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A MODEL PROTEOLIPOSOMAL SYSTEM FOR PROLINE TRANSPORT USING A PURIFIED PROLINE CARRIER PROTEIN FROM *MYCOBACTERIUM PHLEI*

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SUMMARY: The proteoliposomes prepared from purified proline carrier protein isolated from membrane vesicles of *Mycobacterium phlei* exhibited an uptake of proline, which was dependent upon a proton gradient generated across the lipid bilayer. Although a proton gradient was generated by the reduction of the entrapped ferricyanide by ascorbate oxidation with benzoquinone serving as a lipid soluble hydrogen carrier, transport of proline was dependent on the addition of sodium ion. The movement of sodium and proline across the artificial membrane resulted in a simultaneous collapse of the proton gradient.

INTRODUCTION: The uptake of proline into membrane vesicles of *M. phlei* is an active transport process (1,2) and exhibits a strict requirement for substrate oxidation as well as the presence of Na^+ or Li^+ (1,3). However, the active transport of proline appears to be independent of the formation of high energy phosphorylated intermediates since it proceeds in the absence of coupling factor-latent ATPase or phosphate and in the presence of arsenate (4,5).

Carrier proteins responsible for the translocation of various nutrients across biological membranes have recently been solubilized from a number of bacterial and mammalial membranes using appropriate detergents (6-13). The reconstitution of active transport with carrier proteins incorporated into liposomes was demonstrated utilizing a membrane potential introduced by K^+ - diffusion via valinomycin (8, 11, 13) as the energy source. This communication describes the reconstitution of proton gradient dependent proline uptake in proteoliposomes containing the purified carrier protein and an artificial electron acceptor, potassium ferricyanide. The uptake of proline into the pro-

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Abbreviations: 9-AA, 9-aminoacridine; CCCP, m-chlorocarbonylcyanide phenylhydrazone

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teoliposomes presumably involves a proton gradient generated as a result of reduction of the entrapped ferricyanide via ascorbate oxidation with benzoquinone as a mediator. The uptake of proline exhibits an obligatory requirement for Na^+; however, the collapse of the proton gradients requires both Na^+ and proline.

MATERIALS AND METHODS: Sodium cholate, Triton X-100 and 9-Aminoacridine were purchased from Sigma Chemical Co. and [^{14}C]proline from New England Nuclear Corp. Ascorbate and benzoquinone were obtained from Matherson Coleman and Bell. Potassium ferricyanide was purchased from General Chemical Division, New York. All other chemicals were of reagent grade purity.

Solubilization and purification of proline carrier protein- *Mycobacterium phlei* (ATCC 354) was grown as described previously (14), and the electron transport particles (ETP) were prepared by sonication of cells as described by Brodie (15). Depleted ETP (DETP, depleted of the coupling factor-latent ATPase) were prepared by washing the ETP with 0.25 M sucrose in the absence of cations (16,17). The carrier protein was solubilized from the DETP by treatment with cholate and Triton X-100 by a procedure previously described (18). The Triton X-100 extract of the sodium cholate treated DETP was concentrated in an Amicon Diaflo apparatus with a PM 10 membrane. The crude detergent extract was further fractionated by sucrose density gradient centrifugation, and proline carrier protein was purified by gel filtration and isoelectric focusing as described earlier (19).

Preparation of proteoliposomes- Soybean phospholipids (Asolectin from Associate Concentrates, N.Y.) partially purified by acetone extraction (20) were suspended in 0.1 M Tris-HCl buffer (pH 8.0) containing 1.0 mM EDTA and 0.2 M $K_3Fe(CN)_6$, and sonicated for 10 min. in a 10 KC Raytheon Sonic Oscillator at 1.0 amperage (100% output) at 2° under N_2 gas to give a suspension of 50 mg/ml. The proteoliposomes were prepared by combining the phospholipid suspension with the same volume of the purified carrier protein and then sonicating an additional 30 seconds under N_2 gas. The mixture was centrifuged at 166,000 X g for 10 min. using a Beckman Airfuge (Beckman Instrument). The pellets were washed twice with 10 mM Tris-HCl buffer (pH 8.0) containing 0.2 mM EDTA, 10 mM $MgCl_2$ and 0.15 M KCl and resuspended in a small volume of the same buffer. Under these conditions about 50% of the carrier protein was incorporated into the liposomes.

