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# RECONSTITUTION OF TRANSMEMBRANE $K^+$ TRANSPORT WITH A 53 KILODALTON MITOCHONDRIAL PROTEIN

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A 53 kDa protein has been purified from a Triton X-100 extract of liver mitochondrial membranes, by affinity chromatography on immobilized quinine, a $K^+$ transport inhibitor. KCl-containing lipid vesicles reconstituted with this protein lose $K^+$ to a medium low in $K^+$ faster than vesicles lacking protein. With bacteriorhodopsin reconstituted in vesicles containing $K^+$, light induces faster development of a pH gradient if the 53 kDa protein is included during vesicle preparation. This effect is like that of valinomycin, which catalyzes $K^+$ efflux, dissipating the membrane potential arising from $H^+$ entry. Evidence that vesicles containing the 53 kDa protein are permeable to $K^+$, but exhibit low permeability to $H^+$, indicates that this protein acts as a $K^+$ uniporter.



$K^+$ enters mitochondria via a specific, proteinaceous transporter, located in the inner membrane. Unidirectional $K^+$ influx, measured via $^{42}K$, occurs by a saturable mechanism (1-3), sensitive to inhibition by the $K^+$ analogs $Tl^+$ and $Ba^{++}$ (1,4). $K^+$ flux into mitochondria stripped of their outer membranes exhibits dependence on external $K^+$ similar to that of whole mitochondria (5). Sulfhydryl reactive compounds including mercurials (3,6,7), $Cd^{++}$ (8), dibutylchloromethyltin chloride (9), and phenylarsine oxide (10,11) stimulate $K^+$ entry. The carboxyl group reagent, DCCD, inhibits $K^+$ influx (12,13). DCCD does not fully block $K^+$ entry, but increases the apparent $K_m$ for $K^+$ (12). Treatment with DCCD does not prevent subsequent activation of $K^+$ influx by mersalyl or phenylarsine oxide (11). Both $K^+$ influx and $Ca^{++}$-activated net $K^+$ uptake are partially inhibited by the organic cation quinine (14,15).

Rates of both influx and efflux of $K^+$ and other cations are diminished when respiration is blocked (16-18). This respiration dependence has been explained relative to the chemiosmotic theory (19) by the proposal that $K^+$ enters via a

Abbreviations: DCCD, dicyclohexylcarbodiimide; SMP, submitochondrial particles; CCCP, carbonyl cyanide m-chlorophenylhydrazone; BR, bacteriorhodopsin.

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uniporter (3), and exits via a $K^+/H^+$ antiporter (20,21). The pH dependence of $K^+$ efflux (2,21,22) is inconsistent with a $K^+/H^+$ exchange mechanism, but secondary pH effects are not ruled out. $H^+$ may regulate the antiporter (23). Discharge of matrix $Mg^{++}$ with A23187 stimulates net $K^+$ fluxes apparently via the antiporter (23-30). $Mg^{++}$ is proposed to regulate the antiporter (23,29). The physiological significance of a regulatory process expressed only with large changes in matrix $Mg^{++}$ has been questioned (31,32). It has been found that there is no gradient of free $Mg^{++}$ between the cytosol and mitochondria of liver cells (33). Quinine inhibits net $K^+$ fluxes attributed to the antiporter (24,30,31,34) and blocks unidirectional $K^+$ efflux (14). DCCD inhibits antiport activity only if mitochondria are depleted of $Mg^{++}$ before DCCD treatment (23). $Mg^{++}$ and quinine block labeling of an 82 kDa protein with [$^{14}$C]DCCD in $Mg^{++}$-depleted mitochondria (23,35). The kinetics of labeling correspond to the rate constant for inhibition by DCCD of swelling in $K^+$ acetate (36). It was concluded that the 82 kDa protein is the $K^+/H^+$ antiporter (23,36).

