traces e and f) on a time frame which is shifted -1.12 min. This allows both the L.S. trace and the proton traces obtained with the TEA+-pretreated mitochondria to be almost superimposed on the traces obtained with the K+-pretreated mitochondria. This result provides convincing evidence that the lag represents inhibition of the K'/H+ antiporter by matrix protons.

Fig. 6B shows the results of a similar experiment in which nigericin was added to the assay using mitochondria from the same pretreatment incubations. As expected, addition of nigericin induced rapid swelling and a rapid efflux of protons as K'/H+ antiport approaches equilibrium. More importantly nigericin essentially eliminates the differences in the traces. This result provides further evidence that the difference in the H+ traces observed in the absence of nigericin (Fig. 6A) truly reflects a difference in matrix pH.

To test further the hypothesis that it is the matrix pH which regulates the K'/H+ antiporter, we have attempted to devise means by which we can modify the length of the lag in a predictable manner following pretreatment in K+-, NH:-, and TEA+ containing media.

Modification of the Lag Obtained with NH:-pretreated Mitochondria—If the very long lag in the swelling traces following pretreatment in NH3 salts is a consequence of the efflux of NH3, then addition of NH3 to the assay medium to decrease the extent of NH3 efflux should decrease the lag. The data contained in Fig. 7 confirm this prediction. Samples of mitochondria from an NH3 pretreatment were suspended in normal assay medium (trace a) and in assay medium supplemented with 60 mM NH4OAc (trace b). Inclusion of NH4OAc decreases the lag almost 6-fold and prevents the rapid contraction which occurs upon addition of the mitochondria to the assay medium.

Modification of the Lag Obtained with K+-pretreated Mitochondria—According to our hypothesis no lag is observed in
mitochondria pretreated in K+ medium. Therefore, the lag is actually reversed by acidification of the matrix. This is confirmed by the data shown in Fig. 8, which reveal that following preincubation at pH 6.7 for 2 min a lag of 1 min is observed when swelling in KOAc is assayed. Extending the preincubation period results in an increase rather than a decrease in the lag (traces b and c) indicating that a slow rate of Mg2+ efflux cannot be responsible for the lag. Furthermore, measurements of matrix Mg2+ reveal depletion to the same levels observed at pH 7.4 (Table I).

The fact that the lag increases as the preincubation is extended suggests that lowering pretreatment pH to 6.7 has actually reversed the K+/H+ antiport gradient. This is supported by measurements of H+ fluxes and volume changes during pretreatment which reveal an influx of H+ associated with a small shrinkage (results not shown). Thus, when preincubated at pH 6.7, K+/H+ antiport cannot compensate for the acidification of the matrix resulting from depletion of Mg2+.

Modification of the Lag Obtained with TEA+-pretreated Mitochondria—If the lag in K+/H+ antiport activity following pretreatment in K+ free media results from acidification of the matrix, it should be possible to eliminate the lag by realkalinizing the matrix. This can be achieved by allowing the mitochondria to respire in the TEA+ medium for various periods of time prior to the addition of A23187 and rotenone. The data contained in Fig. 10 show the effect of this type of pretreatment on the subsequent swelling trace. In the absence of respiration, a lag of about 1.5 min is observed (trace a). As the period of respiration is increased, the lag declines (trace b) and after 5 min respiration the lag is absent (trace c).

In order to verify that this pretreatment leads to a change in matrix pH, we examined the proton fluxes which take place under these conditions. The data in Fig. 10 show the L.S. and H+ traces recorded during pretreatment which reveal an influx of H+ coupled to electron flow as is drive uptake of TEA+ (2, 11, 22). The TEA+ taken up using this procedure is retained by mitochondria (2, 11, 22), and therefore should not leak out and reacidify the matrix upon dilution into TEA+-free KOAc assay medium. We tested this procedure by permitting the mitochondria to respire in the TEA+ medium for various periods of time prior to the addition of A23187 and rotenone. The data contained in Fig. 9A show the effect of this type of pretreatment on the subsequent swelling trace. In the absence of respiration, a lag of about 1.5 min is observed (trace a). As the period of respiration is increased, the lag declines (trace b) and after 5 min respiration the lag is absent (trace c).

