RESPIRATION DEPENDENT TRANSPORT OF PROLINE BY ELECTRON TRANSPORT PARTICLES FROM MYCOBACTERIUM PHLEI

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SUMMARY: Respiration dependent transport of proline was demonstrated in electron transport particles from Mycobacterium phlei. The uptake proceeded against the concentration gradient with succinate, generated NADH, exogenous NADH or ascorbate-TPD as substrate. The latter two electron donors were most effective for the transport of proline and for oxidation, but they were least efficient for oxidative phosphorylation. Glucose, D-lactate, fumarate and ATP had no effect on the transport process. The transport of proline was sensitive to certain uncoupling agents and respiratory inhibitors. It was independent of oxidative phosphorylation since it proceeded in the absence of coupling factors or phosphate and was not inhibited by arsenate. The transport of proline appears to occur with membranes orientated as in the intact cell.

Active transport phenomena have been widely studied in bacterial systems. Little is known, however, about the relationship between the transport process and oxidative phosphorylation. Kaback and his collaborators have described the coupling of membrane bound D-lactic dehydrogenase to amino acid and β-galactoside transport in isolated membrane preparation from E. coli (1, 2). Their evidence indicated that the active transport of amino acids and β-galactoside depends on electron transport but not on oxidative phosphorylation. Similar findings were obtained by Klein et al., (3).

Abbreviations: TPD, N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride; PMS, phenazine methosulfate; NQNO, 2-n-nonylhydroxyquinoline-N-oxide; m-ClCCP, m-chlorocarbonylcyanide phenylhydrazone; DCCD, N, N'-dicyclohexylcarbodiimide; DDA^+, dimethyldibenzylammonia; TPB^-, tetraphenylboron

However, with the system from E. coli it is difficult to obtain information concerning the relationship between the amino acid transport and oxidative phosphorylation as the level of oxidative phosphorylation in isolated E. coli membranes is low. Since the respiratory pathways and coupling process have been well characterized in the resolved system from M. phlei (4) this system was used to determine if any relationship exists between amino acid transport and oxidative phosphorylation.

MATERIALS AND METHODS. The growth conditions and the preparation of the electron transport particles (ETP) from M. phlei (ATCC 354) have been previously described (5). The ETP were suspended in 2 x 10^{-3}M $MgCl_2$. ETP depleted of particle bound coupling factor(s) (BCF_4) were prepared by the method of Higashi et al., (6). Proline uptake was measured by the method of Kaback and Milner (1) except that $MgCl_2$ was substituted for $MgSO_4$.

In order to determine the intravesicular space of the membrane vesicles water-permeable and inulin-impermeable space of the ETP preparation was determined by the method of Hunter et al., (7) using ^3H_2O and carboxyl-^{14}C-inulin. Following incubation the ETP were removed from the reaction mixture by filtration on a membrane filter, washed with 0.05M K-phosphate buffer (pH 7.0), and extracted with 2 ml of boiling water for three times. The extracts were combined and lyophilized, dissolved in water and chromatographed on filter paper (Whatman No. 3) with proline as a marker. The solvent system consisted of n-butanol:acetic acid:water (4 : 1 : 2). The radioactivity was located and determined with a Packard Radiochromatogram Scanner.

RESULTS AND DISCUSSION. The effects of respiratory substrates on proline uptake by ETP from M. phlei were studied. As shown in Fig. 1 the initial rate of uptake as well as the steady state level of proline accumulation was enhanced by the addition of ascorbate-TPD, exogenous NADH, generated NADH or succinate. Following exhaustion of substrate, transport ceased and proline was released