An approximately 53 kDa protein has been isolated from rat liver SMP by affinity chromatography on immobilized quinine (35). When mitochondria were treated with [$^{14}$C]DCCD under conditions which result in altered kinetics of $K^+$ influx (12), the 53 kDa protein was labeled (35). Because of the similarity in size to the largest subunit of the $F_1$ ATPase (37), a major mitochondrial protein, reactivity with antibodies against $F_1$ was tested. Immunoblots have shown that antibodies to bovine heart $F_1$, prepared by Dr. Saroj Joshi, cross react with SDS gel bands of rat liver SMP in the appropriate size range; but the 53 kDa protein isolated from rat liver SMP fails to bind the anti-$F_1$ antibodies (38). Thus the 53 kDa protein is not an $F_1$ subunit. Results to be described support the conclusion that the 53 kDa protein is a $K^+$ uniporter.

## MATERIALS AND METHODS

Protein purification by affinity chromatography. Epoxy activated Sepharose 6B (Pharmacia Fine Chemicals) was incubated overnight (about 16 h) in the dark at 38 °C in 50 mM quinine in 40% tetrahydrofuran, adjusted to pH 12 with NaOH. The gel beads were then incubated 1 week in 100 mM $Na_2CO_3$, pH 11, to promote hydrolysis of unreacted oxirane groups (39). Mitochondria, isolated from livers of male, albino, retired breeder rats, in 250 mM sucrose, 2 mM Hepes, 1 mM Na EDTA, & 0.5 g/l BSA (defatted), pH 7.4, were sonicated, and centrifuged at low speed to remove unbroken mitochondria. SMP were then sedimented by standard procedures (40). SMP (100-280 mg protein) were dissolved at 13-20 mg protein per ml in a pH 7.5 solution of 50 mM NaCl, 2 mM Hepes, 2% Triton X-100, and 1 mM EDTA. This extract (7-14 ml) was applied at 8-15 min per ml to a 2 ml column (0.8 cm diameter) of the quinine-sepharose. The column was washed with about 20 ml of 100 mM NaCl, 2 mM Hepes, 1 mM EDTA, 2% Triton X-100 (pH 6.6-6.7) during about 2 hours. Elution media (see Fig. 1) and other solutions usually included 0.5 ng/ml leupeptin & 0.7 ng/ml pepstatin.

SDS PAGE was carried out by the procedures of Laemmli (41). Protein of mitochondrial and SMP samples was measured by the biuret technique (42). The protein content of affinity column eluates was measured with the bicinchoninic

acid protein assay reagent from Pierce Chemical Co. Biochemicals were obtained from Sigma Chemical Co. and most other reagents from Fisher Scientific Co.

Vesicle preparation. The protein eluted from the affinity column in the cholate medium listed in Fig. 1B was concentrated by ultrafiltration with an Amicon stirred cell (YM30 filter). About 600 ul concentrated eluate (1.3-1.7 mg protein), or the same volume of elution medium for controls, was added to 45 mg purified (43) asolectin in 1.5 ml of 400 mM octylglucoside, 5 mM Tris $P_i$ (pH 7.5). The mixture, diluted to 3 ml with 5 mM Tris $P_i$, pH 7.5, was dialyzed against 1 l of 5 mM Tris $P_i$, pH 7.5, with 25 mM KCl for 16 h at 4 °C. The buffer was changed, 4 g Bio-Beads (Bio-Rad SM-2) was added in a dialysis sac, and dialysis continued for 24 h more. The vesicles were centrifuged 2 X $10^7$ g(min), and resuspended in 50 µl of 5 mM Tris $P_i$ (pH 7.5) with 25 mM KCl.