In order to verify that this pretreatment leads to a change in matrix pH, we examined the proton fluxes which take place under these conditions. The data in Fig. 10 show the L.S. and H+ traces recorded during a typical experiment. The recording was begun after the pH gradient was allowed to stabilize for 5 min. Addition of succinate induces proton ejection (trace b) and concomitant swelling (trace a), presumably due to uptake of TEA+. After 5 min antimycin A was added to block respiration followed by A23187 to deplete matrix Mg2+. Mg2+ depletion is accom-
The most important finding of this experiment is that by the end of the pretreatment the mitochondria which have been allowed to respire have a matrix which is at least 25 nmol of H⁺/mg more alkaline and possesses a larger matrix volume. This difference could become as great as 40 nmol of H⁺/mg if K⁺/H⁺ antiport reached equilibrium in the non-respiring mitochondria, i.e. the difference observed in the presence of nigericin. Thus, this pretreatment leads to alkalization of the matrix, a result which fully supports the hypothesis that it is matrix protons which inhibit the K⁺/H⁺ antiporter and cause the lag.

**DISCUSSION**

We have presented evidence that the mitochondrial K⁺/H⁺ antiporter is regulated by matrix protons. This property explains a number of phenomena associated with the process of K⁺/H⁺ antiport, most notably the failure of matrix divalent cation depletion alone to induce maximum transport rates. The finding that the rate of K⁺/H⁺ antiport is independent of the concentration of A23187 over a wide range (Fig. 2) supports earlier conclusions (3, 4) that A23187 itself does not catalyze K⁺/H⁺ antiport. Only at concentrations below 1-2 nmol of A23187/mg (0.1-0.2 μM) does the maximum rate of K⁺/H⁺ antiport observed in a given trace appear to be dependent on A23187 concentration. A similar dose dependence for equilibration of Mg²⁺ is observed when inhibition of the mitochondrial inner membrane anion channel by added Mg²⁺ is examined in the presence of A23187 (18). In this case, the anion channel is inhibited by matrix Mg²⁺ and it is found that at least 1-2 nmol of A23187/mg protein is necessary to obtain an IC₅₀ which is independent of the dose of A23187. As noted in Ref. 18, the sharp dose dependence is not unexpected since diuretic A23187 translocates Mg²⁺ meaning that a 10-fold decrease in concentration should lead to a 100-fold decrease in the Mg²⁺ conductance.

Pretreatment of mitochondria with A23187 to remove endogenous divalent cations can be used to activate the K⁺/H⁺ antiporter; however, the characteristics of antiport activity are highly dependent on the conditions used in the pretreatment. Under all pretreatment conditions tested, Mg²⁺ depletion was essentially complete, so in agreement with Jung and Brierley (11) we conclude that the observed lags in activity are independent of Mg²⁺. Attributing the lag phase to inhibition of the K⁺/H⁺ antiporter by matrix protons is consistent with these observations. Thus, when mitochondria are depleted of Mg²⁺ with A23187, which catalyzes Mg²⁺(2H⁺) exchange, one inhibitor is simply replaced by another. Consequently, before maximal rates of K⁺/H⁺ antiport can be observed the matrix pH must be raised.

During pretreatment in a K⁺-containing medium, matrix pH can be raised either by influx of K⁺ on the K⁺/H⁺ antiporter or by loss of endogenous acids; for example, endogenous anions could exchange for OH⁻ on the anion uniporter (16). Support for the involvement of K⁺/H⁺ antiport is provided by the finding that the efflux of H⁺ (Fig. 5) is accompanied by an increase in matrix volume following addition of A23187 (Fig. 4, A and B) at pH 7.4 or higher. The anion uniporter also seems to be involved, since the matrix contracts following Mg²⁺ depletion at pH 7.8, and this contraction can be blocked by DCCD. DCCD treatment leads to a larger increase in volume, because K⁺ is the sole osmolyte moving. Without DCCD, K⁺ goes in but anions leave, and final volume is smaller. When the pretreatment pH is lowered to 6.7, the gradient for K⁺/H⁺ exchange now favors the influx of H⁺ and the gradient for loss of other endogenous acids will be smaller. Thus, consistent with regulation of the antiporter by matrix H⁺, a lag is observed in the subsequent swelling assay. A similar result is obtained if the pH is maintained at 7.4.
or higher and exogenous K\(^+\) is eliminated. Under these conditions, both loss of Mg\(^{2+}\) and subsequent loss of K\(^+\) acidify the matrix (Fig. 10) and inhibit the K\(^+\)/H\(^+\) antiporter. In sucrose or TEA\(^+\) salts loss of K\(^+\) is partially limited by the pH gradient generated. In NH\(_3\) salts, net exchange of NH\(_3\) for both Mg\(^{2+}\) and K\(^+\) can take place, but subsequent transfer to an NH\(_3\)-free assay medium will lead to a very rapid loss of NH\(_3\) and intense matrix acidification.

