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K^+ TRANSPORT IN MITOPLASTS

HSIAO-SHENG CHANG AND JOYCE JOHNSON DIWAN *

Department of Biology, Rensselaer Polytechnic Institute, Troy, NY 12181 (U.S.A.)

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K^+ transport into mitoplasts, prepared by digitonin disruption and removal of the outer membranes from rat liver mitochondria, has been studied. Unidirectional K^+ influx has been measured by means of ^{42}K, in the presence of the respiratory substrate succinate. K^+ influx is inhibited by CN^-, antimycin A and dicyclohexylcarbodiimide, but is insensitive to oligomycin. A linear dependence of the reciprocal of the K^+-influx rate on the reciprocal of the external K^+ concentration is observed. Under the conditions studied, the apparent K_m for K^+ of the transport mechanism is approx. 6 mM, while the V_{max} of K^+ influx is approx. 5 μmol K^+/g protein per min. The rate of K^+ influx increases with increasing external pH over the range from 6.8 to 8.0. The observed kinetics, pH dependence and inhibitor sensitivity are essentially similar to previously reported characteristics of K^+ transport into intact rat liver mitochondria. It is concluded that the outer mitochondrial membrane does not have a role in controlling K^+ flux into rat liver mitochondria.

Introduction

Electron microscopic observations of changes in mitochondrial matrix volume accompanying cation translocation [1–4], and measurements of solute penetration and associated osmotic volume changes [5–9], have been interpreted as indicating that the inner mitochondrial membrane is the major permeability barrier of the mitochondrion and the site of ion-transport mechanisms. Earlier studies have indicated that the outer mitochondrial membrane is generally permeable to low molecular weight solutes [5–10], but impermeable to various polymeric substances [11–13]. However, the outer membranes of freshly isolated mitochondria, and isolated outer membrane vesicles, have been found to be impermeable to ADP and glycerol phosphate [14]. Structures observed in electron microscopic and X-ray diffraction studies of outer membranes of plant [15–17] and *Neurospora* [18,19] mitochondria have been interpreted as representing transmembrane pores [16,17,20]. A glycoprotein isolated from outer membranes of mitochondria of *Paramecium* [21], rat liver [22] and *Neurospora* [23] forms voltage-sensitive pores when inserted into planar lipid bilayers. This material, designated voltage-dependent anion-selective channels (VDAC) [20–23], resembles the channel-forming protein porin which has been isolated from the outer membranes of gram-negative bacteria [24,25]. However, while porin exhibits little voltage dependence below about 150 mV [24,25], VDAC undergoes a transition to a lower conducting state at transmembrane potentials of 10–30 mV [20].

It has been suggested that the variable permeability of VDAC may regulate the flow of metabolites between the cytoplasm and the mitochondria

* To whom correspondence should be addressed.

Abbreviations: VDAC, voltage-dependent anion-selective channels; DCCD, *N,N'*-dicyclohexylcarbodiimide; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

[22]. Studies of permeability to various nonelectrolytes of liposomes containing VDAC have indicated a pore radius of approx. 20 Å [20,23]. This is presumably the radius of the open channel. While the radius of the closed channel has not been as clearly defined, permeability to K^+ of VDAC in planar lipid membranes has been observed to decrease 25–50%, as Cl^- permeability decreases 70–90%, when VDAC closes [20].

Unidirectional K^+ flux into mitochondria is known to be respiration dependent [26], saturable with respect to external K^+ [27,28], pH dependent [27] and sensitive to inhibition by DCCD [29]. The present studies of K^+ flux into rat liver mitoplasts were undertaken to evaluate whether the absence of the outer mitochondrial membrane might result in altered K^+ transport.

Materials and Methods

Rat liver mitochondria were prepared by standard procedures in medium containing 220 mM D-mannitol, 70 mM sucrose, 2.0 mM Hepes KOH buffer (pH 7.4) and 0.5 mg/ml crystalline bovine serum albumin [30]. Mitoplasts were then prepared by essentially the method of Schnaitman and Greenawalt [31], as modified by Pedersen et al. [30]. Mitochondria were treated with 0.11–0.12 mg digitonin/mg protein and the mitoplasts separated by centrifugation, as described previously [30]. Monoamine oxidase was assayed in Lubrol-treated mitochondria and mitoplasts by the procedure of Schnaitman et al. [31,32]. Respiration was monitored by a Clark-type membrane-covered electrode connected to a potentiometric recorder. Protein was assayed by the biuret technique [33]. The K^+ content of acid extracts of mitoplasts was assayed by atomic absorption spectroscopy.