In two experiments, BR was reconstituted by detergent dialysis (44-46) with and without the 53 kDa protein. Purified BR (47,48), provided by Dr. Bonnie Ann Wallace, was solubilized (49) for 24 h at 23 °C at 0.3-0.5 mg/ml in 25 mM $KH_2PO_4$, 2 mM Na EDTA, & 40 mM octylglucoside, pH 6.9. Asolectin (20 mg), dried from chloroform as a thin film in a glass tube, was exposed to vacuum for 2 h. Two ml of the BR solution was added and the lipids dissolved. 0.35 ml concentrated affinity column eluate (or elution medium for controls) was added to yield a ratio of 0.6-0.9 mg BR: 20 mg asolectin: about 0.9 mg 53 kDa protein. The mixture was dialyzed 54 h at 4 °C against 125 mM NaCl, 25 mM KCl, 2 mM Na EDTA, pH 6.9, with the 1 l of buffer changed after 16, 28, and 40 h. Four g Bio-Beads in a dialysis sac was added with the last two buffer changes. The vesicles were loaded on a 4.5-40% sucrose gradient in 25 mM KCl, 1 mM Na EDTA, pH 6.9. After centrifugation [1.4 X $10^8$ g(min) in the experiment of Fig. 3], the purple vesicle bands were harvested by removal of fractions from the top.

Electrode recordings. pH was recorded with a combination pH electrode, pH meter, and potentiometric recorder. A Corning monovalent cation electrode with a double junction reference electrode was used to record $pK^+$. KCl leaking from a standard reference electrode is significant at the concentrations studied. A Kodak slide projector, with Kodak Wratten gelatin filter #4, was used as light source. The reaction vessel was jacketed with water at 30 °C. Electrodes were masked from the light except where protected by the water jacket.

## RESULTS AND DISCUSSION

Fig. 1 shows the purity of the protein as eluted from the affinity column. It is referred to as the "53 kDa protein," although the size estimate is based only on SDS PAGE as shown. Fig. 1B is typical of isolates used for reconstitution. Minor bands are seen, but the one at 53 kDa is always the major protein. As shown by elution with SDS cocktail (Fig. 1A lane 3), little else remains on the column after the washing with high ionic strength Triton solution. Thus binding of the 53 kDa protein to quinine on the column appears to be specific.

When 6 µl vesicles (in and loaded with 25 mM $K^+$) are added to 4 ml medium with only 9 µM $K^+$, accompanying external $K^+$ yields a total of less than 47 µM. At such low $K^+$, efflux from as little as 1 ul intravesicular volume (6 µM) is measurable. The monovalent cation electrode response is non-linear at low $K^+$, but results under the same conditions can be compared. When vesicles lacking protein are added, the electrode records the added external $K^+$ (full deflection not shown), and stabilizes in about 1 min, indicating little continuing $K^+$ release (Fig. 2, trace 3). CCCP in some experiments has caused a slow $K^+$ release; in some experiments no effect has been seen. That the vesicles still contain $K^+$ is shown as $K^+$ is discharged by nigericin and/or Triton X-100.