Measurement of 9-aminoacridine fluorescence- Proton gradient was measured by the method of Schuldiner et al. (21) using 9-aminoacridine as a fluorescence probe. The fluorescence change of 9-aminoacridine was measured in 2.5 ml of reaction mixture containing 50 mM Hepes-KOH (pH 7.5), 0.15 M KCl, 10 mM $MgCl_2$, 10 mM NaCl, 4 µM 9-aminoacridine and 5 mg of the proteoliposomes. The quenching of 9-aminoacridine fluorescence was monitored with MPF-4 Perkins-Elmer Spectrofluorometer equipped with a thermostatic cell holder. Excitation was carried out at 365 nm wavelength while the emission was set at 451nm. A thermostatic bath was used to control the temperature of the cuvette.

Measurement of ferricyanide reduction- Reduction of the internal ferricyanide was assayed in 2.5 ml of reaction mixture containing 50 mM Hepes-KOH, (pH 7.5), 0.15 M KCl, 10 mM $MgCl_2$, 10 mM NaCl and 5 mg of the proteoliposomes. Absorbance changes of ferricyanide by the addition of ascorbate and benzoquinone was measured with a Cary Model 14 Spectrophotometer at 420 nm.

Assay of amino acid uptake- The proteoliposomes (5 mg) were suspended in 0.75 ml of reaction mixture containing 50 mM Hepes-KOH (pH 7.5), 0.15 M KCl,

10 mM $MgCl_2$, and 10 mM NaCl. After 5 min. of preincubation at 30°, proline (final concentration 25 µM containing {^{14}C}proline, 1.0 µCi/ml) and ascorbic acid (17 µM) as substrate were added to the reaction mixture. The reaction was started by the addition of benzoquinone at a final concentration of 33 µM. At indicated time intervals 0.1 ml aliquots of the mixture were removed and immediately diluted in 2.0 ml of 50 mM Hepes-KOH (pH 7.5). The suspension was rapidly filtered on a membrane filter (Millipore 0.22 µm), and the filters were washed twice with the same buffer. The radioactivity retained on the filters was counted in a Nuclear Chicago Liquid Scintillation detector using a scintillation fluor with Beckman Bio-Solv (BBS-3).

Protein estimation- Protein concentration was determined by the method of Lowry et al. (22), or a modification of the Biuret method (23) with bovine serum albumin as standard.

RESULTS AND DISCUSSION: Uptake of proline by reconstituted proteoliposomes- The proteoliposomes prepared from the purified carrier protein and potassium ferricyanide showed a rapid uptake of proline. As shown in Fig. 1, ascorbate alone did not induce the uptake of proline. The addition of benzoquinone, however, resulted in a rapid uptake of proline. Benzoquinone is a lipid soluble hydrogen carrier and presumably crosses the membrane freely in an electrically neutral cycle, transferring hydrogen atoms into the vesicles (24-26). The maximal uptake of proline was observed about 10 seconds after addition of benzoquinone (Fig. 1). The membrane vesicles from *M. phlei* are capable of active transport of only three amino acids; proline, glutamine and glutamic acid. This uptake was specific for proline. Glutamine and glutamic acid were not transported. The proline uptake into the proteoliposomes also required Na^+ or Li^+. A requirement for Na^+ or the stimulation by Na^+ of active transport of amino acids and various substrates has been reported in a number of bacterial and encaryotic systems. These have been considered to represent a symport with Na^+. Previous studies with the *M. phlei* system have indicated that the Na^+ requirement is not due to an alteration in the affinity of the carrier for proline by this cation (3).

Proline uptake into proteoliposomes as a function of the amount of carrier protein added- In proteoliposomes reconstituted with various amounts of the purified proline carrier protein and a fixed amount of phospholipid (10 mg of asolectin), the initial rates of proline uptake were nearly proportional to the amount of protein over the range of 1 to 25 µg (Fig. 2). With concentrations over 20 µg, the uptake of proline reached a plateau, suggesting that the maximal level of transport occurs with a ratio of phospholipids to carrier protein of 500 to 1. Phospholipids extracted from *M. phlei* were equally effective for the reconstitution of proline transport when proteoliposomes are prepared as described under "Materials and Methods".