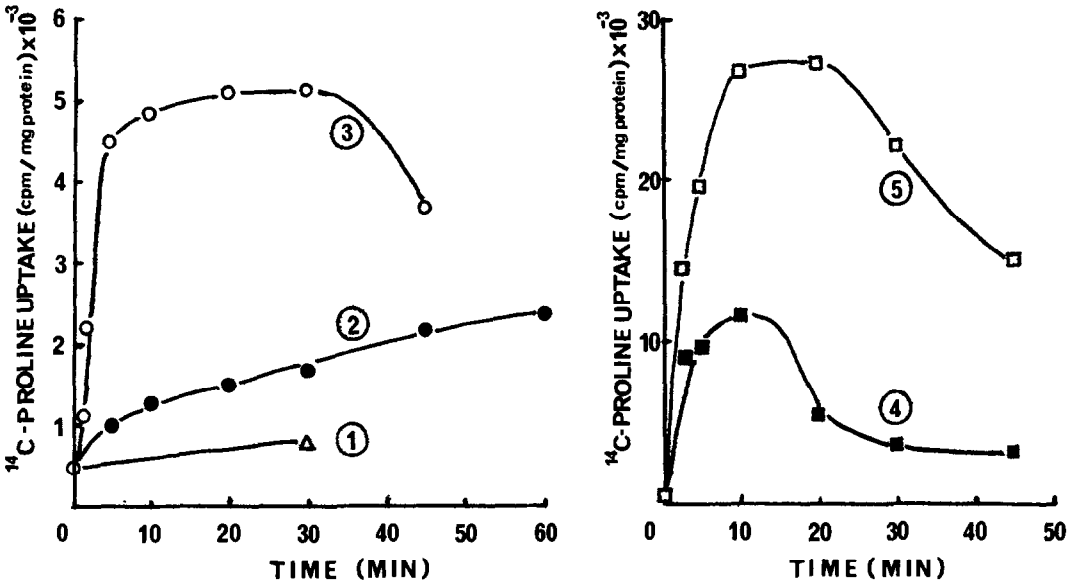

Figure 1. Effects of Respiratory Substrates on Proline Transport by ETP. The reaction mixture (0.1 ml) contained 5 x 10^{-2}M potassium phosphate buffer (pH 7.0), 10^{-2}M $MgCl_2$, substrate, 9 x 10^{-6}M L-Proline-U-^{14}C (200mCi/mmole) and ETP (0.17 mg for curves 1, 2 and 3, 0.21 mg for curves 4 and 5). After preincubation for 15 min at 30°, the reaction was started by the addition of substrate and ^{14}C-Proline at 0 min. Amount of substrate used was 2.5 µmoles succinate (Curve 2), 0.4 µmoles ethanol, 0.01 µmole semicarbazide, 0.1 µmole NAD^+ and 5 µg alcohol dehydrogenase (Curve 3), 1 µmole NADH (Curve 4), and 1.7 µmoles ascorbate and 0.015 µmoles TPD (Curve 5). Curve 1 shows the control experiment without substrate.

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from the ETP. Of the substrates tested ascorbate-TPD was most effective, while exogenous NADH, generated NADH, and succinate followed in decreasing order of effectiveness. It is of interest that the most effective substrates for transport are the least effective for oxidative phosphorylation. For example, with ascorbate and TPD only one site of phosphorylation occurs whereas with generated NADH three phosphorylation sites were demonstrated. Transport of proline was observed with ascorbate-PMS; however, this electron mediator was not as effective as TPD. Other substrates, such as glucose, fumarate, D-lactate, NAD^+ or ATP failed to stimulate the uptake of proline (Table I). The uptake of proline does not appear to depend on its incorporation into protein since almost all of the proline in the ETP was recovered as the free

TABLE I

Effect of Various Substrates on Proline Transport by ETP

Substrate	cpm/mg protein	
	Expt. I	Expt. II
None	366	665
Ascorbate-TPD	12,610	16,000
Ascorbate-PMS	7,290	---
Ascorbate	---	775
TPD	---	713
NADH	7,260	8,340
Generated NADH	2,680	2,390
NAD^+	208	---
Succinate	1,050	1,710
Fumarate	525	---
Glucose	412	---
D-Lactate	---	600
ATP	---	500

The amounts of substrate were similar to that in Fig. 1. In addition, as indicated the system contained fumarate, 2.5 μmoles; glucose, 10 μmoles; D-lactate, 1 μmole; PMS, 0.001 μmole; NAD^+, 1 μmole; ATP, 2 μmoles. The incubation period was 10 min except for the control and succinate in which the incubation was carried out for 30 min.

amino acid. For determination of the concentration gradient the intravesicular space was estimated. ^{14}C-Inulin impermeable and $^{3}H_2O$ permeable space of ETP was found to be approximately 1.7 μl/mg protein. Assuming that this space corresponds to the intravesicular volume, ETP established a concentration gradient of proline of approximately 35-fold when ascorbate-TPD was used as substrate, 20-fold with exogenous NADH, 6-fold with generated NADH and 3-fold with succinate.

