The Mitochondrial Inner Membrane Anion Channel

REGULATION BY DIVALENT CATIONS AND PROTONS*

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It is now well established that incubation of mitochondria at pH 8 or higher opens up an electrophoretic anion transport pathway in the inner membrane. It is not known, however, whether this transport process has any physiological relevance. In this communication we demonstrate that anion uniport can take place at physiological pH if the mitochondria are depleted of matrix divalent cations with A23187 and EDTA. Using the light-scattering technique we have quantitated the rates of uniport of a wide variety of anions. Inorganic anions such as Cl\(^-\), SO\(_4\)\(^{-2}\), and Fe(CN)\(_6\)\(^{3-}\) as well as physiologically important anions such as HCO\(_3\), P\(_4\), citrate, and malate are transported. Some anions, however, such as gluconate and glucuronate do not appear to be transported. On the basis of the finding that the rate of anion uniport assayed in ammonium salts exhibits a dramatic decline associated with loss of matrix K\(^+\) via K\(^+\)/H\(^+\) antiport, we suggest that anion uniport is inhibited by matrix protons. Direct inhibition of anion uniport by protons in divalent cation-depleted mitochondria is demonstrated, and the apparent pK of the binding site is shown to be about 7.8. From these properties we tentatively conclude that anion uniport induced by divalent cation depletion and that induced by elevated pH are catalyzed by the same transport pathway, which is regulated by both matrix H\(^+\) and Mg\(^{2+}\).

A number of carriers have been identified for the transport of anionic substrates across the inner mitochondrial membrane; however, none involved in a major metabolic pathway catalyzes electrophoretic uniport (see Ref. 1 for a review). Most inner membrane carriers catalyze net transport of the acid or electroneutral exchange of the anion for another anion; however, two catalyze the electrophoretic exchange of anions. The adenine nucleotide translocator catalyzes the exchange of ATP\(^{4-}\) for ADP\(^{3-}\) and the aspartate/glutamate carrier catalyzes the exchange of Asp\(^-\) for Glu\(^+\) + H\(^+\). All of these transport mechanisms are consistent with and provide indirect evidence for the chemiosmotic theory of the coupling of oxidative phosphorylation. This theory predicts that if an anion were transported by electrophoretic uniport, its entry into the mitochondrion would be severely limited or it would be ejected from the matrix by the high membrane potential generated by respiration.

In view of these considerations it is interesting that certain "nonphysiological" conditions, such as high pH (2–10) and low osmolality (6, 11), open up an electrophoretic anion uniport pathway with broad selectivity in the inner membrane of both liver and heart mitochondria. The groups of both Azzone (4, 5) and Brierley (7, 12, 13) have attributed the increase in anion fluxes observed under these extreme conditions to changes in the permeability of the lipid bilayer. On the other hand, Selwyn's group (10, 14) has proposed that a specific "pH-dependent anion conducting pore" is opened up; however, the existence of an anion uniport protein has not become generally accepted. Possible reasons for this are the facts that it has no obvious function and that it only appears to be active under extreme conditions. Furthermore, it follows from chemiosmotic theory that if an anion could be transported both electroneutrally and electrophoretically, as is the case for the classic uncouplers (15), futile anion cycling could uncouple oxidative phosphorylation (16). Despite these considerations, as we have discussed elsewhere (17), a well-controlled anion uniport pathway in mitochondria could serve a number of useful functions.

In this communication we present evidence that an anion uniport pathway can be opened up at neutral pH by depleting mitochondria of divalent cations. We also demonstrate that this pathway has a very broad specificity for anions and that it is regulated by protons. On the basis of these findings, we conclude that divalent depletion and alkaline pH probably activate the same transport pathway, which we refer to as inner membrane anion channel (17).

EXPERIMENTAL PROCEDURES

Mitochondrial Preparations—Rat liver mitochondria, isolated by differential centrifugation as previously described (18), were resuspended to 50 mg of protein/ml in 0.25 M sucrose and stored on ice. Mg\(^{2+}\)-depleted mitochondria were prepared by adding one part of stock suspension to four parts of pretreatment medium. The resulting mitochondrial suspension was 110 mosm and pH 7.4. Rotenone (1 µg/mg) and A23187 (1 nmol/mg) were added, the suspension was incubated at 25 °C for 2 min to allow K\(^+\)/H\(^+\) antiport to come to equilibrium, and then it was placed on ice. Two different pretreatment media were used. One contained the K\(^+\) salts (K\(^+\) pretreatment medium) and the other NH\(_4\)\(^+\) salts (NH\(_4\) pretreatment medium) of TES\(^1\) (44 mM) and EDTA (5.5 mM). The effect of pretreatment on mitochondrial Mg\(^{2+}\) and K\(^+\) levels was determined by centrifuging of the pretreatment suspension and analyzing both the supernatant and pellet for Mg\(^{2+}\) and K\(^+\) by atomic absorption spectroscopy. In both media matrix Mg\(^{2+}\) was depleted from 38 nmol/mg to about 0.6 nmol/mg, and in the NH\(_4\) pretreatment medium mitochondrial K\(^+\) was depleted from 145 nmol/mg to about 9 nmol/mg. It should be pointed out that this work was supported by National Institutes of Health Grants GM 31086 and HL 36573 awarded by the National Institute of General Medical Sciences and the National Heart, Lung, and Blood Institute, United States Public Health Service, Department of Health and Human Services and also by a grant from the Northwestern Ohio Chapter of the American Heart Association. The costs of publication of this article were defrayed in part by the payment of page charges. The abbreviations used are: mosm, milliosmolar; TES, N-tris-[hydroxymethyl]methyl-2-aminoethanesulfonic acid; EGTA, [ethylbyenebis(oxyethyl)enitrilo]tetraacetate acid; CCCP, carbonyl cyanide m-chlorophenylhydrazone; L.S., light scattering; MP\(^+\), divalent cation.
out that when the mitochondria were pretreated with A23187 and EDTA to remove matrix M$^{2+}$, 1 nmol/mg A23187 was routinely used; however, lower doses are equally effective, provided sufficient time is allowed for M$^{2+}$ depletion to take place. Doses of A23187 as high as 5–10 nmol/mg are necessary to achieve maximum rates only when A23187 is added directly to the assay medium in which the mitochondria are present at 0.1 mg/ml.

