A mitochondrial protein fraction catalyzing transport of the K^+ analog Tl^+

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Received 6 September 1990

A protein fraction has been obtained from detergent-solubilized mitochondrial membranes by its affinity for quinine, an inhibitor of K^+ transport. A peptide derived from the predominant 53 kDa protein in this fraction is found to be identical in sequence to a portion of aldehyde dehydrogenase. Antigenically unrelated bands at 97, 77, 57, and 31 kDa are also seen on polyacrylamide gels. Observations utilizing a fluorescent probe entrapped in the lumen of membrane vesicles indicate that the reconstituted protein fraction imparts permeability to the K^+ analog Tl^+. These and other findings suggest that the affinity purified fraction includes a cation transport catalyst.

K^+ transport; Mitochondrial membrane; Quinine; Fluorescence quenching; Tl^+

1. INTRODUCTION

The literature relating to mitochondrial K^+ transport has been reviewed [1,2]. An electrophoretic K^+ uniport mechanism of K^+ uptake is suggested by the finding that swelling of respiring mitochondria in solutions of K^+ salts of acetate or phosphate depends on entry of the free acid [1,3,4]. Unidirectional K^+ flux into respiring mitochondria measured via ^{42}K is saturable with respect to external K^+ [5,6]. K^+ influx is competitively inhibited by Mg^{2+} [6,7] and by the K^+ analog Tl^+ [5]. Tl^+ is itself taken up by respiring mitochondria [8]. The organic cation quinine partially inhibits unidirectional and net K^+ fluxes into respiring mitochondria [9,10]. In contrast, quinine is reported to stimulate the K^+ uniport activity activated by depletion of matrix Mg^{2+} [11].

DCCD inhibits K^+ entry, increasing the apparent K_m for K^+ of the influx mechanism [12,13]. Various sulfhydryl reactive reagents stimulate unidirectional K^+ influx, including mersalyl [6,14], Cd^+ [15], and phenylarsine oxide [16]. Induction of a non-specific permeability pathway by reagents such as diamide, *N*-ethylmaleimide, P_i, and *t*-butyl hydroperoxide under conditions of Ca^{2+} loading leads to loss of endogenous cations [17,18,19,20]. But in the ^{42}K flux experiments, no Ca^{2+} was added and a substantial net loss of K^+ was caused by phenylarsine oxide only at relatively high concentrations or when added along with mersalyl [16].

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Abbreviations: ANTS, 8-amino-1,3,6-napthalene trisulfonate; DCCD, dicyclohexylcarbodiimide

The evidence for saturability, competition, and sensitivity to protein-reactive compounds suggests that K^+ enters mitochondria via a specific proteinaceous transport catalyst. Conversely, it has been argued that such data are consistent with K^+ entry via 'leak' pathways along protein-lipid interfaces [21]. Patch clamping has shown the presence of various ion channels in the inner mitochondrial membrane [22,23,24], as well as in preparations of whole mitochondria [25]. Purification of an ion channel from beef heart mitochondria has been reported [26].

Much evidence suggests that mitochondria also contain a K^+/H^+ antiporter. Separate K^+ uniport and K^+/H^+ antiport mechanisms would account for the repiration-dependence [27] of K^+ influx and efflux [1,2]. Discharge of matrix Mg^{2+} activates non-electrogenic K^+ fluxes attributed to the K^+/H^+ antiporter [e.g. 28,29]. K^+/H^+ antiport activity is sensitive to inhibition by quinine [30,31] and by DCCD [28]. An 82 kDa protein exhibiting Mg^{2+} and quinine sensitive labeling with[^{14}C]DCCD has been identified as the K^+/H^+ antiporter [28,32].

A fraction obtained from detergent-solubilized mitochondrial membranes by affinity chromatography on immobilized quinine contains protein of about 53 kDa, which is antigenically unrelated to the ATPase subunit of similar size [33,34,35]. This protein is labeled when mitochondria are treated with [^{14}C]DCCD under conditions that result in altered ^{42}K flux kinetics [33]. Both the K^+ uniporter and antiporter exhibit sensitivity to quinine (see above). However, electrode recordings have shown that membrane vesicles reconstituted with the affinity-purified protein are leaky to K^+, but not to H^+ [34]. It was concluded that the quinine affinity columns had purified the uniporter.