In order to investigate possible relationships between oxidative phosphorylation and proline transport, the effects of various inhibitors were studied with generated NADH (Table II). Similar results were obtained with other substrates. Proline transport was inhibited by anaerobiosis, cyanide, azide, NQNO and near UV irradiation (360 nm). The transport of proline was inhibited by uncoupling agents such as m-ClCCP, and pentachlorophenol, while DCCD, an inhibitor of energy transfer in mitochondria (8) and in M. phlei (9), slightly stimulated the uptake of proline. Potassium arsenate in the absence of potassium phosphate had no effect on the transport

TABLE II

Effect of Inhibitors on Proline Transport

Additions	Concentration	cpm/mg protein	%
None		6,770	100
Anaerobic		1,080	15.9
m-ClCCP	5×10^{-5}M	660	9.7
DCCD	5×10^{-5}M	7,850	116.0
Chloramphenicol	100 μg/ml	5,500	81.3
None		2,760	100
Cyanide	10^{-2}M	1,540	55.8
Azide	10^{-2}M	1,610	58.3
Pentachlorophenol	10^{-4}M	823	30.0
None		2,390	100
Irradiation		418	17.5
None		3,220	100
Arsenate	5×10^{-2}M	3,260	101.0
None (2% ethanol)		4,060	100
Valinomycin	2×10^{-5}M	320	7.9
Nigericin	4×10^{-6}M	3,160	78.0
TPB^-	5×10^{-5}M	3,080	75.9
DDA^+	5×10^{-3}M	546	13.4

Generated NADH was used as a substrate. The generating system was similar to that described in Fig. 1. Inhibitors were added with the substrate. The anaerobic experiment was performed in an atmosphere of argon. Valinomycin and nigericin in 2% ethanol solutions were used. Light treated ETP were prepared by irradiation with 360 nm light for 20 min at 4°. Potassium arsenate buffer (5×10^{-2}M, pH 7.0) was used instead of potassium phosphate buffer as indicated.

process. In addition, the reaction was inhibited by the sulfhydryl agent p-chloromercuribenzoate. Other amino acids, alanine, valine, aspartic acid, lysine, tryosine, tryptophan and histidine did not have any inhibitory effect on proline transport.

It was of interest to determine if the removal of particle bound coupling factor influenced the transport process. Transport of proline was observed with the depleted ETP and was dependent on substrate oxidation but not on ATP synthesis (Table III). Furthermore, the addition of coupling factor(s) failed to enhance the proline uptake. However, the addition of AMP, hexokinase and glucose slightly inhibited proline uptake, suggesting some competition between the phosphorylative process and amino acid transport.

The ETP preparation may consist of a mixture of vesicles, some with membranes oriented as in the intact cell and others in which the membrane

TABLE III

Proline Uptake by Coupling Factor Depleted ETP

	O_2 uptake (μatom)	Pi esterified (μmole)	P/O	Proline uptake cpm/mg protein
ETP	9.7	11.2	1.15	2,645*
Depleted ETP	4.6	0	0	8,950
Depleted ETP + BCF_4	8.3	13.1	1.54	7,220

Generated NADH was used as a substrate. The ETP were depleted of particulate bound coupling factor(s) (BCF_4) by the method of Higashi, et. al., (6). Oxygen uptake was measured in a Gilson respirometer at 30° and inorganic phosphate was estimated by the method of Fiske and SubbaRow (17). *The depleted ETP comprise approximately 30% of the ETP preparation.

orientation has been reversed. The removal of coupling factor from the ETP (6), inhibition of phosphorylation by specific antibody to the coupling factor(s) (10) and electron microscopic observation of structures similar to the repeating units of the mitochondrial inner membrane (11, 12) indicated that the ETP preparation contains structures in which the inner surface of the membrane appears on the outside of the vesicle. However, the inhibitions of proline transport by directional specific uncoupling agent (13, 14, 15) suggest the possibility of the presence of "rightside-out" vesicles in the ETP preparation. For example, proline transport was inhibited by either valinomycin in the presence of K^+ or DDA^+ whereas TPB^- was a poor inhibitor (Table II).

In conclusion, amino acid transport in sonicated vesicles of M. phlei was shown to occur against a concentration gradient. Evidence (16) indicates that the active transport process is related to a proton gradient formed by electron transport. If one assumes that a proton gradient is also necessary for oxidative phosphorylation then these lack of correlation in M. phlei between transport and oxidative phosphorylation are inconsistent with the chemiosmotic hypothesis. The transport of proline in M. phlei was dependent on oxidation but not related to a phosphorylated high energy intermediate or ATP synthesis. Furthermore, the transport process appears to occur across membranes which are oriented as in the intact cell.

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