Light Scattering (L.S.) Measurements—Uptake of salts and water into the mitochondrial matrix results in matrix swelling and a consequent decrease in the light scattered by the mitochondrial suspension (19, 20). The light scattering variable $\beta$ normalizes reciprocal absorbance ($A^{-1}$) for mitochondrial concentration, $P$ (mg/ml),

$$\beta = \frac{P}{P_0} (A^{-1} - a)$$  

(1)

where $P_0$ (equals 1 mg/ml) is introduced to make $\beta$ a scaled dimensionless quantity and $a$ is a machine constant equal to 0.25 with our apparatus (18).

Absorbance was measured at 520 nm and sampled 0.01-min intervals with a Brinkmann PC 700 probe colorimeter connected to a Cyborg 41A analog/digital converter. The digitalized signal was passed to an Apple IIe computer for conversion to inverse absorbance, real time plotting, and storage. A linear regression routine was used to obtain rates, $d\beta/dt$ (min$^{-1}$), from light scattering traces. Over the range studied, $\beta$ and matrix water ($W_\text{m}$) are both linear with inverse osmolality (18),

$$\beta = \beta_0 + \frac{\Phi}{\phi}$$  

(2)

$$W_\text{m} = W_\text{m} + \frac{S_0}{\phi}$$  

(3)

where $\Phi$ is medium osmolality, $b$ (osmolal) and $S_0$ (osmol/mg) are the observed slopes of the corresponding equilibrium osmotic curves, and $\beta_0$ and $W_\text{m}$ (mg/mg) are the corresponding intercepts. $W_\text{m}$ is matrix water content (sucrose-free water) determined from the distribution of $[^{14}\text{C}]$sucrose and $^3\text{H}_2\text{O}$ in parallel gravimetric experiments (18). Since water uptake is much faster than salt transport (18), the rate of salt uptake, $J_s$ (nmol/min/mg), is proportional to the L.S. kinetic, $d\beta/dt$ (min$^{-1}$), and the proportionality constant is defined by Equations 2 and 3. For any salt, ignoring the osmotic coefficient of the matrix solutions (21),

$$J_s = \frac{\Phi S_0}{nb} \frac{d\beta}{dt}$$  

(4)

where $n$ is the number of moles of osmotically active particles which make up 1 mol of the salt. Since $S_0$ is reproducibly found to be 190 mosmol/mg in our laboratory and $b$ is approximately 15 mosmol with our equipment, at $\Phi = 110$ mosmol $\Phi S_0/b$ is about 1400 nmol/mg.

All assay media were made up to be of equal osmolality (110 mosmol), e.g. the pH 7.4 KCl and potassium malonate media contained the K$^+$ salts of Cl$^-$ (55 mM) or malonate (37 mM), respectively, plus the K$^+$ salts of TES (5 mM), EDTA (0.1 mM), and EGTA (0.1 mM). Rotenone (2 $\mu$g/mg) was added just prior to the mitochondria, and the media were maintained at 25°C by a circulating water bath. For experiments at other pH values, the concentration of TES was maintained constant and the salt concentration was adjusted slightly to maintain constant osmolality. For assays in ammonium salts the media were identical except that NH$_4^+$ was substituted for K$^+$.

RESULTS

Divalent Cation Depletion Activates an Anion Uniport Pathway—Fig. 1 contains L.S. traces of mitochondria suspended in 110 mosmol KCl. In the absence of A23187 and valinomycin no swelling takes place (Fig. 1A, trace a). A combination of valinomycin (1A, trace a; and B, trace a) improves the L.S. kinetics exhibited by mitochondria (0.1 mg/ml) suspended in KCl assay medium are shown. A: trace a, control; trace b, valinomycin (Val, 1 nmol/mg) was added at 1 min; trace c, valinomycin (1 nmol/mg) was added at 2 min. B: trace a, control; trace b, A23187 (10 nmol/mg) was added at 1 min; trace c, A23187 (10 nmol/mg) was added at 1 min, and valinomycin (1 nmol/mg) was added at 2 min. The assay medium is described under "Experimental Procedures."

DEPLETION OF MATRIX DIVALENT CATIONS ACTIVATES A UNIPORT PATHWAY FOR Cl$^-$—L.S. kinetics exhibited by mitochondria (0.1 mg/ml) suspended in KCl assay medium are shown. A: trace a, control; trace b, valinomycin (Val, 1 nmol/mg) was added at 1 min; trace c, valinomycin (1 nmol/mg) was added at 2 min. B: trace a, control; trace b, A23187 (10 nmol/mg) was added at 1 min; trace c, A23187 (10 nmol/mg) was added at 1 min, and valinomycin (1 nmol/mg) was added at 2 min. The assay medium is described under "Experimental Procedures.