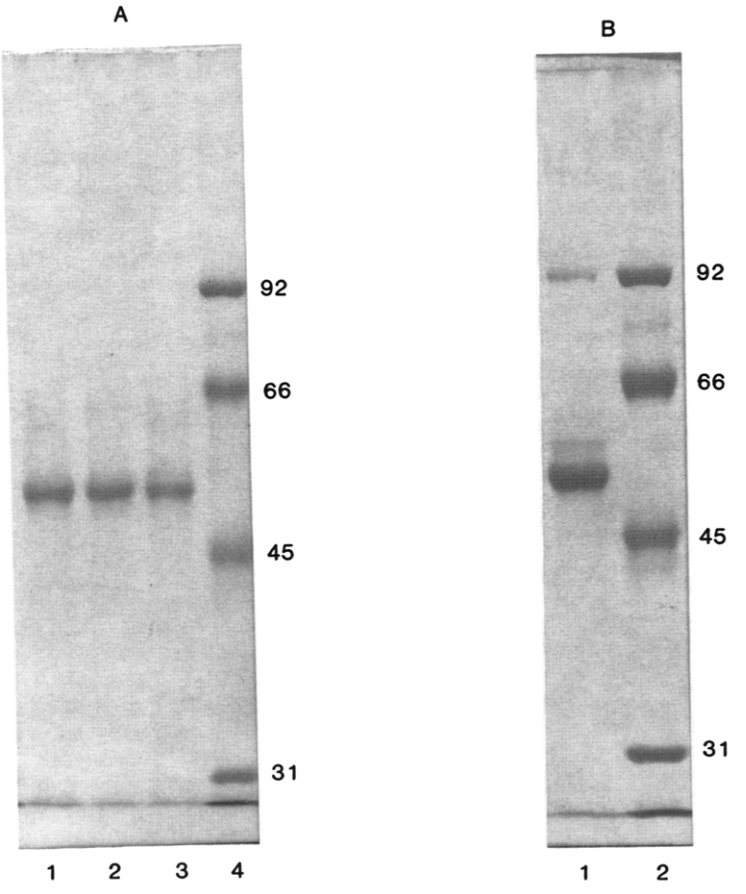
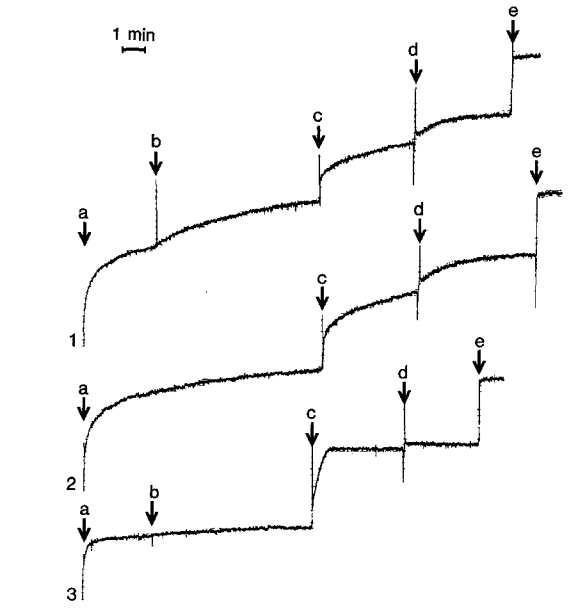
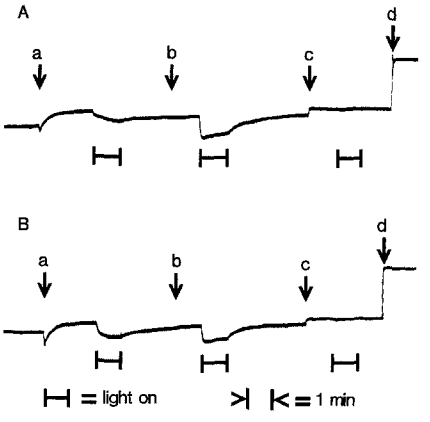



Fig. 1. SDS PAGE analysis of affinity column eluates. A: Each eluate was precipitated with cold acetone and dissolved in 200 ul SDS cocktail [139 mM TrisCl, pH 6.8, 2.3% SDS, 11% glycerol (v/v), 5.6% 2-mercaptoethanol (v/v)], and 4 µl loaded on the gel. Lane 1, initial elution with 10 mM quinine, 500 mM KCl, 2 mM Hepes, & 2% Triton X-100, pH 6.0, combined with a 1 ml elution with the same solution lacking quinine. Lane 2, subsequent 2ml elution with 500 mM KCl, 2 mM Hepes, & 1% sodium cholate, pH 6.2, combined with a 1 ml elution with the same solution at pH 6.8. Lane 3, final elution with 3 ml SDS cocktail. Lane 4, MW standards. Expt. B: Protein eluted with 500 mM KCl, 2 mM Hepes, and 1% sodium cholate, pH 6.2, was precipitated with cold acetone, dissolved in SDS cocktail, and 7 µg loaded in Lane 1. Lane 2, MW standards.

