STIMULATION OF PROLINE TRANSPORT BY CUPRIC ION IN MEMBRANE VESICLES FROM MYCOBACTERIUM PHLEI

S.A. Yankofsky* and Arnold F. Brodie

Department of Biochemistry
University of Southern California
School of Medicine
Los Angeles, California 90033

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SUMMARY: Membrane vesicles of *M. phlei* actively take up proline in the absence of exogenously provided electron donors. Evidence is provided that endogenous transport is coupled to the oxidation-reduction of low-potential electron carriers, but does not involve the cytochromes. The endogenous transport was found to be enhanced under either aerobic or anaerobic conditions by the addition of certain artificial electron acceptors such as Cu^{2+}.

INTRODUCTION: Like other bacterial transport systems, the uptake of amino acid by membrane vesicles of *Mycobacterium phlei* requires substrate oxidation, does not require high energy phosphate bonds and is inhibited by anaerobic conditions (1-4). In the *M. phlei* system, the uptake of proline requires, in addition to substrate oxidation, Na^+ ion (5), and specific phospholipids (6), but does not require a proton gradient (7). It is of interest that substrates such as generated NADH or succinate are less effective for proline uptake than electrons derived from artificial electron donors. Thus, one might conclude that active transport of proline into membrane vesicles of *M. phlei* requires the input of an oxidizable substrate and involves the participation of the respiratory chain. This communication demonstrates that the active transport of proline in the *M. phlei* system can be mediated by Cu^{2+} under both aerobic and anaerobic conditions.

*On leave from Department of Microbiology, University of Tel-Aviv, Ramat Aviv, Tel-Aviv, Israel

MATERIALS AND METHODS: The growth conditions and the preparation of the electron transport particles (ETP) from *M. phlei* (ATCC 354) have been previously described (8). The uptake of proline, glutamine and glutamic acid was measured as previously described (3,5) except that NaCl was omitted from the assay system when glutamine and glutamic acid were used as the solute. The rate of oxidation was measured polarographically at 30° with a Clark oxygen electrode. The identification of radioactive materials accumulated by membrane vesicles was carried out as described by Hirata *et. al.* (2).

RESULTS AND DISCUSSION: The relationship between vesicular volume and total protein in *M. phlei* membrane vesicles has been shown to be 1.7×10^{-3} ml/mg protein (9). Thus, incubation of membrane vesicles with 25 nmoles/ml of proline should, in the absence of energy transduction, result in an uptake of 42.5 pmoles of amino acid per mg protein. Under anaerobic conditions, uptake of proline does not occur. In contrast, aerated membrane vesicles in the absence of added substrate accumulated some proline. This endogenous uptake may range from 40 to 80 pmoles of proline/mg protein. Under these conditions, the uptake of proline is ten times less than the corresponding values obtained with an electron donor such as ascorbate-tetramethyl phenylene-diamine. However, the endogenous levels of transport appear to establish a two-fold concentration gradient.

Endogenous proline uptake is coupled to endogenous respiration, suggesting that inhibitors of endogenous respiration should also inhibit the transport process. It was observed that amytal, atebrin and o-phenanthroline abolish both endogenous respiration and endogenous transport (Table I).

However, inhibitors of the cytochromes such as NHQNO or sodium azide completely inhibit the endogenous respiration, but not endogenous proline transport. The results suggest that the energy for the endogenous transport process is derived from electron flow through low potential carriers (flavoprotein or nonheme iron).

Abbreviations: NHQNO, 2-n-nonyl-hydroxy-quinoline-N-oxide.

TABLE I

Effect of Various Respiratory Inhibitors on the Endogenous Proline Transport

Inhibition	Inhibitor Concentration	Proline Uptake pmoles/mg protein	Proline Uptake Percent Inhibition
Expt. I			
None	----	88	----
Atebrin	5mM	41	100*
Sodium amytal	10mM	42	100
o-Phenanthroline	5mM	41	100
NHQNO	25ug/ml	85	6.5
NaN_3	10mM	78	1.2
KCN	5mM	79	13.1

Incubation mixtures contained, per ml, 25nmoles ^{14}C-L-proline, (10,000 CPM/nmole), 50μmoles HEPES buffer, pH7.5, 10μmoles $MgCl_2$, 20μmoles NaCl and 1.86 mg of ETP protein. The reaction was carried out with vigorous stirring at 30° for ten minutes and then terminated by 20-fold dilution into 0.05M potassium phosphate buffer, pH7.5.

*An uptake of 40-45 pmoles of proline/mg protein is observed in the absence of energy transduction and may be due to diffusion and nonspecific binding of the amino acid.

Endogenous proline transport appears to be coupled to an electron transport pathway involving direct reoxidation of relatively low potential carriers by oxygen. Thus, it should be possible to drive active transport of proline under anaerobic conditions by simply replacing oxygen with an artificial electron acceptor system of appropriate oxidation-reduction potential. A suitable electron acceptor for this purpose was found to be Cu^{2+}.