Depletion of matrix Mg$^{2+}$ (22–25) (see "Appendix"). A major contribution by electroneutral Cl$^-$/OH$^-$ antiport is, however, ruled out by the findings that A23187 alone only induces a slow rate of swelling (Fig. 1B, curve b) and that subsequent addition of valinomycin is necessary to induce rapid swelling (Fig. 1B, trace c). Although the transport observed in the absence of valinomycin could reflect Cl$^-$/OH$^-$ exchange, the increment induced by valinomycin must represent Cl$^-$ uniport.

The Rate of Anion Uniport in NH$_4^+$ Salts Is Highly Variable—Experiments similar to those shown in Fig. 1 were carried out with ammonium salts using the protonophore CCCP to provide the counterion for Cl$^-$ transport (not shown). In these experiments, however, we observed that the rate of swelling was much faster when we added CCCP first than when we added A23187 first. Moreover, even faster rates were obtained when we added A23187 and CCCP together at zero time. To investigate the possibility that the rate of swelling was related to the length of time between depletion of mitochondrial M$^{2+}$ and initiation of swelling, we devised a pretreatment medium containing A23187 and the K$^+$ salts of TES prior to the assay (see "Experimental Procedures"). When these mitochondria were transferred to assay medium containing CCCP, to our surprise we still observed very rapid swelling and found that the length of the pretreatment period had virtually no effect on the rate (not shown). Because these mitochondria swelled only very

FIG. 1. Depletion of matrix divalent cations activates a uniport pathway for Cl$^-$. L.S. kinetics exhibited by mitochondria (0.1 mg/ml) suspended in KCl assay medium are shown. A: trace a, control; trace b, valinomycin (Val, 1 nmol/mg) was added at 1 min; trace c, valinomycin (1 nmol/mg) was added at 2 min. B: trace a, control; trace b, A23187 (10 nmol/mg) was added at 1 min; trace c, A23187 (10 nmol/mg) was added at 1 min, and valinomycin (1 nmol/mg) was added at 2 min. The assay medium is described under "Experimental Procedures.

2 Portions of this paper (including "Appendix" and Figs. 10-12) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 67M-1686, cite the authors, and include a check or money order for $4.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
slowly in the absence of CCCP, we were also able to examine
the effect of the duration of exposure of M^2+-depleted mito-
chondria to the assay medium on the subsequent rate of
CCCP-induced anion transport. Fig. 2 shows the rate of Cl-
transport as a function of the time interval between the
addition of the mitochondria to the assay and the addition of
the CCCP to the mitochondria. As the interval increases the
rate declines dramatically until it reaches about 15% of the
maximum rate, at which point it becomes constant. Note that
the addition of both A23187 and CCCP to the pretreatment
has only a small effect on the initial rate of Cl^- transport.
Thus, activity of the uniport pathway appears to decline upon
exposure of M^2+-depleted mitochondria to the assay medium.

Variability of Anion Uniport Fluxes in NH4Cl Salts Is Asso-
ciated with Exchange of Exogenous K^+ for Medium NH4—
When we found that the decline in uniport activity only
appears to take place in the assay medium, it occurred to us
that the decline in activity may be related to the exchange of
matrix K^+ for medium NH4+, since it is known that Mg^2+
depletion activates the K^+/H^+ antipporter (22–25). Three
experiments were designed to investigate this possibility. In the
first we examined the effect of substituting NH4+ for K^+ in the
pretreatment medium. This should permit matrix K^+ to
be exchanged for NH4^+ and, therefore, if the decline in uniport
activity is related to the exchange of K^+ for NH4^+, the decline
in activity should take place prior to transferring the mito-
chondria to the assay medium. The data in Fig. 3 confirm this
prediction. When CCCP is included in the medium from zero
time, mitochondria pretreated in the K^+ medium swell rapidly
(trace a), whereas those pretreated in the NH4 medium swell
more slowly (trace b). Moreover, the rate of swelling of mito-
chondria pretreated in the NH4+ medium is independent of
the time of addition of CCCP (compare traces b and c) and,
furthermore, it is identical to the rate observed when CCCP
is added at 1 min to mitochondria pretreated in K^+ medium
(trace d). Thus, consistent with the exchange of K^+ for NH4^+
being related to the decline in uniport activity, inclusion of
NH4+ in the pretreatment medium allows the decline in uni-
port activity to take place in the pretreatment mix.

In the second experiment we looked at the effect of adding
nigericin to the assay medium. If the decrease in uniport
activity is related to the exchange of matrix K^+ for medium
NH4+, then the period over which the decline takes place
should be the period required for equilibration of K^+/H^+
exchange across the inner membrane. Thus, addition of ni-
gericin should accelerate the decline in anion uniport activity.
The data shown in Fig. 4 confirm this prediction. When both
A23187 and CCCP are included in the medium from zero time
very rapid swelling is observed (trace a); however, if nigericin
is also included in the medium (trace b) the maximum rate of
swelling is much slower. More importantly, the rate observed
under these conditions is very close to that observed when
CCCP is added at 1 min in the absence (trace c) or presence
(trace d) of nigericin. The lack of effect of nigericin on the rate
of swelling induced by the late addition of CCCP suggests
that nigericin per se has no effect on the Cl^- conductance.
Thus, like pretreatment in NH4+ medium, nigericin in the
assay medium simply appears to accelerate the decline in
anion uniport activity.