When vesicles prepared with the 53 kDa protein are added to fresh medium, after the rapid increase due to external $K^+$, a progressive increase in $K^+$ is consistently recorded (traces 1,2). In the experiment of Fig. 2, the average rate of $K^+$ loss from protein-containing vesicles from 1 to 2 min after vesicle addition, based on the recorder deflection, was at least 4 times the rate of $K^+$ loss from control vesicles. The protonophore CCCP increases the rate of $K^+$ release from the protein-containing vesicles. This could be due to dissipation of a diffusion potential, if $K^+$ transport is by a uniport type of mechanism. The effect of CCCP also indicates limited permeability of the membranes to $H^+$.




(2)




(3)

Fig. 2. $K^+$ efflux from membrane vesicles. Traces 1 and 2, vesicles prepared with the 53 kDa protein; Trace 3, control vesicles lacking protein. Additions to 4 ml containing 5 mM Tris $P_i$, pH 7.5, 9 μM KCl, and 50 mM sucrose: a, 6 μl vesicles in 5 mM Tris $P_i$, pH 7.5, with 25 mM KCl; b, 1 μM CCCP; c, 50 ng/ml nigericin; d, 0.01% Triton X-100; e, 6 μM KCl (calibration).

Fig. 3. $H^+$ uptake by vesicles containing BR. Trace A, vesicles prepared with BR alone; Trace B, vesicles prepared with BR plus the 53 kDa protein. To 2.3 ml of 150 mM NaCl plus 2 mM sodium EDTA, pH 6.9, was added: a, 200 μl vesicles in sucrose (from density gradient) with 25 mM KCl and 1 mM sodium EDTA, pH 6.9; b, 40 ng/ml valinomycin; c, 1.6 μM CCCP; d, 40 nmoles HCl (calibration).

BR reconstitutes into vesicle membranes in the reverse orientation (44). Light activates slow $H^+$ uptake by vesicles containing BR alone (Fig. 3A). With valinomycin added, light activates more rapid buildup of a pH gradient (44), as the membrane potential due to $H^+$ uptake is dissipated by outward $K^+$ flux. Vesicles including the 53 kDa protein show a more rapid light-activated $H^+$ uptake in the absence of valinomycin (Fig. 3B). With 1 min illumination in the experiment of Fig. 3, the time to attain the maximum observed pH gradient was 0.9-1.0 min with BR alone, and 0.4-0.6 min for vesicles with BR and the 53 kDa protein. The faster development of a pH gradient indicates that vesicles with the 53 kDa protein have a pathway for counterion flux. But they are not leaky to $H^+$, as shown by their ability to develop a pH gradient, and by the slow decay of this gradient when the light ceases. The 53 kDa protein, like valinomycin, appears to be a uniporter. Incorporation of a $K^+/H^+$ exchanger or a non-specific leak into the membrane would have inhibited pH gradient development.

$K^+$ efflux was not recorded during $H^+$ uptake via BR, due to extreme light sensitivity of the monovalent cation electrode. Mild uncoupling by quinine

(31,50) prevented testing for effects on $H^+$ pumping by BR in vesicles with the 53 kDa protein. Quinine (600 μM) blocks formation of a pH gradient, even in the absence of the 53 kDa protein. When the effect of quinine on $K^+$ gradient-driven $K^+$ loss was tested, all $K^+$ flux rates increased, even those mediated by ionophores, in vesicles lacking protein, and in the presence of CCCP. The basis for this effect is unclear. The stereoisomer quinidine is reported to affect membrane fluidity (51). Although the inhibitor sensitivity of the 53 kDa protein remains to be determined, the results obtained so far suggest that this protein is the uniporter postulated to mediate $K^+$ flux into mitochondria.

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