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00145793/90/$3.50

2. MATERIALS AND METHODS

Quinine was linked to epoxy activated sepharose 6B (Pharmacia), and columns were loaded with Triton X-100 extracts of rat liver submitochondrial particles and washed as described [34]. The elution medium contained 400 mM KCl, 2 mM Hepes, 1% sodium cholate, 0.5 μg/ml leupeptin and 0.7 μg/ml pepstatin, pH 6.5. The eluate derived from 16 rat livers was concentrated to 3–4 ml by ultrafiltration (Amicon YM30 filter). The concentrate (or same volume of elution medium for controls), was combined in a total of 14 ml with 200 mM octyl glucoside, 15 mg/ml asolectin, and 5 mM Tris P_i, pH 7.5, and dialyzed at 4°C against 4 l of 25 mM KCl with 5 mM Tris P_i, pH 7.5. During 46 h of dialysis, the medium was changed 3 times, with 8 g BioBeads (BioRad) added in a dialysis sac each time after the initial 16 h. The resulting vesicles were sedimented at 40000 RPM for 2 h 20 min in a Beckman SW40Ti rotor.

ANTS was incorporated into the vesicle lumen by 3 cycles of freezing in liquid nitrogen followed by thawing at 0°C, in solution containing 50 mM ANTS and 10 mM Hepes adjusted to pH 7.4 with NaOH [36]. After brief sonication, external ANTS was removed by sequential passage of each 300 μl of suspension through 5 ml (12 cm) columns of BioGel P-6DG and BioBeads equilibrated with 10 mM Na Hepes, pH 7.4, and 200 mM sucrose. With vesicles in this medium, fluorescence emission was recorded at 515 nm, with excitation at 370 nm, at room temperature, with a Perkin Elmer LS-5B spectrofluorimeter with 3600 Data Station.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out by the method of Laemmli[37]. Vesicles were delipidated with chloroform-methanol prior to electrophoresis [38]. Gels stained with Coomassie blue were dried between cellophane sheets and scanned with a Hoefer GS300 densitometer with GS-370 Data System. The method of O'Farrall [39] was used for 2D gels. After electroblotting from the 2nd dimension gel onto Immobilon (Millipore) or nitrocellulose, the amino acid composition of excised bands was analyzed, and a peptide derived from the protein in one of these blots was sequenced, by the Harvard Microchemistry Facility.

Antisera were prepared by injecting into rabbits the 53 kDa protein purified by preparative 1D gel electrophoresis. Immunoblots were carried out by standard procedures [40].

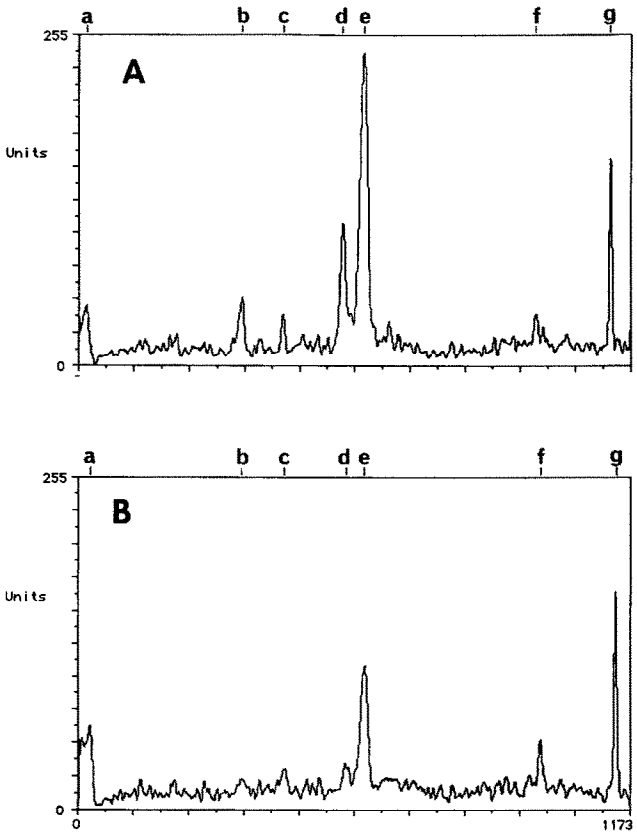

Fig. 1. Scans of electrophoresis gel. (A) Affinity column eluate; (B) Delipidated vesicles reconstituted with the eluate shown in A. Peaks: a,top of gel; b, 97 kDa; c, 77 kDa; d, 57 kDa; e,52 kDa; f, 31 kDa; g, dye front.

3. RESULTS AND DISCUSSION

Fig. 1A shows a scan of an electrophoresis gel loaded with the affinity column eluate. In this experiment, the most prominent protein band, which is usually estimated to be 53 kDa was calculated by the Data System to be 52 kDa. The bands at 56 and 97 kDa are consistently seen. Minor bands at 77 and 31 kDa are usually present. Gels such as that of Fig. 1B have confirmed incorporation of the major proteins of the eluate into membrane vesicles by detergent dialysis.