In the third experiment we looked for a change in anion
uniport activity in KCl assay medium. If our explanation for
the changes observed in NH4Cl assay medium was correct the
rate of swelling should not decline upon exposure of the
mitochondria to the assay medium. The data contained in
Fig. 5 confirm this prediction. The rate of swelling is in fact
slower when valinomycin is added to the assay at zero time.
quantitative analysis, we have adopted a nonequilibrium ther-
fluxes for a variety of different anions. For the purpose of ex-
periment shown in Fig. 6, in which we have plotted the rate 
selectively of the anion uniporter by determining the maximal 
yields a $J_{A,max}$ for $\text{Cl}^-$ transport of $434$ nmol/min $\cdot$ mg, and 
the slope yields a $a$ for valinomycin of 
tron. However, this difference also appears to represent a change in anion uniport activity associated with $\text{K}^+/\text{H}^+$ ex-
change, since addition of nigericin stimulates the rate swelling 
when valinomycin is added at zero time (trace c) but has no 
effect when valinomycin is added at 1 min (trace d). Thus, a 
change in anion uniport activity is observed in both NH$_4$Cl and 
KCl, but the change occurs in opposite directions.

**Determination of Maximal Anion Fluxes**—Having established 
conditions under which reasonably reproducible rates of 
swelling could be determined, we wished to examine the selectivity of the anion uniporter by determining the maximal fluxes for a variety of different anions. For the purpose of quantitative analysis, we have adopted a nonequilibrium thermodynamic model to describe the coupling between the fluxes of the anion and cation. This model is described in the "Appendix" and predicts that for $\text{K}^+$ salts there should be an hyperbolic relationship between the rate of transport and the square of the concentration of $\text{K}^+$ on both sides of the membrane is relatively high, the pH gradient should be at a minimum. Fig. 7A strongly suggests that anion uniport activity is influenced by net exchange of matrix $\text{K}^+$ for $\text{NH}_4^+$ in $\text{NH}_4$ salts and the redistribution of $\text{K}^+$ via $\text{K}^+/\text{H}^+$ antiport in $\text{K}^+$ salts. Two factors contributing to these effects could be: 1) regulation of anion uniport by matrix pH and 2) a change in $\Delta_\phi$ for chloride. Although it is difficult to demonstrate direct regulation by matrix pH, in $\text{K}^+$ salts nigericin can be used to equilibrate $\text{K}^+/\text{H}^+$ antiport, thereby ensuring that the matrix pH will vary as the medium pH is varied. Furthermore, since the concentration of $\text{K}^+$ on both sides of the membrane is relatively high, the pH gradient should be at a minimum. Fig. 7A contains data showing that the rate of chloride and malonate transport in $\text{Mg}^{2+}$-depleted mitochondria is strongly dependent on pH. Note that at all pHs studied the rate of anion transport is much lower in normal $\text{Mg}^{2+}$-containing mitochondria. In

![Fig. 5. The effect of nigericin on mitochondrial swelling in KCl. L.S. kinetics exhibited by $\text{Mg}^{2+}$-depleted mitochondria in KCl medium are shown. The mitochondria (0.1 mg/ml) were added to the assay medium which contained A23187 (10 nmol/ml). Valinomycin (0.1 nmol/ml) was added at zero time (traces a and c) or at 1 min (traces b and d). Nigericin (0.05 nmol/ml) was added at zero time in trace a and at 0.9 min in trace d. The rates of anion transport calculated from traces a–d are 230, 375, 425, and 370 nmol of $\text{Cl}^-$/min $\cdot$ mg, respectively. The assay medium is described under "Experimental Procedures."](image1)

![Fig. 6. Determination of $J_{A,max}$ for $\text{Cl}^-$ uniport. The rate of $\text{Cl}^-$ transport ($J_a$, pmol/min $\cdot$ mg) is plotted versus ($J_a - J_{a,0}$)/|[valinomycin]| (pmol/nmol $\cdot$ min) where $J_{a,0}$ (equal to 0.02 pmol/min $\cdot$ mg) is the rate observed in the absence of valinomycin. Analysis of the data yields a $J_{A,max}$ of 434 nmol Cl$^-$/min $\cdot$ mg and a $K_{a,0}$ for valinomycin of 0.049 pmol/mg. Mitochondria (0.1 mg/ml) were added to the KCl assay medium (described under "Experimental Procedures") containing nigericin (1 nmol/mg) and A23187 (10 nmol/mg). Valinomycin was added at 0.2 min. The net salt transport was followed using the L.S. technique, and the rate of $\text{Cl}^-$ transport was calculated from the L.S. kinetics as described under "Experimental Procedures."](image2)
these data are consistent with the existence of two independent pathways for SCN\(^-\) transport, one sensitive to M\(^{2+}\) and pH and another insensitive to M\(^{2+}\) and pH.

When we first found that M\(^{2+}\) depletion activated anion uniport at neutral pH, we thought that alkaline pH may simply lead to the loss of matrix M\(^{2+}\) rather than having a direct effect on anion permeability. The data in Figs. 7 and 8 show that this is not the case; however, the possibility remained that elevated pH leads to the loss of some other regulatory factor from the mitochondria. If some factor other than protons was involved, it should not be possible to block swelling after it has reached a maximum rate by adding acid. Curve \(a\) of Fig. 9 shows that mitochondria suspended in KC\(_1\) and J\(_2\) according to Equation A16 of the "Appendix." These measurements were made by following L.S. kinetics of mitochondria (0.1 mg/ml) added to assay medium containing nigericin (0.5 nmol/mg). When present A23187 (10 nmol/mg) was added at zero time and valinomycin (Val, 1 nmol/mg) was added at 0.5 min. The method used for calculating the transport rates from the L.S. kinetics and the composition of the assay medium are described under "Experimental Procedures." For the measurements of HCO\(_3\) transport acetazolamide (40 \(\mu\)M) was added to the medium to block carbonic anhydrase. For measurements of P\(_4\) transport the mitochondria were pretreated with N-ethylmaleimide (40 nmol/mg) to block the electroneutral P\(_4\)\,-\,H\(^+\)symporter. For measurements of ferrocyanide transport 0.5 mM CN\(^-\) was added to prevent oxidation of ferrocyanide, and 0.5 mM ascorbate was added to reduce any ferricyanide formed.

