MEMBRANE ORIENTATION AND ACTIVE TRANSPORT OF PROLINE

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SUMMARY. Active transport of proline with ascorbate-TPD as the electron donor has been demonstrated in both the protoplast ghosts and the electron transport particles (ETP) from Mycobacterium phlei. The steady state level of proline uptake in the ETP, generated with ascorbate-TPD, was found to decrease on the further addition of succinate. The succinate induced efflux of proline from the ETP was inhibited by malonate. In contrast, with ghost preparations, in which the membrane orientation is right side out, no efflux of proline was observed on addition of succinate. Evidence was obtained that indicates that succinic dehydrogenase is located on the outer surface of the membrane in the ETP and on the inner surface of the ghost membrane. It would appear that the direction of active transport is influenced by the location of specific respiratory components.

Active transport of proline in the electron transport particles (ETP) derived from Mycobacterium phlei is dependent on the oxidation of substrate (1). The ETP preparations from M. phlei are also capable of coupling phosphorylation to the oxidation of substrates. However, the bioenergetic process involved in active transport exhibits different requirements from those necessary for oxidative phosphorylation suggesting that the bioenergetic mechanisms for these two membrane related phenomena differ (1). Kaback and his collaborators (2-5) have described a membrane-bound D-lactic dehydrogenase which was coupled to the transport of various amino acids and sugars in isolated membrane vesicles from E. coli. They have proposed a mechanism for active transport in which the carrier protein(s) is one of the electron transport mediators located between D-lactic dehydrogenase and cytochrome b_1 (3-5).

The membrane orientation and consequent localization of respiratory carriers may influence the direction of transport. The membrane orientation of the bulk of the ETP preparation was found to be "inside out" whereas the orientation of the protoplast ghosts was found to be "right side out" as in the intact cell (6). Thus, it was possible to determine whether membrane orientation influences the direction of active transport.

MATERIALS AND METHODS. The growth conditions and the preparation of the ETP from M. phlei (ATCC 354) have been described (7). The ETP were suspended in 2×10^{-3} M $MgCl_2$ or water. Protoplast ghosts were prepared by a modification of the method of Mizuguchi and Tokunaga (8). Proline uptake was measured by the method described previously (1). In some experiments 0.1 M Tris-HCl buffer (pH 7.4) was used for both the incubation mixture and the washing medium instead of 0.05 M potassium phosphate buffer (pH 7.0). Similar results were obtained with either of the buffers described.

RESULTS AND DISCUSSION. Ascorbate-TPD was found to be the most effective electron donor for supporting the transport of proline in the ETP (1). Nevertheless, the P/O ratios with ascorbate-TPD are lower than those observed with other electron donors. Ascorbate-TPD enters the electron transport chain at the level of cytochrome c and elicits one phosphorylative site. With succinate as the electron donor the transport of proline in the ETP was found to be low (1). In contrast, with the ghost preparations, succinate was found to be equally as effective as ascorbate-TPD in supporting proline transport. Thus, it was of interest to examine the effects of a combination of electron donors on proline transport with membrane structures which differ in membrane orientation.

Although the rate of oxidation with ascorbate-TPD and succinate increased 2-fold compared to that observed with ascorbate-TPD alone, the steady state level of proline uptake was found to decrease 60 per cent (Table I). Malate-vitamin K reductase (MKR), a membrane-bound enzyme, is solubilized from

TABLE I

Effect of Combination of Substrates on Proline Transport by ETP

Substrate	Proline uptake pmoles/mg protein	%
Ascorbate-TPD	418.2	100
Ascorbate-TPD + Succinate	169.0	40.4
Ascorbate-TPD + Malate*	249.5	59.7

*Partially purified malate-vitamin K reductase (96 μg) and FAD (25 μM) were added as indicated.

TABLE I.

The reaction mixture (0.1 ml) contained 0.05 M potassium phosphate buffer (pH 7.0), 0.01 M $MgCl_2$, substrate, 9×10^{-6}M L-Proline-U-^{14}C (200 mCi/nmole) and ETP (0.14 mg). After preincubation for 10 min at 30°, the reaction was started by the addition of substrate and ^{14}C-Proline. The electron donors were 1.7 μmoles Na-ascorbate and 0.015 μmoles TPD, 2.5 μmoles Tris-succinate, and 2.5 μmoles Tris-L-malate and 0.0025 μmoles FAD. After 10 min the reaction was stopped by the addition of 2 ml of 0.05 M potassium phosphate buffer (pH 7.0), followed by filtration on a membrane filter (Millipore HA025). After washing with 2 ml of the same buffer, the filter was glued on planchettes and dried. Radioactivity was measured by a Nuclear-Chicago gas flow counter.

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the protoplast ghosts by sonic oscillation. This enzyme requires phospholipid and catalyzes the dehydrogenation of malate via FAD (9). Electron transport from MKR enters the respiratory chain of the ETP at the level of vitamin K_9 (II-H). It is of interest that the addition of MKR, FAD and malate to the ETP resulted in a decrease in the steady state level of proline uptake generated by ascorbate-TPD (Table I). Since the rate of oxidation increased on the addition of succinate or malate to the ETP with ascorbate-TPD, vigorous shaking was employed to maintain aerobiosis. The aerobic state was monitored with a Clark oxygen electrode. Thus, the decrease in the steady state level of proline on addition of succinate or malate to the ascorbate-TPD system can not be attributed to a limitation of oxygen.