For measurements of K^+ influx, mitoplasts were incubated at 20°C in medium, unless otherwise indicated, containing 70 mM sucrose, 220 mM mannitol, 31 mM Tris, 7.8 mM succinate, variable amounts of KCl, and the radioisotopes ^{42}K (approx. 0.6 μCi/ml), ^3H_2O (approx. 2.6 μCi/ml), and [^{14}C]sucrose (approx. 0.4 μCi/ml), with the pH adjusted to 7.5 with HCl. At timed intervals, mitoplast samples were separated from incubation media by rapid centrifugation through silicone [9]. ^{42}K was assayed by liquid scintillation counting of the Cerenkov radiation in aqueous dilutions of acidified mitoplast samples and supernates, and the counts were corrected for decay. Following decay of the ^{42}K, total K^+ levels were assayed by atomic absorption spectroscopy, and ^3H and ^{14}C were counted using a standard liquid scintillation cocktail.

The mitoplasts' content of labeled K^+ was calculated from the ^{42}K counts sedimented with the mitoplasts and the initial (0.75 min) supernatant specific activity. The contaminating external K^+ sedimenting with the mitoplasts was calculated as the product of the [^{14}C]sucrose distribution space and the measured supernatant K^+ concentration. The unidirectional K^+-influx rate was calculated from the change in mitoplast content of labeled K^+ between samples taken after 0.75 and 7 min of incubation. These procedures and calculations are similar to those used in studies of K^+ flux into intact mitochondria (e.g., see Refs. 27 and 29).

^{42}K was obtained from New England Nuclear. The silicone used (SF1154) was a gift from the General Electric Co. Digitonin was obtained from Fisher Scientific Co. DCCD, oligomycin and antimycin A were purchased from Sigma Chemical Co.

Results and Discussion

Characterization of mitoplasts

Observations of the dependence of mitoplast respiration on ADP in pH 7.4 medium containing P_i and succinate have yielded estimates of acceptor control ratios of 1.8 ± 0.3 (means of six determinations in two experiments ±S.D.). Such values are low, e.g., compared to the average respiratory control ratio of 5.6 determined for intact mitochondria in the same experiments. However, they are close to values of 1.7–2.5 reported previously for mitoplasts [30]. Approx. one-third each of mitochondrial protein and K^+ is lost in the preparation of mitoplasts. For example, in three experiments the yield of protein in the prepared mitoplasts was $67 \pm 4\%$ of the protein content of the starting mitochondrial suspension (mean ±S.D.). The corresponding yield of K^+ was $64 \pm 3\%$. The relative K^+ concentration, in units of μmol K^+/g protein, thus remained similar, being

115 ± 8 for the starting mitochondria and 112 ± 6 after removal of the outer membrane. The retention of substantial quantities of endogenous K^+ is consistent with maintenance of normal permeability properties of the inner membrane following digitonin treatment.

Assays for activity of monoamine oxidase, an outer mitochondrial membrane marker enzyme [32], have confirmed removal of most of the outer membrane. The mitoplasts were estimated to contain $6.8 \pm 4.5\%$ of the total monoamine oxidase activity of the starting mitochondria (mean of seven determinations in three experiments $\pm$S.D.). In the same experiments the average specific activity of monoamine oxidase, measured by assay of benzaldehyde produced from benzylamine, was 26 nmol benzaldehyde/mg protein per min for the starting mitochondria and 3.3 nmol/mg protein per min for the mitoplasts.

K^+-influx measurements

The amount of labeled K^+ taken up by the mitoplasts, in the presence and absence of CN^-, is plotted as a function of incubation time in Fig. 1. The ^{42}K uptake is biphasic. There is an intitial rapid uptake of labeled K^+, followed by a slower increase in the content of labeled K^+. In similar

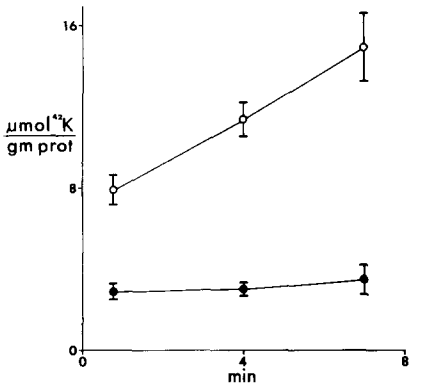

Fig. 1. Effect of CN^- on the time course of uptake of labeled K^+. The concentration of mitoplast protein was 4.3 mg/ml. The K^+ concentration of the medium was 3.0 mM. The labeled K^+ taken up, in units of μmol/g protein, is plotted against the incubation time (min). All values are corrected for contaminating external K^+, calculated as specified in Materials and Methods. The values shown are means of six determinations. Standard deviations are shown by the vertical bars. (○) The medium included 1 mM NaCl (controls), (●) the medium included 1 mM NaCN.

