Reconstitution of the Na\(^+\)K\(^+\) Pump of Ehrlich Ascites Tumor and Enhancement of Efficiency by Quercetin*

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Plasma membranes from Ehrlich ascites tumor cells were solubilized by octylglucoside in the presence of phospholipids. The Na\(^+\)K\(^+\)-ATPase was purified from this extract by adsorption and elution from thiol-Sepharose 4B. The enzyme (specific activity, 7 μmoles of ATP hydrolyzed min\(^{-1}\) mg\(^{-1}\) of protein\(^{-1}\)) was reconstituted into liposomes by the octylglucoside dilution procedure. An ATP-dependent Na\(^+\) influx with low efficiency was observed. On addition of appropriate amounts of quercetin, the Na\(^+\) flux/ATP hydrolysis ratio was increased from 0.4 to 1.4.

The high aerobic glycolysis of tumors (1) is supported by different ATPases (2). In Ehrlich ascites tumor cells, the sensitivity of glycolysis to ouabain pointed to the Na\(^+\)K\(^+\)-ATPase as the major supplier of ATP for the process of aerobic glycolysis. The efficiency of the Na\(^+\)K\(^+\)-ATPase is increased by the action of quercetin (3). However, the observation that quercetin at very low concentrations inhibits Rb\(^+\) uptake into Ehrlich ascites tumor cells (4) complicated the interpretation of the mode of action of these versatile compounds.

It became imperative to test the effect of quercetin on the Na\(^+\)K\(^+\) pump itself. Studies of purified Na\(^+\)K\(^+\)-ATPase from eel eel localized the action of quercetin to the step $E_1 \sim P \Leftrightarrow E \setminus P$ (7). Both formation and hydrolysis of $E_2 \sim P$ were highly sensitive to quercetin while the formation of $E_1 \sim P$ with ATP was unaffected. The action of quercetin again resembled that of a regulatory subunit that protects the enzyme against illicit entry of water as in the case of the ε subunit of chloroplast ATPase (8).

We now wish to report the purification of the Na\(^+\)K\(^+\)-ATPase from Ehrlich ascites tumor cells and its incorporation into liposomes. The reconstituted proteoliposomes catalyzed an ATP-dependent influx of Na\(^+\). The efficiency of pumping was very low and was increased severalfold by addition of quercetin.

**MATERIALS AND METHODS**

Thiol-Sepharose 4B. 4N-(2-hydroxyethyl-1-piperazine-ethanesulfonic acid (Hepes), ouabain, and quercetin were obtained from Sigma. Octyl-$β$-D-glucopyranoside was from Calbiochem, and asolectin was from Associated Concentrates (Woodside, NY). $^{2}$P, for preparation of $[^{32}]\text{P}\text{ATP}$ (9) and $^{23}$Na were purchased from New England Nuclear.

Plasma membranes were isolated from Ehrlich ascites tumor cells according to the procedures of Brunette and Till (10) with the following modifications. The cells were swollen in 10 mM CaCl\(_2\) (instead of 1 mM ZnCl\(_2\)) and 100 mM CaCl\(_2\) was substituted for 10 mM ZnCl\(_2\) in the preparation of the polyethylene glycol-Dextran two-phase system. The membranes were stored at $-90^\circ$C at a protein concentration of 6 to 10 mg/ml.

The Na\(^+\)K\(^+\)-ATPase was extracted by incubating 5 mg of plasma membranes in 1 ml of medium containing 2% octylglucoside (pH 7.4), 400 μg of sonicated asolectin, and buffered sucrose (40 mM 4-[(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)-HOH (pH 7.4), 200 mM sucrose, and 6 mM MgCl\(_2\)) at 4°C for 10 min. The suspension was centrifuged at 30,000 rpm (50,000 × g) for 20 min at 0°C in a Beckman Ti-50 rotor. The supernatant, containing most of the Na\(^+\)K\(^+\)-ATPase activity, was then centrifuged at 45,000 rpm (144,000 × g) for 2 h at 0°C in a Beckman type 65 rotor. Most of the Na\(^+\)K\(^+\)-ATPase activity was found in the pellet, which was resuspended in 0.2 ml of buffered sucrose, 0.1% octylglucoside.

The Na\(^+\)K\(^+\)-ATPase was further purified by chromatography on a thiol-Sepharose 4B column (0.5 × 2 cm) which was equilibrated with buffered sucrose plus 0.1% octylglucoside. The enzyme (110 mg) was applied to 2.5 ml bed volume of the gel. The column was washed with 25 ml of buffered sucrose plus 0.1% octylglucoside and the Na\(^+\)K\(^+\)-ATPase eluted with Na\(^+\) at a linear gradient of 0 to 25 mM dithiothreitol in buffered sucrose plus 0.1% octylglucoside.

