Active Transport of Amino Acids by Membrane Vesicles of *Thiobacillus neapolitanus*

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SUMMARY

Membrane vesicles of *Thiobacillus neapolitanus* take up amino acids at 25 °C in the presence of the nonphysiological electron donor, ascorbate-*N,N,N',N'*-tetramethyl-*p*-phenylenediamine. The amino acids accumulate inside the membrane vesicles against a concentration gradient. Inhibitors of the electron transport chain inhibit the accumulation; therefore active transport of amino acids in *T. neapolitanus* is coupled to the electron transport chain. The K_m values for the transport of glycine and L-serine in this organism are 2.5 and 5 μ M respectively.

INTRODUCTION

The inability of obligate chemolithotrophs to grow in completely organic media is difficult to understand. These organisms are permeable to a wide variety of organic compounds which are extensively assimilated and metabolized when supplied in the growth medium (Rittenberg, 1969; Kelly, 1971) and can, under certain conditions, actually increase growth yield (Kuenen & Veldkamp, 1973). These findings have disproved the hypothesis (Winogradsky, 1890) that organic compounds are generally and uniquely toxic to chemolithotrophs, and recent work (Kelly, 1969; Lu, Matin & Rittenberg, 1971) has shown that the toxicity of specific organic compounds towards these organisms resembles that in certain heterotrophs (Gladstone, 1939; Umbarger, 1969).

The Krebs cycle is incomplete in certain chemolithotrophs (Smith, London & Stanier, 1967), and NADH oxidation may not be coupled with oxidative phosphorylation (Hempfling & Vishniac, 1965; Johnson & Abraham, 1969). However, *Thiobacillus neapolitanus* and *Thiobacillus thioparus* possess high concentrations of the enzymes of carbohydrate metabolism (Johnson & Abraham, 1969; Matin & Rittenberg, 1970, 1971) which should make ATP synthesis by substrate-level phosphorylation possible, and hence allow growth on sugars.

Transport mechanisms in these organisms have received little attention, despite their possible role in obligate chemolithotrophy. Our studies were undertaken to determine whether *T. neapolitanus* can actively transport amino acids. Membrane vesicles prepared from this organism were used so that transport characteristics could be studied with minimal interference from other processes (Kaback, 1972).

METHODS

Growth conditions and preparation of membrane vesicles. *Thiobacillus neapolitanus* (kindly supplied by S. C. Rittenberg, University of California, Los Angeles, U.S.A.) was grown at 28 °C in a chemostat of 1.5 l working volume, which was equipped with devices for

automatic pH control. The medium (Matin & Rittenberg, 1971) used contained per 100 ml: NH_4Cl , 0.1 g; MgSO_4 , 0.05 g; K_2HPO_4 , 0.5 g; KH_2PO_4 , 0.5 g; solution of trace elements (Vishniac & Santer, 1957), 0.1 ml; and $\text{Na}_2\text{S}_2\text{O}_3$, 1.0 g. Growth was limited by thiosulphate; see Kuenen & Veldkamp (1973) for details. Cultures were frequently checked for heterotrophic contaminants by streaking on nutrient agar plates.

The organism was grown at a dilution rate of 0.27 h^{-1} and the effluent culture was collected in a reservoir placed in a refrigerator at 4°C until about 18 l had accumulated (approx. 45 h). Cells stored under these conditions for this duration had only about 30 % less transport activity than those freshly harvested. Membrane vesicles were obtained by the general method of Kaback (1971): cells were collected at 16000 g using a Sorvall continuous flow rotor, washed twice in 0.01 M-tris-HCl buffer (pH 8.0), and the pellet was taken up in the same buffer supplemented with 0.01 M-EDTA and 20 % (w/v) sucrose to give 1 g wet wt of cells/80 ml buffer. Lysozyme (Merck) ($500 \mu\text{g/ml}$) was added and the mixture incubated at 37°C for 2 h. Concentration and osmotic shock of the lysozyme-treated cells was as described by Kaback (1971). A significant number of spheroplasts failed to lyse on osmotic shock; these were removed by centrifugation at 10000 g for 10 min. The final preparation contained no more than 2 spheroplasts/microscopic field of $400\times$ magnification. The membrane vesicles were washed and stored in liquid nitrogen in 1 ml portions containing 5 to 10 mg membrane protein.