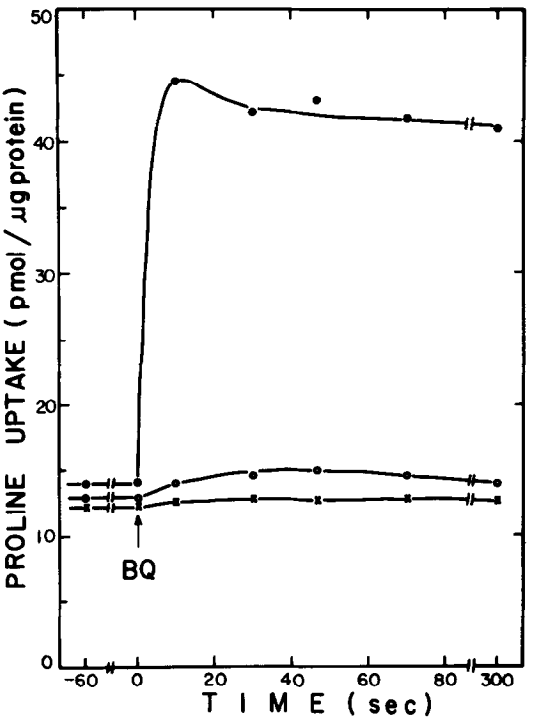

Fig. 1 Uptake of proline by reconstituted proteoliposomes.
The reaction mixture contained 50 mM Hepes-KOH (pH 7.5), 0.15 M KCl, 10 mM $MgCl_2$, 10 mM NaCl and 0.15 ml of proteoliposomes (reconstituted from 40 µg of carrier protein/ 10 mg of phospholipid) in a total voluem of 1.5 ml. After 5 min. of preincubation at 30°C, proline (final concentration, 25 µg) containing 1 µCi/ml of [^{14}C]-proline and ascorbic acid (17 µM) as substrate were added to the reaction mixture. The reaction was started by the addition of benzoquinone (BQ) to a final concentration of 33 µM. At indicated time intervals, 0.1 ml of the reaction mixture were removed and assayed for proline uptake as described under "Materials and Methods". ●——●, complete system; o——o, complete system minus ascorbate; x——x, complete system minus NaCl.

Proton accumulation into proteoliposomes- Proton accumulation inside the vesicles was measured by fluorescence change of 9-AA. As shown in Fig. 3, a and b, a large decrease in the fluorescence was observed by the addition of benzoquinone. This decrease in fluorescence reflects the accumulation of protons and the generation of a pH gradient across the membrane as a result of the oxidation of benzohydroquinone by ferricyanide. After 2 to 3 min. the fluorescence levels reached a steady state level, a rapid efflux of proton occurred upon the addition of proline and Na^+ to the medium, suggesting the uptake of proline induces proton extrusion (Fig. 3,a). When Na^+ or proline alone was added separately, no proton extrusion was observed (Fig. 3,b). This induction of proton efflux was again specific for proline addition; glutamine and

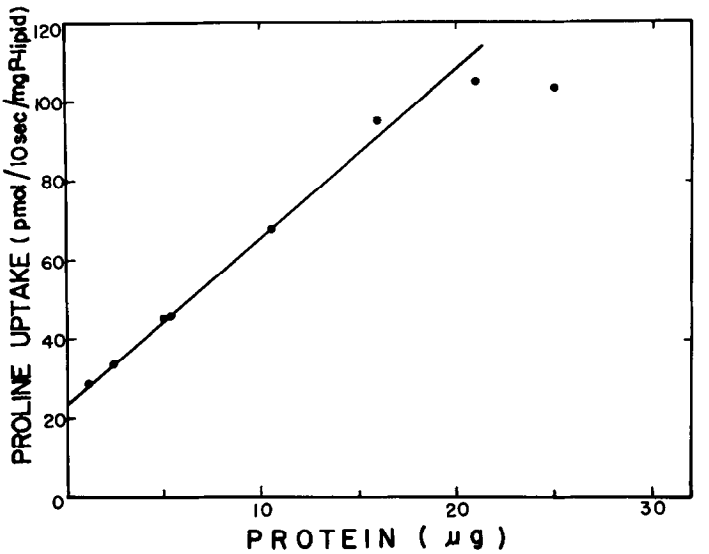

Fig. 2 Proline uptake into reconstituted proteoliposomes with various amounts of carrier protein.

Proteoliposomes were prepared as described under "Materials and Methods" with various amounst of carrier protein as indicated above, and 10 mg of phospholipids (asolectin). The reaction mixture and the assay of proline uptake were as described in Fig. 1.