The Na\(^+\)K\(^+\)-ATPase activity was measured with $[^{2}]\text{P}\text{ATP}$ as described (9). Reconstitution of the Na\(^+\)K\(^+\)-ATPase was by octylglucoside dilution (11). Measurement of $\text{Na}^+$ uptake into the proteoliposomes (12) was performed by incubating 0.05 ml of reconstituted vesicles (generally containing about 10 μg of protein) with buffered sucrose plus 0.1% octylglucoside and the Na\(^+\)K\(^+\)-ATPase was eluted with a linear gradient of 0 to 25 mM dithiothreitol in buffered sucrose plus 0.1% octylglucoside.

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**TABLE I**

* Purification of ascites tumor Na\(^+\)K\(^+\)-ATPase

The purification and assays were performed as described under "Materials and Methods." Specific activities were calculated by subtracting ouabain-insensitive activity from the total activity.

| Fractions | Na\(^+\)K\(^+\)-ATPase | Yield | Ouabain sensitivity % |
|-----------|-----------------|-------|-----------------------|
| Crude membranes | 0.17 | 100 | 42 |
| Octylglucoside extract | 1.1 | 97 | 65 |
| Pellet (48,000 rpm) | 2.2 | 81 | 78 |
| Eluate (SH-Sepharose 4B) | 7.0 | 61 | 92 |

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Reconstitution of a Tumor Na⁺K⁺ Pump

1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins from plasma membrane and fractions during purification of ascites tumor Na⁺K⁺-ATPase. Lane 1, plasma membranes, 75 μg of protein; Lane 2, plasma membranes, 50 μg of protein; Lane 3, octylglucoside extract, 50 μg of protein; Lanes 4 and 5, 48,000 rpm pellet, 50 μg of protein; Lanes 6, 7, and 8, fractions of ATPase eluted from a thiol-Sepharose column, containing 50 μg of protein each. The specific activity of Fractions 6, 7, and 8 were 5.2, 6.1 and 7.0 μmoles min⁻¹ mg of protein⁻¹, respectively.

2. Optimum phospholipid/protein ratio and detergent concentration for reconstitution of the Na⁺K⁺-ATPase. Reconstitution was done with asolectin by the octylglucoside dilution method (11) at the various detergent concentrations and with varying protein and phospholipid concentrations.

RESULTS AND DISCUSSION

The recoveries and specific activities of fractions during the purification of the Na⁺K⁺-ATPase are shown in Table 1. On sodium dodecyl sulfate-polyacrylamide electrophoresis gels (Fig. 1), there were two major bands in the best preparation.
Reconstitution of a Tumor Na⁺K⁺ Pump

**Table II**

**Na⁺/ATP ratios of vesicles reconstituted with Na⁺K⁺-ATPases from tumor, brain, and electric eel**

In Experiment 1, reconstitution was performed with 80 μg of the ATPase preparation in the presence of 2.8 mg of asolectin in 50 mM imidazole-HCl (pH 7.5), 75 mM K₂SO₄, 50 mM Na₂SO₄, 20 mM 2-mercaptoethanol, and 2% octylglucoside, in a final volume of 0.12 ml. After 15 min at 0°C, 3 ml of the same cold buffer (without octylglucoside) was added. Centrifugation at 49,000 rpm (144,000 × g) for 30 min in a Ti-50 rotor of a Spinco centrifuge yielded a pellet which was suspended in 0.15 ml of the same buffer, and 50 μl were used for each assay. Quercetin solutions (1 to 3%) were prepared in dimethylsulfoxide and kept at 0°C in the dark. Controls were run with the same amounts of dimethylsulfoxide. Experiment 2 was performed the same as Experiment 1 except that 1.4% deoxycholate was used instead of 2% octylglucoside.

| Source of Na⁺K⁺-ATPase | Quercetin | Na⁺/ATP ratio |
|------------------------|-----------|---------------|
|                        | μg/mg lipid + protein | nmoles min⁻¹ mg protein⁻¹ | nmoles min⁻¹ mg protein⁻¹ | ATPase | Na⁺/ATP ratio |
| Ascites tumor          | 0         | 471           | 985             | 514     | 1510 | 0.34 |
|                        | 6         | 455           | 965             | 510     | 1420 | 0.36 |
|                        | 10        | 407           | 955             | 548     | 922  | 0.67 |
|                        | 16        | 466           | 1017            | 551     | 642  | 0.86 |
|                        | 20        | 436           | 942             | 507     | 402  | 1.26 |
|                        | 24        | 530           | 924             | 394     | 275  | 1.43 |
|                        | 30        | 422           | 682             | 260     | 275  | 0.96 |
| Electric eel           | 0         | 418           | 798             | 390     | 210  | 1.81 |
|                        | 24        | 426           | 701             | 275     | 156  | 1.76 |

**Fig. 3.** Effect of quercetin on the sodium-pumping efficiency of the reconstituted Na⁺K⁺-ATPase. The experimental conditions were as described in the legend to Table II for Experiment 1.