Measurements. Transport of amino acids was measured as described by Matin & Konings (1973). Reaction mixtures contained, in a total volume of $100 \mu\text{l}$ at 25°C : 50 mM-potassium phosphate buffer, pH 6.6; 10 mM- MgSO_4 ; 0.15 to 0.2 mg membrane vesicle protein; an electron donor; and a radioactive amino acid.

The methods used for oxygen measurements, extraction of radioactivity from membrane vesicles, and thin-layer chromatography were as described by Matin & Konings (1973) and Konings & Freese (1972). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Radioactive amino acids (Radiochemical Centre, Amersham, Buckinghamshire) had the following specific activities: glycine, 108 mCi/mmol; L-serine, 159 mCi/mmol; L-threonine, 208 mCi/mmol; and L-leucine, 331 mCi/mmol.

RESULTS

To examine the possibility that amino acid transport in *T. neapolitanus* could be linked to the electron transport chain (Kaback, 1972), the membrane vesicles were tested for their ability to take up glycine in the presence of the non-physiological electron donor, sodium ascorbate, plus one of several compounds which could mediate electron flow from ascorbate to the electron transport chain of the vesicles. The presence of ascorbate alone in the reaction mixture stimulated amino acid transport by the membrane vesicles (Fig. 1). With the exception of methylene blue, the concurrent presence of any one of the electron mediators tested enhanced the stimulation caused by ascorbate, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. 2HCl (TMPD) being the most effective in this respect, followed by phenazine methosulphate (PMS). These results are in agreement with the finding that *T. neapolitanus* extracts oxidize ascorbate-TMPD faster than ascorbate (Sadler & Johnson, 1972).

The kinetics of ascorbate-TMPD-energized transport of L-leucine, L-serine, and L-threonine are presented in Fig. 2. Both the initial rates of uptake and the maximum amounts of each of the three amino acids taken up were greater in the presence of the electron donor than in its absence. The transport of L-arginine and L-aspartate was also stimulated by the electron donor in the reaction mixture (data not presented).

Fig. 1. Time course of glycine uptake by membrane vesicles of *T. neapolitanus* in the presence of 40 mM-sodium ascorbate (pH 6.6) plus one of the electron mediators in concentration specified: (○), none; (△), 0.1 mM-methylene blue; (◻), 0.5 mM-FeCl₃; (●), 0.1 mM-dichlorophenol-indophenol; (▲), 0.1 mM-PMS; (▼), 0.15 mM-TMPD. Uptake in the absence of an electron donor (▽) was also determined. The initial concentration of glycine in the reaction mixture was 23 μ M.

The fate of transported L-serine, L-leucine and L-threonine was determined by thin-layer chromatography. In all cases, the radioactivity recovered from the vesicles chromatographed with the original amino acid. These amino acids were therefore concentrated 5.6-, 5.5- and 1.5-fold, respectively, inside the membrane vesicles; these values are calculated from the data in Fig. 2, on the assumption that 1 mg of vesicle protein corresponds to an internal vesicle volume of 3 μ l (Konings & Freese, 1972).

As a preliminary to investigating whether electron donors which are likely to be available to this organism during growth would energize amino acid transport, we examined whether a number of such donors were oxidized by the membrane vesicles. The vesicles possessed a low endogenous rate of respiration (Table 1). Among the substrates tested, only NADH and thiosulphate were oxidized; succinate, L-lactate and D-lactate were not. The oxidation of sulphide and sulphite by the vesicles was not determined because of the relatively high spontaneous rate of oxidation of these compounds.