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<th>(J_1) (+Val)</th>
<th>(J_2) (+A23187)</th>
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<th>(J_2/J_1)</th>
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**Table I**

**Effect of divalent cation depletion on anion transport in mitochondria**

\(J_0\), \(J_1\), and \(J_2\) are rates of anion transport (nmol/min\,-mg) determined under various conditions. \(J_0\) is the rate in normal mitochondria in the presence of valinomycin and A23187; and \(J_2\) is the rate in the presence of A23187 alone. \(J_1\) is the maximum rate of electrophoretic OH\(^-\) uniport or the maximum rate of endogenous K\(^+\) uniport calculated from \(J_0\) and \(J_2\) according to Equation A16 of the "Appendix." These measurements were made by following L.S. kinetics of mitochondria (0.1 mg/ml) added to assay medium containing nigericin (0.5 nmol/mg). When present A23187 (10 nmol/mg) was added at zero time and valinomycin (Val, 1 nmol/mg) was added at 0.5 min. The method used for calculating the transport rates from the L.S. kinetics and the composition of the assay medium are described under "Experimental Procedures." For the measurements of HCO\(_3\) transport acetazolamide (40 \(\mu\)M) was added to the medium to block carbonic anhydrase. For measurements of P\(_4\) transport the mitochondria were pretreated with N-ethylmaleimide (40 nmol/mg) to block the electroneutral P\(_4\)\,-\,H\(^+\)symporter. For measurements of ferrocyanide transport 0.5 mM CN\(^-\) was added to prevent oxidation of ferrocyanide, and 0.5 mM ascorbate was added to reduce any ferricyanide formed.

Both the groups of Azzone (4, 5) and Brierley (7, 12, 13) have attributed the increase in permeability to anions induced by alkaline pH to a non-specific change in the permeability of the lipid membrane. In a preliminary attempt to determine whether the effect of pH on anion uniport reported above reflects a non-specific change in permeability of the lipid bilayer, we have investigated the effect of pH on the transport of SCN\(^-\), since SCN\(^-\) can be transported at rapid rates through the lipid bilayer in normal M\(^{2+}\)-containing mitochondria. The data in Fig. 8 (closed circles) show that in the presence of A23187, the transport of SCN\(^-\) exhibits a strong dependence on pH. In the absence of A23187, however, the rate is almost independent of pH (Fig. 8, open circles). Furthermore, in the presence of A23187, the transport of SCN\(^-\), unlike Cl\(^-\), cannot be completely blocked by lowering the pH but becomes equal to the rate in the absence of A23187. These data indicate that, at least in the presence of endogenous M\(^{2+}\), the permeability of the lipid bilayer is unaffected by the pH of the medium in the range from 6.6 to 8.4. Furthermore, these data are consistent with the existence of two independent pathways for SCN\(^-\) transport, one sensitive to M\(^{2+}\) and pH and another insensitive to M\(^{2+}\) and pH.
Anion Uniport in Mitochondria

The pH dependence of Cl⁻ and malonate uniport in mitochondria. A, the rate of anion transport (Jₐ, µmol/min·mg) is plotted versus the pH of the assay medium. Valinomycin (0.5 nmol/mg) and nigericin (1 nmol/mg) were added to the assay media at zero time, and A²3187 (10 nmol/mg) when present, was added at 0.2 min. The rate of anion transport was determined from the L.S. kinetics as described under “Experimental Procedures.” B, Hill plots of the data shown in A. The [Jₐ,max] for each anion was estimated from a plot of [Jₐ]/[Jₐ,max] versus [H⁺]. Values obtained were 1.35 µmol/min·mg for Cl⁻ and 0.49 µmol/min·mg for malonate. The slopes equal 1.24 and 1.20, and the pIso values obtained equal 7.83 and 7.69 for Cl⁻ and malonate, respectively. The assay media are described under “Experimental Procedures.”

The concentration is much higher than that of Ca²⁺ (36), we tentatively conclude that Mg²⁺ is the endogenous inhibitor of this transporter. Brierley’s group (12, 13) has previously suggested that Mg²⁺ may be involved in maintenance of the integrity of the inner membrane and that removal of bound Mg²⁺ simply induces a generalized increase in membrane permeability. On the other hand, Selwyn’s group (37) have shown that uptake of ³⁶Cl⁻ into respiring submitochondrial particles is stimulated by the addition of 10 mM Mg²⁺. Furthermore, they have also proposed that low levels of matrix Ca²⁺ activate anion uniport (10). These results suggest that under certain conditions divalent cations do not block anion uniport. It should be noted, however, that the rate of uptake they observe in the presence of Mg²⁺ (37) is lower than the rate we observe prior to Mg²⁺ depletion. Thus, the stimulatory effect of Mg²⁺ observed by Selwyn’s group (37) may result from inhibition of ³⁶Cl⁻ efflux during the wash prior to isotope counting. In view of these findings, the precise role of Mg²⁺ in the regulation of anion transport is being investigated further.