studies with intact mitochondria, most of the initial rapid ^{42}K binding was found to be insensitive to antimycin A, while the respiratory inhibitor blocked the slower progressive uptake of labeled K^+ [27]. It was concluded that the initial K^+ binding is attributable at least in part to adsorption, while the slower rate of ^{42}K uptake corresponds to respiration-dependent entry of K^+ into the mitochondrial matrix [27]. As shown in Fig. 1, CN^- blocks part of the initial rapid ^{42}K uptake by mitoplasts, and largely eliminates the slower influx of K^+. The nature of the rapid CN^--sensitive K^+ binding is unclear. As shown in Fig. 1, ^{42}K uptake in the absence of the respiratory inhibitor is an essentially linear function of incubation time during the 7-min incubations, although in some experiments a slight slowing of the uptake rate with time has been observed. The K^+-influx rates are thus considered to approximate to initial rates.

Under the conditions of these experiments, the mitoplasts are close to but not quite in a steady state with respect to total K^+ content. For example, for control samples in Fig. 1, the average rate of unidirectional K^+ influx, estimated from the slope of the time course of ^{42}K uptake, was 1.3 μmol K^+/g protein per min. This was balanced by a somewhat larger rate of unidirectional K^+ efflux such that there was an average net efflux of 1.5 μmol K^+/g protein per min. Under some conditions of other experiments, e.g., at the higher external K^+ levels tested in the experiment of Fig. 3 depicted below, where the rates of unidirectional K^+ influx are higher, there was either a smaller net efflux or a net influx. Under all of the conditions tested, the amounts of K^+ moving into or out of the mitoplasts during the 6.25-min time course of the influx measurements were modest compared to the total endogenous K^+ content which, for example, averaged 102 μmol/g protein in the experiment of Fig. 1.

The inhibitor sensitivity of K^+ influx is explored further in the experiment shown in Table I. The inhibitory effect of the respiratory inhibitor antimycin A is consistent with the effect of CN^- shown in Fig. 1. Consistent with results obtained with intact mitochondria [29], the oxidative phosphorylation inhibitor oligomycin has no apparent effect on K^+ flux into respiring mitoplasts, while DCCD is inhibitory.

TABLE I

EFFECTS OF SOME METABOLIC INHIBITORS ON K^+ INFLUX

The concentration of mitoplast protein was 8.5 mg/ml. The medium included 2.4 mM K^+ and where indicated 0.25 μg/ml antimycin A or 2.5 μg/ml oligomycin. The DCCD-treated samples were preincubated at 0°C for at least 40 min with 31 nmol DCCD/mg protein. The values shown are means of three determinations ± S.D.

Additions	K^+ influx (μmol/g protein per min)
None	1.7±0.2
Antimycin A	0.4±0.1
Oligomycin	1.9±0.2
DCCD	0.6±0.1

The pH dependence of K^+ influx is examined in the experiment shown in Fig. 2. The dependence of K^+ flux into mitoplasts on pH is similar to that described for intact mitochondria [27]. An effect of the sulfhydryl a reagent *N*-ethylmaleimide in increasing the pH dependence of K^+ flux into intact mitochondria has been attributed [27] to the inhibitory effect of *N*-ethylmaleimide on H^+-linked phosphate exchange [34]. In the presence of *N*-ethylmaleimide, a linear dependence of K^+ flux into intact mitochondria on the OH^- concentration of the medium is observed [27]. As shown in Fig. 3, *N*-ethylmaleimide also increases the pH dependence of K^+ flux into mitoplasts. However, a linear dependence on OH^- is not observed. Consistent with the results obtained with intact mitochondria, the rate of K^+ flux into mitoplasts increases with increasing pH of the medium over the range from 6.8 to 8.0.

The concentration dependence of the rate of K^+ flux into mitoplasts is examined in the experi-

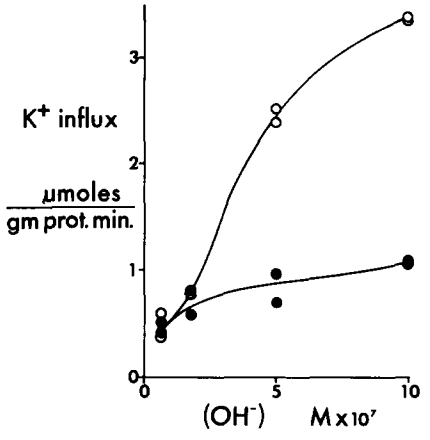

Fig. 2. The pH dependence of K^+ influx in the presence and absence of *N*-ethylmaleimide. The concentration of mitoplast protein was 5.0 mg/ml. The K^+ concentration in the medium was 2.6 mM. The pH of the medium was varied from 6.8 to 8.0 by titration with HCl. The K^+-influx rate, in units of μmol K^+/g protein per min, is plotted against the molar concentration of OH^- in the medium. (●) Control samples, (○) the medium included 500 μM *N*-ethylmaleimide.