(Fraction 8) corresponding to 100,000 and approximately 50,000 daltons as well as several minor bands including one in the region of proteolipids (6,000 to 10,000).

Reconstitution was performed by the octylglucoside dilution procedure (11). The effects of varying phospholipid/protein ratios and optimal detergent concentration for reconstitution are shown in Fig. 2. Simultaneous measurements of ATPase activity and Na⁺ flux in the presence of ouabain, which inhibited unincorporated ATPase, but not the inverted ATPase, revealed a low efficiency of pump action. Values of 0.3 to 0.5 for the Na⁺/ATP ratio were observed compared to ratios of 1.3 to 1.5 for a reconstitution of a Na⁺K⁺-ATPase from electric eel or mouse brain. As shown in Table II and Fig. 3, the efficiency of pumping with the tumor enzyme was greatly increased by the addition of appropriate concentrations of quercetin. It should be noted that the amount of quercetin added is expressed in terms of the phospholipid plus protein concentration, since both interact with the bioflavonoids and influence the effective concentration. Quercetin had little or no effect on the Na⁺/ATP ratio in proteoliposomes reconstituted with active electric eel or mouse brain Na⁺K⁺-ATPase (Table II, Experiment 2).

Although in this paper we have reported only values for the 2-min flux of "Na⁺", we have established that the efficiency of pumping in the absence and presence of quercetin remains constant up to 10 min. The possibility of a Na⁺/Na⁺ exchange reaction contributing to the [³²Na] flux measurements was ruled out by substituting either choline chloride or choline sulfate for the internal NaCl. Within 15%, the [³²Na] flux data were identical. It should be noted that the "ouabain-insensitive ATPase" of reconstituted vesicles is completely inhibited by ouabain which is incorporated inside the vesicles.

These experiments strongly support the hypothesis that the Na⁺K⁺-ATPase in these tumor cells is defective and operates inefficiently. There are several possibilities with respect to the type of lesion. The most likely and most easily explored explanation is a defective regulatory subunit analogous to the e subunit of the H⁺-ATPases. During the past years, we have searched for such a subunit without success. However, we have used the highly purified eel enzyme in the search for an ATPase inhibitor and this may have been an inadequate assay system. Now that highly purified preparations of the tumor enzyme are available, we can resume our search with better hope of success. It is encouraging that a natural inhibitor of the Na⁺K⁺-ATPase has been discovered recently (15, 16). According to the published data (15), the fraction isolated from brain does not seem to conform to the expected properties of a regulatory subunit since it inhibited ATPase activity and ion transport to the same extent, an action similar to that of ouabain. However, further tests are required to establish whether a differential effect can be induced.

**REFERENCES**

1. Warburg, O. (1926) *Über den Stoffwechsel der Tumoren*, Springer-Verlag, Berlin
2. Racker, E. (1976) *A New Look at Mechanisms in Bioenergetics*, Academic Press, New York
3. Scholnick, H., Lang, D., and Racker, E. (1973) *J. Biol. Chem.* 248, 5175-5182
4. Soolinna, E.-M., Lang, D. R., and Racker, E. (1974) *J. Natl. Cancer Inst.* 53, 1515-1519
5. Pullman, M. E., and Monroy, G. C. (1963) *J. Biol. Chem.* 238, 3762-3769
6. Belin, J. A., Thomas, J. A., Buchbaum, R. N., and Racker, E. (1979) *Biochemistry* 18, 3506-3511
7. Kuriki, Y., and Racker, E. (1975) *Biochemistry* 14, 4951-4956
8. Nelson, N., Nelson, H., and Racker, E. (1972) *J. Biol. Chem.* 247, 7657-7662
9. Nelson, N., Nelson, H., and Racker, E. (1972) *J. Biol. Chem.* 247, 6506-6510
10. Brunette, D. M., and Till, J. E. (1971) *J. Membr. Biol.* 5, 214-224
11. Racker, E., Violand, B., O’Neal, S., Alfonso, M., and Telford J. (1979) *Arch. Biochem. Biophys.* 198, 470-477
Reconstitution of a Tumor Na\textsuperscript{+}K\textsuperscript{+} Pump

12. Gasko, O., Knowles, A., Shertzer, H., Suolinna, E.-M., and Racker, E. (1976) Anal. Biochem. 72, 57-65

13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275

14. Chua, N.-H., and Bennoun, P. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 2175-2179

15. Fishman, M. C. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4661-4663

16. Haupert, G. T., Jr., and Sancho, J. M. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4658-4660