The next question which must be answered is: by what mechanism or mechanisms does net salt transport occur in M⁺-depleted mitochondria? The most significant finding is that valinomycin stimulates the rate of anion transport (Fig. 1B, Fig. 6, Table I). Stimulation is only expected under three circumstances: 1) when an electrophoretic anion uniport pathway is open (see models 1–3 in the “Appendix”); 2) when both

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**Fig. 7. The pH dependence of Cl⁻ and malonate uniport in mitochondria.** A, the rate of anion transport (Jₐ, µmol/min·mg) is plotted versus the pH of the assay medium. Valinomycin (0.5 nmol/mg) and nigericin (1 nmol/mg) were added to the assay media at zero time, and A²3187 (10 nmol/mg) when present, was added at 0.2 min. The rate of anion transport was determined from the L.S. kinetics as described under “Experimental Procedures.” B, Hill plots of the data shown in A. The [Jₐ,max] for each anion was estimated from a plot of [Jₐ]/[Jₐ,max] versus [H⁺]. Values obtained were 1.35 µmol/min·mg for Cl⁻ and 0.49 µmol/min·mg for malonate. The slopes equal 1.24 and 1.20, and the pIso values obtained equal 7.83 and 7.69 for Cl⁻ and malonate, respectively. The assay media are described under “Experimental Procedures.”

**Fig. 8. The effect of pH on the rate of SCN⁻ transport.** The rate of SCN⁻ transport (Jₐ, µmol/min·mg) is plotted versus the pH of the KSCN assay medium. Valinomycin (0.5 nmol/mg) and nigericin (1 nmol/mg) were included in the assay medium from zero time. A, data obtained with A²3187 (10 nmol/mg) included in the assay from zero time. O, data obtained in the absence of A²3187. The method used to determine the rate of SCN⁻ transport from the L.S. kinetics and the composition of the assay medium are described under “Experimental Procedures.”

**Fig. 9. Inhibition of Cl⁻ uniport by H⁺.** L.S. kinetics exhibited by M⁺-depleted mitochondria in KCl medium are shown. The mitochondria (0.1 mg/ml) were added to a KCl medium at pH 8.3 containing valinomycin (0.5 nmol/mg) and nigericin (0.1 nmol/mg). Trace a is the control and shows the effect of adding A²3187 (10 nmol/mg) at 0.2 min. Traces b, c, and d show the effect of the subsequent addition of a small volume of 1 M TES (final concentration, 4.3 mM) to lower the pH to 7.06. The rates of anion uniport calculated from traces a-d are 1400, 117, 68, and 24 nmol of Cl⁻/min·mg, respectively. The basic assay medium contained the K⁺ salts of Cl⁻ (55 mM), EDTA (0.1 mM), and EGTA (0.1 mM) and Tris (2 mM) to buffer the pH at 8.3.
electroneutral anion/OH\(^-\) antiport and electrophoretic proton uniport pathways are open; 3) when the anion is able to form neutral lipid-soluble ion pairs with the K\(^+\)-valinomycin complex. If mechanism 2 were the major mechanism for net salt transport, one would predict that it would be unnecessary to add CCCP to observe maximum swelling rates in NH\(_2\) salts, and similarly, nigericin would be sufficient to induce maximum rates in K\(^+\) salts. The data presented in this paper demonstrate that this is not the case. Furthermore, the finding that net salt transport is induced in NH\(_2\) salts by a protonophore also rules out mechanism 3 as a major pathway for most anions. This leaves electrophoretic anion uniport as the major pathway for anion transport in Mg\(^{2+}\)-depleted mitochondria.

Valinomycin-independent salt transport can be explained by a number of different mechanisms. The simplest is model 1 shown in the "Appendix." In this model electrophoretic influx of the anion drives the electrophoretic efflux of OH\(^-\) (\(J_{OH}\)), with both processes being catalyzed by the anion uniporter. In turn, this Cl\(^-\)/OH\(^-\) exchange drives electroneutral K\(^+\)/H\(^+\) antiport via the endogenous K\(^+\)/H\(^+\) antiporter or nigericin. A plausible alternative is model 2 shown in the "Appendix," in which the electrophoretic influx of the anion drives electrophoretic influx of K\(^+\) (\(J_{Kb}\)) via an endogenous K\(^+\) uniporter. For different anions the maximum rates calculated for these processes (\(J_b\), Table I) vary from about 20 to 900 nmol of K\(^+\) or OH\(^-\) /min·mg. This variation suggests that there is an interaction between anion uniport and OH\(^-\) or K\(^+\) uniport. Interaction between anion and OH\(^-\) transport is easily envisaged if both are carried by the same uniporter; however, interaction between anion transport and K\(^+\) transport could also be possible if the uniporter were able to co-transport K\(^+\) with certain anions. A third model which must be considered is one in which both electrophoretic anion uniport pathways and electroneutral anion/OH\(^-\) antiport pathways exist (see "Appendix," model 3). This model requires one to postulate that Mg\(^{2+}\) depletion activates both these pathways or that valinomycin or CCCP converts the antiporter into a uniporter. We consider this the least plausible model.