The steady state level of proline accumulation in the ETP established with ascorbate-TPD as a substrate was rapidly decreased upon subsequent

addition of succinate (Fig. 1a). In contrast, in the ghost preparations, the addition of succinate resulted in a further uptake of proline for two minutes followed by a return to the same level as that observed with ascorbate-TPD alone. These results suggested that succinate induced the efflux of proline from the ETP but not from the ghosts. Kinetic studies showed that the $V_{max.}$

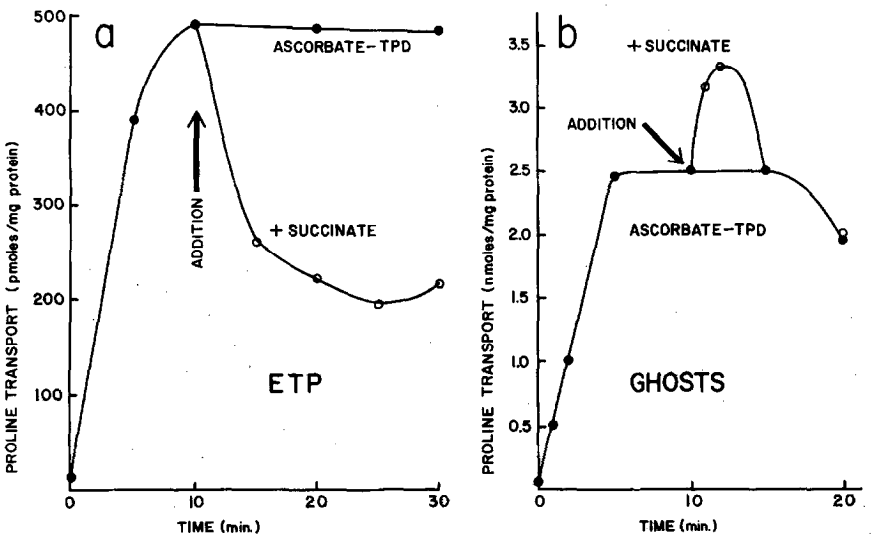

Figure 1. Effect of addition of succinate on steady state level of proline transport.

The assay mixture was similar to that in Table I. Ghosts (0.13 mg) and chloramphenicol (10 μg) were added instead of ETP in the experiment shown in Fig. 1b. Addition of succinate is indicated with an arrow.

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of the succinate-induced efflux was almost identical to that of influx with ascorbate-TPD as the substrate (104 pmoles/min/mg protein), while the apparent K_m for proline was several hundred fold higher for efflux than for influx.

The effect of inhibitors of succinic dehydrogenase and succinoxidase on the efflux phenomena in ETP was measured (Fig. 2). The succinate induced efflux was inhibited by malonate, a competitive inhibitor of succinic dehydrogenase, whereas it was not affected by the addition of low concentrations (2 μg/mg protein) of nonylhydroxyquinoline-N-oxide (NHQNO). The latter inhibitor is known to block electron transport between cytochromes b and c (9);

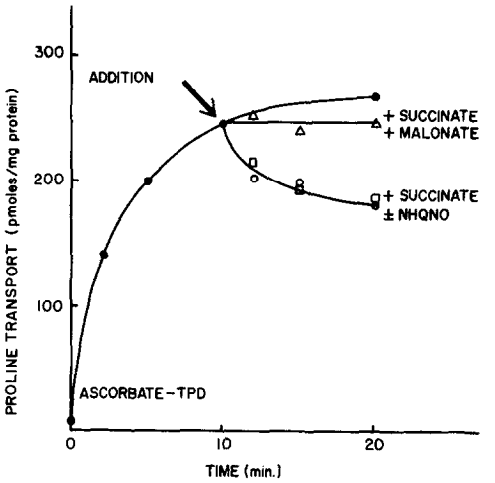

Figure 2. Succinate dependent efflux of proline from ETP.

The reaction mixture was similar to that in Table I, except 0.1 M Tris-Cl buffer (pH 7.4) and 0.01 M NaCl were used instead of potassium phosphate buffer. Addition of succinate (2.5 μmoles), succinate plus malonate (5 μmoles), and succinate plus NHQNO (1 μg) is indicated with an arrow. Substrates were neutralized with Tris-HCl buffer, pH 7.4.

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however, succinate can be oxidized through the Wurster's blue shunt by TPD (10-12). Thus, it appears that succinic dehydrogenase plays a role in the efflux process in the ETP from M. phlei.

Succinic dehydrogenase activity in the ghost preparations was found to be low when compared to that observed with the ETP. However, the succinic dehydrogenase activity of the ghost preparation was increased several fold by the addition of a non-ionic detergent (Triton X-100). In contrast, the ETP exhibit high succinic dehydrogenase activity without any treatment. These results suggest that the succinic dehydrogenase in M. phlei is located on the inner surface of the cytoplasmic membrane. Succinic dehydrogenase in mammalian mitochondria is localized on the inner membrane (13).

A number of types of membrane structures which differ in size and membrane orientation have been isolated from M. phlei. The different membrane vesicles were found to transport proline; however, the steady state level was dependent on the degree of intactness and orientation of the membrane.

The direction of active transport across the membrane appears to be influenced by the location of certain respiratory components such as succinic dehydrogenase and cytochrome oxidase.

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