NADH, thiosulphate and sulphide, but not sulphite, caused a weak stimulation of the transport of L-serine by the membrane vesicles (Fig. 3). However, the uptake of L-threonine, which is weakly transported even in the presence of ascorbate-TMPD (Fig. 2), was not

Fig. 2. Uptake of L-serine ($10 \mu\text{M}$; $\square$, $\blacksquare$), L-threonine ($14 \mu\text{M}$; ∇ , $\blacktriangledown$), and L-leucine ($9 \mu\text{M}$; $\circ$, $\bullet$) by membrane vesicles of *T. neapolitanus* in the absence (open symbols), or presence (solid symbols) of 40 mM-sodium ascorbate–0.15 mM-TMPD.

Table I. Oxidation of various substrates by membrane vesicles of *T. neapolitanus*

Concentration of all substrates in the reaction mixture was 20 mM, and the amount of membrane protein was 2.5 mg.

Substrate	Rate of oxygen uptake (nmol/min/mg membrane protein)
None	1.8
NADH	21.0
Thiosulphate	9.4
Succinate	N.D.
D-Lactate	N.D.
L-Lactate	N.D.

N.D., not detected.

stimulated by any of the above electron donors. In addition, ATP failed to stimulate transport of either of the amino acids.

In contrast to membrane vesicles, transport of L-serine by whole cells of *T. neapolitanus* was strongly stimulated by thiosulphate and little by ascorbate–PMS (Fig. 4). The uptake in the presence of thiosulphate was followed by a rapid efflux of radioactivity; the reason(s) for this is not known. The lack of uptake of L-serine by whole cells of *T. neapolitanus* in the

Fig. 3

Fig. 3. Time course of L-serine uptake by membrane vesicles of *T. neapolitanus* in the absence (Δ), or presence of one of the following potentially physiological electron donors: $\circ$, 20 mM-NADH; $\square$, 20 mM-sodium thiosulphate; ∇ , 20 mM-sodium sulphide. The initial concentration of L-serine in the reaction mixture was 25 μ M.

Fig. 4

Fig. 4. Time course of L-serine uptake by washed whole cells of *T. neapolitanus* in the absence ($\blacksquare$), or presence of one of the following electron donors: $\bullet$, 20 mM-sodium thiosulphate; $\blacktriangle$, 40 mM-sodium ascorbate-0.15 mM-PMS. The initial concentration of L-serine in the reaction mixture was 20 μ M, and the amount of cell protein was 0.1 mg/100 μ l.

absence of the inorganic energy source is in agreement with previous reports (Kelly, 1967*a*, 1971) and is in contrast to the behaviour of heterotrophic organisms, washed suspensions of which take up amino acids and other substrates without addition of an exogenous source of energy.

To obtain conclusive evidence on the involvement of the electron transport chain in amino acid transport by the membrane vesicles, the effect of some common inhibitors of the electron transport chain on ascorbate-TMPD-energized transport of glycine and L-serine was investigated. The results are presented in Table 2. All of the inhibitors tested caused almost complete inhibition of amino acid transport.

The kinetic constants for L-serine and glycine transport by *T. neapolitanus* membrane vesicles were determined by averaging the results of two experiments using different membrane vesicle preparations (the amino acid concentration ranged from 1 to 25 μ M, the electron donor system contained 40 mM-sodium ascorbate-0.15 mM-TMPD, and the uptake time for each determination was 2 min). As with membrane vesicles of other organisms (Konings

Table 2. *Effect of electron transport chain inhibitors on the initial rate of uptake of two amino acids by membrane vesicles of Thiobacillus neapolitanus*

Initial rates of uptake (3 min incubation time) were determined using sodium ascorbate (40 mM)-TMPD (150 μ M) as electron donor as described in Methods. Initial concentrations of amino acids in the reaction mixture were as follows: glycine, 23 μ M; L-serine, 25 μ M. Amytal was dissolved in dimethyl sulphoxide (DMSO), the final concentration of the latter being 1 % (v/v). Percentage inhibitions were calculated with respect to initial rates of uptake in the absence of the inhibitor, except that the controls contained 1 % DMSO in the case of amytal.