Most of our early studies of anion uniport were carried out using NH\(_2\) salts. We frequently observed, however, that the rate of swelling varied substantially depending on the precise experimental protocol and on the age of the mitochondrial preparation. Recently both types of variability have also been noted, but not explained, by Selwyn et al. (38). We have now provided evidence that the first type of variability is related to the exchange of endogenous matrix K\(^+\) for medium NH\(_2\). The cause of the decline in anion uniport rates associated with net NH\(_2\)/K\(^+\) exchange has not been established with certainty; however, we believe that a major factor is a change in the matrix pH. Due to the very high permeability of the inner membrane to NH\(_3\), mitochondria suspended in 55 mM NH\(_3\) must have a relatively alkaline matrix. As matrix K\(^+\) is lost via the K\(^+\)/H\(^+\) antiporter (22-25) and NH\(_3\) is taken up, the NH\(_3\) gradient, and consequently the pH gradient, will decline and the matrix pH will fall. Thus, the decline in the rate of anion uniport which accompanies NH\(_3\)/K\(^+\) exchange could result from inhibition of the uniporter by matrix protons. Inhibition of anion uniport by matrix protons is also able to explain the results obtained in KCl medium shown in Fig. 5. Thus, the low rate observed when both A23187 and valinomycin are added at zero time is explained by inhibition of anion uniport by protons which enter the matrix in exchange for Mg\(^{2+}\). Delaying the addition of valinomycin or adding nigericin permits these inhibitory protons to escape from the matrix by K\(^+\)/H\(^+\) antiport. On the basis of other experiments Brierley (6, 7) has suggested that the pH-dependent anion-conducting pathway may be regulated by matrix pH. The probability that both the Mg\(^{2+}\)-dependent and the pH-dependent uniport pathways are regulated by matrix pH provides further evidence that these pathways are the same.

Although our data are consistent with the regulation of anion uniport by matrix pH, it is difficult to be certain that the effects observed do not arise from changes in the pH gradient. We have, however, been able to demonstrate that anion uniport in Mg\(^{2+}\)-depleted mitochondria is inhibited directly by protons and that the proton pKa values for the transport of chloride and malonate differ by about 0.14 unit. This difference is reproducible and can be explained by the difference in the K\(^+\) concentration in the two media if the H\(^+\) binding site is located on the matrix side of the membrane. Since the media were of equal osmolality, the K\(^+\) concentration in the malonate medium was higher than in the chloride medium (76 versus 58 mM). Therefore, in the presence of nigericin to equilibrate K\(^+\)/H\(^+\) antiport, this difference would make the matrix mitochondria suspended in the malonate medium 0.12 pH unit more alkaline than those suspended in chloride medium of the same pH. Thus, if the H\(^+\) binding site were located on the matrix side of the membrane, one would expect the pKa in malonate to be 0.12 unit lower than the pKa in chloride.

It has not yet been proven that anion uniport is catalyzed by a specific membrane protein and does not occur by simple diffusion through the lipid bilayer. The groups of Azzone (4, 5) and Brierley (7, 12, 13) have proposed that high pH induces a nonspecific change in membrane permeability. In contrast Selwyn's group (10) and Beavis and Garlid (17, 39) have proposed that a specific membrane protein is involved. It is well accepted that SCN\(^-\) can be transported through lipid bilayers electrophoretically (26, 27) or electroneutrally as a lipid-soluble ion pair complex with K\(^+\) and valinomycin (28, 29). Therefore, our finding that SCN\(^-\) transport in Mg\(^{2+}\)-containing mitochondria is essentially unaffected by medium pH in the range from 6.8 to 8.4 suggests that the permeability of the lipid bilayer, at least in the presence of matrix Mg\(^{2+}\), is unaffected by pH.

We have demonstrated that depleting mitochondria of matrix Mg\(^{2+}\) makes them permeable to a wide variety of anions (Table I). Between them the groups of Azzone (4), Brierley (7, 40), and Selwyn (10) have shown that also alkaline pH makes mitochondria permeable to many different anions. Although no quantitative data are available on the relative rates of anion uniport through this "pH-dependent" channel, the finding that both the pH-dependent and "Mg\(^{2+}\)-dependent" channels have very broad selectivities provides further evidence that these pathways are identical. The voltage-dependent anion channel located in the outer mitochondrial membrane also has a very high specificity for anions but, unlike the anion uniport pathway in the inner membrane, it is also able to transport nonelectrolytes (see Ref. 41 for a review).

The physiological function of anion uniport in mitochondria is unknown; however, we have suggested a number of possible roles (17). One of these is the regulation of energy transduction. Examination of Table I reveals that one of the most rapidly translocated physiological anions is HCO\(_3\). Since liver and skeletal muscle mitochondria contain a very active carbonic anhydrase (42) and they are permeable to CO\(_2\), uniport of HCO\(_3\) completes a potentially potent energy-con-
Anion Uniport in Mitochondria

suming futile cycle, which could convert respiratory energy to heat (14). HCO₃⁻ unport could, therefore, be important in nonshivering thermogenesis and obesity. It is noteworthy that in heart and brain mitochondria in which the maximum efficiency of oxidative phosphorylation is desired, there is no carbonic anhydrase in the mitochondrial matrix (42). Phosphate could also undergo futile cycling, and Gainutdinov et al. (43) have recently suggested that this may be the mechanism by which thyroid hormone is able to raise the basal metabolic rate.

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REFERENCES

SUPPLEMENTAL MATERIAL TO THE MITOCNDOCHRIAL IMAGO MEMBRANE ANION CHANNEL: REGULATION BY SIMILAR CATIONS AND PROTONS

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APPENDIX

Model used for Quantitative Analysis

We will consider three simple models. In models 1 and 2 (Figs. 10 and 11) anion transport can only occur via electrochemical gradient whereas $\Delta G$ transport can take place via electrochemical gradient or electrochemical $\Delta G$ gradient. In model 1, net salt transport in the absence of any membrane potential results from electrochemical gradient of ions for hydrogen ions, together with electrochemical $\Delta G$ gradient catalyzed by the anion-specific $\Delta G$ transporter, whereas in model 2, it results from electrochemical gradient of both the anion and $\Delta G$ each catalyzed by an endogenous transporter.