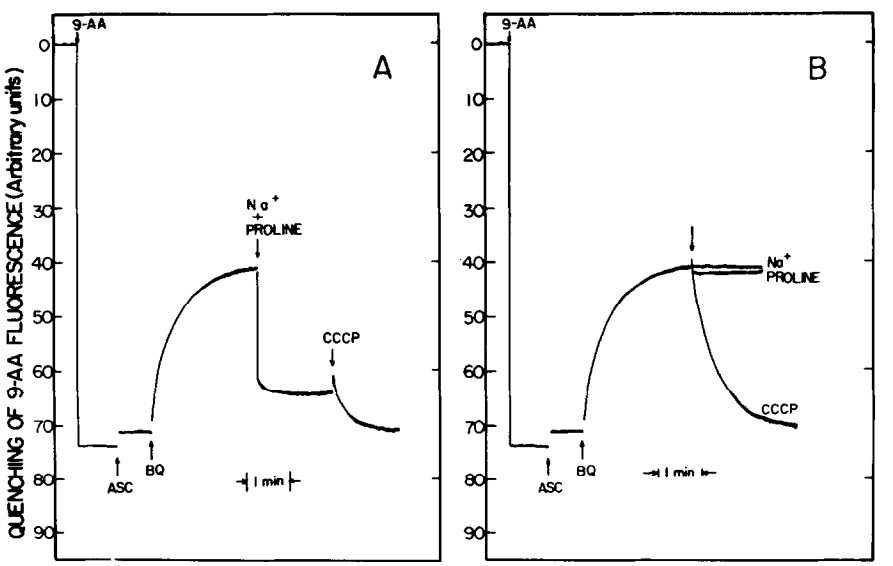

Fig. 3 Fluorescence change of 9-AA.

The reconstituted proteoliposomes and the reaction mixture were as described under "Materials and Methods". At the time indicated 17 μM ascorbate (Asc), 33 μM benzoquinone (BQ), 10 mM NaCl, 25 μM proline, and 10 μM CCCP were added as a final concentration.

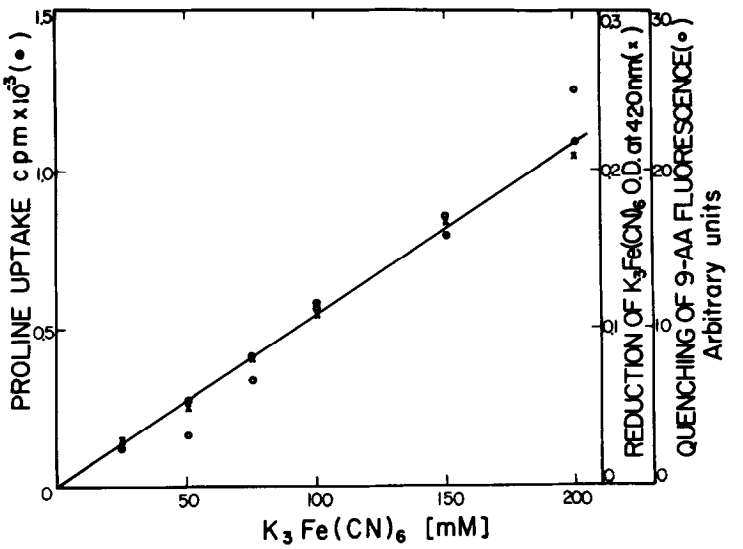

Fig. 4 Relationship between quenching of 9-AA fluorescence, reduction of ferricyanide and proline uptake.

The reconstituted proteoliposomes were prepared as described under "Materials and Methods" using 40 µg of carrier protein per 10 mg of phospholipids, except with various amounts of potassium ferricyanide as indicated. The assay of proline uptake was similar to that described in Fig. 1 and the cpm was obtained from 1.0 mg of phospholipid. Reduction of ferricyanide and quenching of 9-AA fluorescence were similar to those described under "Materials and Methods". All the values were obtained from a steady state level with a subtraction of control.

hydroxy-proline did not induce proton efflux. Further addition of proton conducting uncoupler, CCCP, caused an increase in the fluorescence to the original level indicating a complete collapse of the proton gradient (Fig. 3,a). In the absence of benzoquinone the addition of proline or preincubation with CCCP resulted in no change in the fluorescence. In order to confirm that the uptake of proline into this proteoliposomal system is dependent on the proton gradient, proline uptake was redemonstrated after the proton gradient was established by ascorbate and benzoquinone.(data not shown).

Correlation between quenching of 9-AA fluorescence, reduction of ferricyanide and proline uptake- In proteoliposomes prepared from various concentrations of potassium ferricyanide and a fixed amount of carrier protein and phospholipids, quenching of 9-AA fluorescence, reduction of ferricyanide and the proline uptake were measured. As shown in Fig. 4. The uptake of proline as well as the reduction of ferricyanide and the fluorescence quenching of 9-AA were increased with increasing amounts of ferricyanide. Thus there appears to be a direct relationship between amount of reduction of the entrapped ferri-

cyanide, extent of proton accumulation inside the vesicles and the level of proline uptake into the vesicles.

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