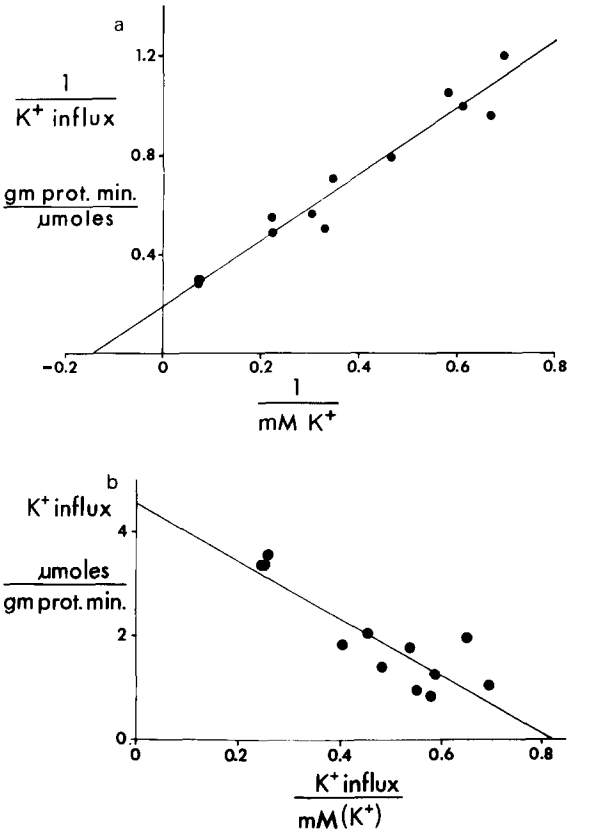

Fig. 3. The dependence of K^+ influx on external K^+ concentration. The concentration of mitoplast protein was 2.8 mg/ml. The K^+ concentration of the medium was varied from 1.5 to 13.7 mM. The lines drawn were calculated by the method of least squares. (a) The reciprocal of the K^+-influx rate, in units of g protein·min per μmol K^+, is plotted against the reciprocal of the external K^+ concentration (mM^{-1}). (b) The K^+-influx rate, in units of μmol K^+/g protein per min, is plotted against the K^+-influx rate divided by the external K^+ concentration (mM), in units of ml/g protein per min.

TABLE II

APPARENT KINETIC CONSTANTS

Kinetic constants were calculated from lines fitted to each of the plots indicated by the method of least squares. The values shown were obtained in the experiment depicted in Fig. 3 and another equivalent experiment.

Plot	K_m (mM K^+)	V_{max} (μmol/g protein per min)
Lineweaver-Burk	6.4	5.0
	6.9	5.2
Eadie-Hofstee	5.3	4.4
	5.6	4.6

ment shown in Fig. 3. The Lineweaver-Burk plot of the reciprocal of the K^+-influx rate against the reciprocal of the external K^+ concentration is linear, as shown in Fig. 3a, as are similar plots obtained with intact mitochondria [27,29]. An Eadie-Hofstee plot of the same data is shown in Fig. 3b. Kinetic constants calculated in this and another similar experiment are summarized in Table II. These compare to values of approx. 7 mM apparent K_m for K^+ and 3 μmol K^+/g protein per min estimated V_{max} of K^+ flux into intact mitochondria under equivalent conditions [29]. On a protein basis, the estimated V_{max} of K^+ flux into mitoplasts is thus higher than the V_{max} of K^+ flux into intact mitochondria. However, when one takes into account the fact that on average 33% of mitochondrial protein is lost with removal of the outer membrane, the values of V_{max} in terms of activity per fraction of the total preparation are actually very similar for the mitoplasts and intact mitochondria.

Thus, it is concluded that the K^+-transport properties of rat liver mitochondria are little affected by removal of the outer mitochondrial membrane. At least under the conditions studied, any variation in the size of outer membrane pores does not appear to be a significant factor controlling K^+ permeability. These findings support the conclusions of Saint Macary et al. [10], based on studies of the kinetics of substrate-anion exchange rates in mitochondria and mitoplasts. The mitoplast preparation is found to be a suitable model system for studying mitochondrial transport reactions.

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