Antisera prepared against the 52–53 kDa protein fail to recognize the other major protein bands of the affinity column eluate (Fig. 2D). Thus, for example, the band estimated to be 97 kDa is not a dimer of the 53 kDa protein. No antibody reactivity is seen with any protein in submitochondrial particles in the 82 kDa size range expected for the K^+/H^+ antiporter (Fig. 2B).

A 2D gel loaded with the affinity column eluate shows multiple bands at approximately 53 kDa, as shown in Fig. 3. Table I shows the animo acid composition of the most prominent band (the apparently double band labeled 'A'), and a second band ('B') obtained in smaller quantities, in blots from such gels. Tryptophan

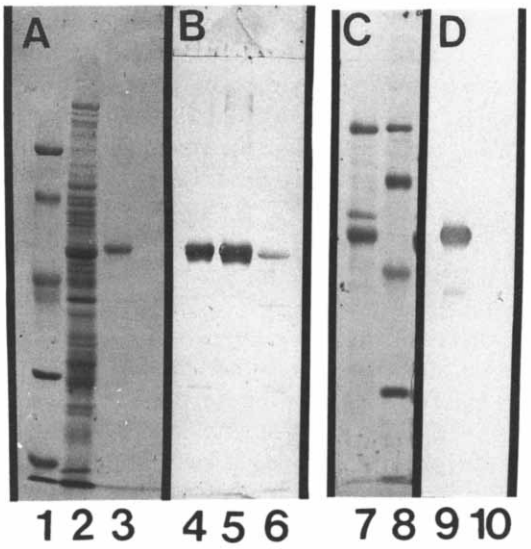

Fig. 2. Immunoblot. Nitrocellulose strips A&C were stained for protein with Amido black. Strips B&D were stained for antibody binding. Lanes 1, 8, 10: protein standards (97, 66, 45, 31 kDa); lanes 2, 6: rat liver submitochondrial particles; lanes 3, 4, 5: purified 53 kDa protein against which antibodies were raised; lanes 7, 9: quinine affinity column eluate.

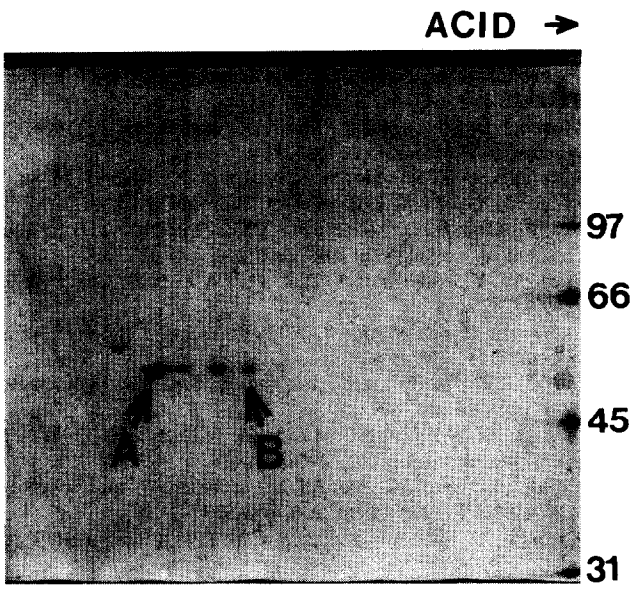

Fig. 3. Photograph of a 2D gel of the affinity column eluate. Horizontal axis: isoelectric focusing; vertical axis: sodium dodecyl sulfate polyacrylamide gel electrophoresis.

and cysteine were not assayed. The data suggest similar amino acid contents. Most differences are smaller than the discrepancies between duplicate samples. Amino acid sequence analysis was carried out on a 14-amino acid peptide derived from the protein in a pooled blot of band A. The sequence obtained (Thr-Phe-Val-Gln-Glu-Asp-Val-Tyr-Asp-Glu-Phe-Val-Glu-Arg) exactly matches part of the published sequence for the mitochondrial aldehyde dehydrogenase [41], an enzyme unlikely to have a role in mediating ion transport. The amino acid composition of aldehyde dehydrogenase is also consistent with that of bands A and B, suggesting that another protein is not a significant part of the bands at 53 kDA in gels loaded with the affinity column eluate.