Inhibitor	Inhibition of amino acid transport (%)	
	Glycine	L-Serine
Cyanide (10 mM)	98	97
Azide (10 mM)	100	100
Amytal (10 μ M)	100	97

& Freese, 1972), the K_m values varied little with different preparations – 5.0 ± 0.1 μ M for L-leucine and 2.5 ± 0 μ M for glycine; however, the V_{max} values showed significant variation from preparation to preparation, 0.16 ± 0.06 nmol/min/mg protein for L-serine and 0.72 ± 0.30 nmol/min/mg protein for glycine. A similar K_m value was obtained for L-serine when whole cells were used (5.0 ± 1.7 μ M; average of four determinations), but the V_{max} was higher (25 ± 8 nmol/min/mg protein). These K_m values are of the same order of magnitude as those reported for *Bacillus subtilis* (40 μ M for L-serine and 9 μ M for glycine) by Konings & Freese (1972).

DISCUSSION

The transport of several amino acids in membrane vesicles of *T. neapolitanus* occurs actively by transport systems which are coupled to the electron transport chain. The vesicles take up amino acids only in the presence of an electron donor, the transported amino acid is accumulated inside the membrane vesicles against a concentration gradient, and inhibitors of the electron transport chain almost stop transport of the amino acid. In this respect, *T. neapolitanus* is similar to the heterotrophic organisms, *Escherichia coli* (Barnes & Kaback, 1971), *Bacillus subtilis* (Konings & Freese, 1972), *Staphylococcus aureus* (Short, White & Kaback, 1972), and others (Konings, Barnes & Kaback, 1971; Sprott & McLeod, 1972).

The vesicle preparation described here was active primarily with the nonphysiological electron donors, ascorbate-TMPD or ascorbate-PMS. Relatively little stimulation of transport was observed with any of the potentially physiological electron donors tested. The reason for this is not known. Effective coupling with the latter donors may require some soluble enzyme, or the molecular integrity of the cell membrane may have been disturbed during the isolation of the vesicles. In this respect it should be noted that the thiosulphate-oxidizing activity of the vesicle preparation was considerably lower than that reported for whole cells of thiobacilli (London, 1964).

This is the first demonstration of active transport in an obligate chemolithotroph. It is not known if *T. neapolitanus* possesses mechanisms for active transport of organic compounds other than amino acids but it seems likely that it does. Lack of active transport mechanisms, therefore, is probably not the reason for obligate chemolithotrophy in this organism. It is possible, as has been suggested by Kelly (1971), that only the lithotrophic energy source can effectively energize the active transport mechanisms. Experiments dealing with the uptake of organic substrates by whole cells of obligate chemolithotrophs tend to

support this suggestion. We have shown that *T. neapolitanus* cells did not take up L-serine unless thiosulphate was present. A similar phenomenon has been reported by Kelly (1967*a, b*) in this organism with respect to amino acid and acetate incorporation. Similarly, *Methylococcus* requires methane for acetate incorporation (Dahl, Mehta & Hoare, 1972), and uptake of alanine by *Nitrosomonas* is doubled in the presence of ammonium ions (Clark & Schmidt, 1967). Further work with improved vesicle preparations of these organisms might show how transport is energized in physiological circumstances.

Growth in the chemostat under conditions of thiosulphate limitation probably did not influence the ability of *T. neapolitanus* to transport amino acids: thiobacilli and related organisms take up amino acids and other organic compounds during growth in a chemostat under conditions of thiosulphate or CO₂ limitation (Kuenen & Veldkamp, 1973), and also in batch cultures (Kelly, 1967*a*, 1969).

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