Incubation of membrane vesicles with 0.15 mM $CuCl_2$ (or $CuSO_4$) for just one minute prior to proline addition resulted in a four to five fold

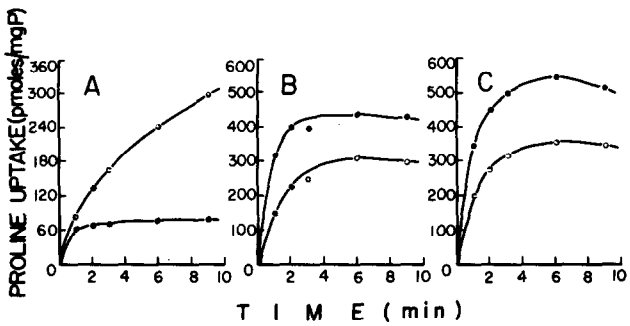

Fig. 1. Effect of Cu^{2+} on aerobic proline transport

The mixture contained in a total volume of 2 ml: 1.8 mg ETP protein, 50 µmoles HEPES, pH 7.5 buffer, 40µmoles NaCl, 20 µmoles $MgCl_2$, and 50 µmoles of ^{14}C-L-proline (10,000 CPM/nmole). Where pertinent, $CuCl_2$ was present at 150 nmoles/ml, potassium succinate (pH 7.5) at 25 µmoles/ml, or NADH (12.5 µmoles/ml).

(A) Without added substrate; (B) With succinate; (C) With NADH. Incubation was at 30° and 0.3 ml samples were taken at the indicated time points. Symbols are: -●-●- without $CuCl_2$, -o-o- with $CuCl_2$.

stimulation of the steady state level of proline uptake (Fig. I). It should, however, be noted that variation of the $CuCl_2$ input or prolongation of the preincubation period reduced the stimulatory effect of Cu^{2+}. The uptake of proline mediated by Cu^{2+} (150-200 µM), is 40 to 50 percent less than that observed with succinate and NADH as electron donors. The addition of Cu^{2+} ion in the presence of energy donors such as succinate and NADH reduced the steady state level of uptake of proline (Fig. 1B & C), as well as the rate of oxidation (Table II). Proline uptake mediated by Cu^{2+} also occurred under anaerobic conditions. Except for Fe^{3+}, other fourth period transition metals were ineffective. However, even at its optimal concentration, (0.6mM), Fe^{3+} is only one-tenth as effective as Cu^{2+}.

That electron flow through low-potential carriers is required for enhancement of endogenous transport by Cu^{2+} is indicated by the fact that amytal, atebrin and o-phenanthroline inhibit this process (Table III). However, irradiation of membrane vesicles at 360nm light does not affect the uptake of proline. It is pertinent to mention that irradiation of

TABLE II

Effect of Cu^{2+} on ETP Respiratory Capacity

Additions	Oxygen Consumption natoms/mg/min	Relative Rates
ETP	10.8	1.00
ETP + $CuCl_2$	7.0	0.70
ETP + Succinate	54.2	5.02
ETP + Succinate + $CuCl_2$	8.1	0.75
ETP + NADH	67.8	6.28
ETP + NADH + $CuCl_2$	21.1	1.95

Reaction mixture compositions are identical to those given in the legend to Fig. 1.

membrane vesicles of *M. phlei* with light at 360nm inactivates the natural naphthoquinone and results in an inhibition of oxidation via the cytochrome (10). Except for cyanide, inhibitors of cytochrome function do not significantly inhibit the Cu^{2+} promoted proline uptake. Inhibition of Cu^{2+}-promoted transport by cyanide is due to the removal of Cu^{2+} from the system as a consequence of its chemical interaction with cyanide (11).

The reduction of cytochromes b,c and $a+a_3$ in the presence of $CuCl_2$ did not occur in the absence of added substrate. Although reduction of the cytochromes occurred on addition of substrate and Cu^{2+}, the rate of reduction was decreased. Cu^{2+} also had no affect on the capacity of *M. phlei* cytochromes to undergo chemical reduction by dithionite. The above results indicate the participation of only low potential carriers (e.g., flavoprotein and nonheme iron) in the cupric-promoted proline uptake process.

TABLE III

Effect of Inhibitors on Cu^{2+} Mediated Proline Transport Under Aerobic Conditions

Inhibitors	$+Cu^{+2}$	$-Cu^{+2}$	(A-B)	Percent of Inhibition
	pmoles/mg protein			
Expt. I				
None	310	55	255	----
Amytal	141	7	134	48
Atebrin	62	0	62	76
o-Phenanthroline	16	0	16	94
NHQNO	270	51	219	14
NaN_3	303	46	257	0
KCN	54	47	7	97
Expt. II				
360nm light	290	31	259	0

The concentrations of inhibitors and ETP protein were as in Table I, the concentration of $CuCl_2$ was 150 nmoles/ml.

The role of Cu^{2+} may be to serve as an efficient bridge between a part of the respiratory chain and the transport process. It is pertinent to mention that Cu^{2+} has been shown to stimulate glucose transport in fat cells (12,13) and to play an important role in copper-containing oxygenases (14). Although the concentrations of Cu^{2+} required to stimulate transport are nonphysiological and membrane vesicles preparations appear to be perturbed systems, the cupric-mediated transport process may serve as a model system for studying the events and the nature of the protein(s) involved in the mechanism of active transport.

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