Table I

The amino acid composition of aldehyde dehydrogenase is based on the known amino acid sequence [41]

	(Percent of total amino acids)		
Amino acid	Aldehyde dehydrogenase	band A	band B
Asx	9.6	17	8–10
Glx	10.6	11	8–12
Ser	5.0	4	5– 6
Gly	9.2	7	7–10
His	1.2	2	1– 2
Arg	3.6	4	5
Thr	5.2	5	4– 6
Ala	10.6	10	12
Pro	4.8	5	5– 6
Tyr	3.6	3	3
Val	9.2	9	8–10
Met	1.8	2	0.4– 2
Ile	4.6	4	4– 7
Leu	6.8	7	6– 7
Phe	4.8	6	5– 6
Lys	6.2	4	7– 9

Bands 'A' and 'B' refer to those labeled in the 2D gel of Fig. 3. The analysis for band A was obtained with a pooled sample of blots of 5 such bands, estimated to contain a total of 100 pmol protein. The ranges of values shown for band B represent separate analyses of 1–5 pooled blots containing 1–2 pmol protein.

Fig. 4 compares the ability of external Tl^+ to quench the fluorescence of ANTS entrapped in phospholipid vesicles, when added before or after valinomycin, a K^+ ionophore which also complexes Tl^+ [42]. In A, Tl^+ added to control vesicles lacking protein quenches only a small portion of the ANTS fluorescence, until valinomycin is added to promote Tl^+ permeability. Tl^+ rapidly quenches a large portion of the ANTS in vesicles reconstituted with the affinity-purified protein,

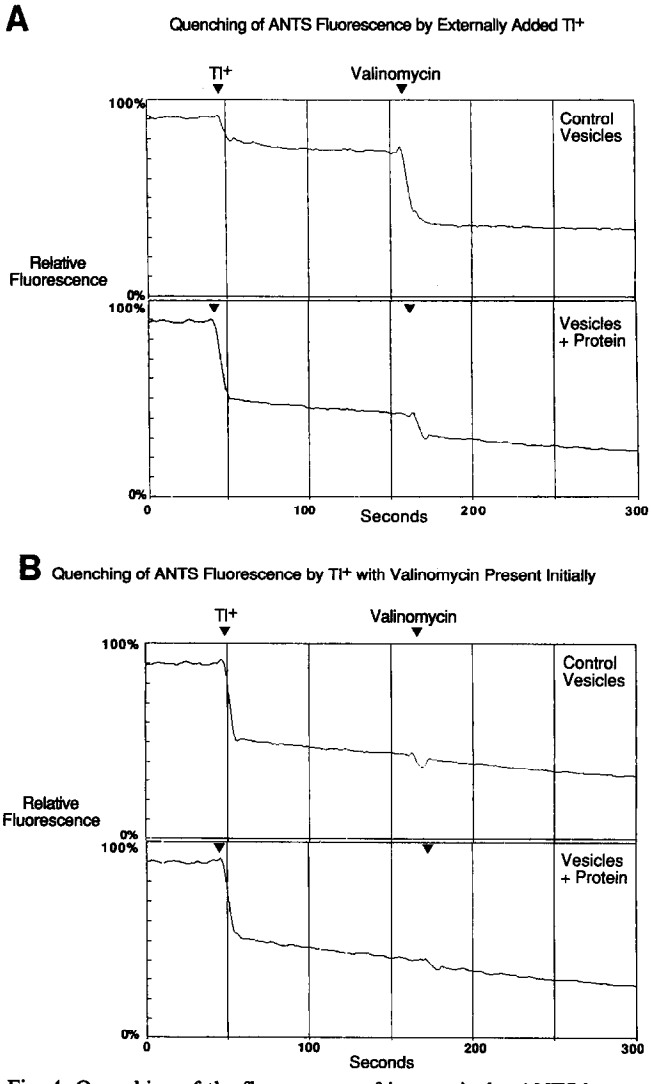

Fig. 4. Quenching of the fluorescence of intravesicular ANTS by externally added Tl^+. Control vesicles lacked protein. Vesicles + Protein were reconstituted with the eluate depicted in Fig. 1. Additions: 39 mM thallium (I) nitrate; 0.2 μg/ml valinomycin. In A, valinomycin was added only when indicated. In B, valinomycin was also added before the start of fluorescence recording.

even in the absence of valinomycin. That fraction of the ANTS which is quenched only after valinomycin addition may be sequestered in vesicles which lack functional transport catalysts. Fig. 4B shows that with valinomycin present initially Tl^+ rapidly quenches the fluorescence of ANTS in both control vesicles and those containing the affinity-purified protein. These studies indicate that incorporation of the affinity-purified protein renders membrane vesicles permeable to the K^+ analog Tl^+. This finding provides additional support for the conclusion that the quinine affinity column eluate contains a cation transport catalyst. Having ruled out such a role for two 53 kDa bands, future work will focus on determining which of the other protein bands in the affinity column eluate may be responsible for the observed cation transport activity.

Acknowledgements: The authors are grateful to D. Rao Sanadi, Saroj Joshi, and William Lane for helpful advice, and to Kathryn Sidman for assistance in searching the Protein Identification Resource database. This work was supported by USPHS Grant GM-20726.

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