These findings lead to the conclusion that K\(^+\)/H\(^+\) antiporter must be inhibited by matrix protons. Furthermore, this must be an allosteric site, since we are assaying influx of K\(^+\) and efflux of H\(^+\), a process which should be facilitated, rather than inhibited, by lowering matrix pH. These findings also enable us to define the best pretreatment conditions for activation of the K\(^+\)/H\(^+\) antiporter in a reproducible manner. Thus, in addition to A23187 and EDTA the pretreatment medium should contain a K\(^+\) salt of an impermanent anion as the major osmolyte and should be maintained at or above pH 7.4.

Our interpretation of the lag is also consistent with the data obtained by Brierley et al. (5) in heart mitochondria, although their interpretation is different. They observed a lag in the rate of swelling in both KNO\(_3\) and KOAc followed pretreatment to remove endogenous Mg\(^{2+}\) in K\(^+\), trimethylammonium, and Na\(^+\) salts and sucrose. In all cases the preparations were washed in sucrose following Mg\(^{2+}\) depletion, and these washes undoubtedly result in net exchange of matrix K\(^+\), trimethylammonium, and Na\(^+\) for H\(^+\). Thus, the slow rates or lags observed can be attributed to an acid matrix. These authors (5) observed rapid swelling with no lag only when depletion of Mg\(^{2+}\) and K\(^+\) was carried out in TEA\(^+\) or tetramethylammonium salts with respiring mitochondria. Since there is no mechanism for rapid TEA\(^+\)/H\(^+\) or tetramethylammonium/H\(^+\) exchange, these cations will be retained and matrix acidification will not take place during washing.

Our finding that the lag in swelling correlates with the change in ΔpH with the finding of Jung and Brierley (11). These authors observed a lag in K\(^+\)/H\(^+\) antiporter activity following depletion of matrix Mg\(^{2+}\); however, they could only detect small changes in ΔpH. We believe that the reason for this is that ΔpH and swelling were not measured simultaneously. From our data (Fig. 6), it is clear that a delay of 1 min or more in measurement of ΔpH would not reveal the true pH gradient during swelling.

Regulation of the mitochondrial K\(^+\)/H\(^+\) antiporter by matrix protons could be of physiological importance. The function of the K\(^+\)/H\(^+\) antiporter in chemiosmotic systems is to provide a pathway for the efflux of K\(^+\) taken up electrochemically under the influence of the high membrane potential maintained by respiring mitochondria (1, 2). Since K\(^+\) uptake driven by respiration, i.e., coupled to H\(^+\) ejection, will lead to an increase in matrix pH, K\(^+\)/H\(^+\) antiporter activity will be increased, augmenting the increase in activity resulting from the decrease in Mg\(^{2+}\) activity secondary to the increase in matrix volume and chelating anions. We have previously provided evidence that the inner membrane anion channel is also regulated by matrix protons (16). Thus, these two pathways were regulated in parallel by both matrix Mg\(^{2+}\) and matrix H\(^+\) consistent with their postulated roles in matrix volume homeostasis (17).

Acknowledgment—We wish to thank Cathleen Wechter for expert technical assistance.

REFERENCES

Evidence for the allosteric regulation of the mitochondrial K+/H+ antiporter by matrix protons.
A D Beavis and K D Garlid


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