The fluxes through these independent pathways can be described by phenomenological force-laws, in which the flux is expressed as a product of the force driving transport $\Delta G$, and $G$, and the conductance $G$, of the pathway (142). For models 1 and 2, the fluxes are given by

\[ \text{flux} = \text{concentration} \times \text{conductance} \]

where $x$ is the charge on the anion, and $G$ is the transmembrane potential. In model 1, since $\Delta G = 0$, the net salt flux $\Delta G$ is given by

\[ \Delta G = \Delta G \times \Delta G \times \Delta G \]

and, since electromotility is not maintained, $\Delta G = 0$. In model 2, since $\Delta G = 0$, the net salt flux $\Delta G$ is given by

\[ \Delta G = \Delta G \times \Delta G \times \Delta G \]

Using these equations, the net salt flux can be expressed in terms of the salt gradient $\Delta G$, and the conductance.

For model 1:

\[ \Delta G = \frac{\Delta G \times \Delta G \times \Delta G}{\Delta G \times \Delta G \times \Delta G} \]

If $\Delta G \gg \Delta G$, i.e., when sufficient electromotility is present, this equation may be simplified to give

\[ \Delta G \times \Delta G \times \Delta G \]
Anion Uniport in Mitochondria

\[ J_a = \frac{\Delta V_a}{R_a + \frac{1}{2} \frac{1}{R_m}} \Delta \alpha_a(A) \]  

(420)

This equation is very similar to the equation for net salt flux for model 2:

\[ J_a = \frac{\Delta V_a}{\left( R_a + \frac{1}{2} \frac{1}{R_m} \right)} \Delta \alpha_a(A) \]  

(421)

For both models if \( R_a \gg R_m \) i.e. when sufficient valinomycin is present these equations reduce to:

\[ J_{a,max} = \lambda_{a,\text{max}} = \lambda_{a,\text{max}} \Delta \alpha_a(A) \]  

(422)

where \( \lambda_{a,\text{max}} \) is limited only by the anion conductance and the gradient.

In the absence of valinomycin, \( V_a = 0 \) and therefore equation 420 for model 2 reduces to:

\[ J_{a,0} = \lambda_{a,0} \Delta \alpha_a(A) \]  

(423)

and similarly equation 421 for model 2 reduces to:

\[ J_{a,0} = \lambda_{a,0} \Delta \alpha_a(A) \]  

(424)

From equations 423 - 424 the following equation may be derived for both models:

\[ J_{a,0} = J_{a,\text{max}} \left( 1 - \frac{V_{a,0}}{V_{a,\text{max}}} \right) \]  

(425)

This equation demonstrates that for both models there is a linear relationship between \( J_a \) and \( \lambda_{a,0} \). Thus, if we assume that \( \lambda_{a,0} \) is a linear function of the valinomycin concentration and that \( \Delta \alpha_a(A) \) is the same for each flux measurement, we would predict a linear relationship between \( J_a \) and \( \lambda_{a,0} \) (Experiment). The intercept in the ordinate gives \( V_{a,\text{max}} \) and the slope provides the \( K_a \) for valinomycin.

In these models the other quantities of interest are the maximum rates of endogenous On transport (\( J_{\text{max}} \)) in model 1, and the maximum rate of \( a^- \) uniport (\( J_{a,\text{max}} \)) in model 2. These fluxes are given by \( \lambda_{a,\text{max}} \Delta \alpha_a(A) \) and \( \lambda_{a,\text{max}} \Delta \alpha_a(A) \), respectively, and can be calculated using the following equation which may be derived from equations 422 or 424:

\[ J_{a,\text{max}} \Delta \alpha_a(A) = \lambda_{a,\text{max}} \left( 1 - \frac{V_{a,0}}{V_{a,\text{max}}} \right) \]  

(426)

In conclusion, these models although not experimentally, cannot be distinguished by the data presented in this paper.

The third model which we will consider is one in which the rate of salt transport observed in the absence of valinomycin is due to electroneutral anion uniport coupled to an electroneutral \( K^+ \) uniport. The fluxes which contribute to net salt transport are shown in Fig. 10. They are described by equations 421, 422 and 423 plus the flux equation for electroneutral anion uniport:

\[ J_a = \lambda_{a,\text{max}} \Delta \alpha_a(A) \]  

(427)

For this model net salt flux is given by:

\[ J_{a,\text{net}} = \lambda_{a,\text{max}} \Delta \alpha_a(A) \]  

(428)

and, in order to maintain electroneutrality:

\[ J_K = \lambda_{K,\text{max}} \Delta \alpha_K(K) \]  

(429)

From these equations it may be shown that:

\[ J_a = \frac{\lambda_{a,\text{max}} \Delta \alpha_a(A)}{\left( \frac{1}{R_a} + \frac{1}{R_m} \right)} \]  

(430)

If \( R_a \gg R_m \) i.e. no valinomycin is present and if \( J_{K,\text{net}} = 0 \) i.e. sufficient valinomycin is present, this equation reduces to:

\[ J_a = \lambda_{a,\text{max}} \Delta \alpha_a(A) \]  

(431)

This is the maximum rate of electroneutral anion uniport.

If \( J_{K,\text{net}} = 0 \) i.e. in the presence of sufficient valinomycin the rate of swelling is limited only by the gradient and anion conductances.