Observations on Methionine Transport in *Pseudomonas fluorescens* UK1

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SUMMARY

Pseudomonas fluorescens UK1 absorbed DL-methionine with a K_m value of 25 μ M, optimum temperature 42 to 45 °C. DL-Ethionine, S-methyl-L-cysteine, S-ethyl-L-cysteine and α -methyl-DL-methionine inhibited methionine transport competitively, with K_i values of 0.10, 0.35, 0.40 and 0.20 mM respectively.

Using lysozyme, EDTA and trypsin, membrane vesicles were prepared from cells grown with methionine. Methionine accumulation in the vesicles was enhanced both by ascorbate-phenazine methosulphate and by several physiological electron donors; the most effective were FAD+malate, malate, succinate or 2-oxoglutarate. Oxidation of succinate, but not of malate, was inhibited by malonate; oxidation of malate was inhibited by azide; FAD did not reverse azide inhibition.

Tracer methionine was recovered from the membranes both as the unchanged amino acid and methionine sulfoxide.

INTRODUCTION

A specific uptake system for methionine has been demonstrated in *Escherichia coli* (Cohen & Monod, 1957; Piperno & Oxender, 1968; Kadner, 1974), in *Salmonella typhimurium* (Ayling & Bridgeland, 1972) and in *Saccharomyces cerevisiae* (Gits & Grenson, 1967). Methionine transport is competitively inhibited by some analogues in *S. typhimurium* (Ayling & Bridgeland, 1972). Little is known, however, about the mechanism of methionine transport and its relationship to amino acid degradation, especially when methionine is the only source of carbon, nitrogen and sulphur. Kay & Gronlund (1969*a, b*) found that, although *Pseudomonas aeruginosa* transports methionine, the amino acid is a source of nitrogen only. Kaback & Milner (1970) reported that methionine is converted to an unidentified metabolite by the isolated membranes from *E. coli* ML308-225. When nitrogen-starved mycelium of *Penicillium chrysogenum* is incubated with high concentrations of labelled methionine, influx is followed by efflux of the corresponding labelled α -keto acid (Hunter & Segel, 1973). However, some aerobic bacteria can utilize methionine as the only source of carbon, nitrogen and sulphur (Segal & Starkey, 1969); methionine is first deaminated and then demethylated.

This paper presents our studies on methionine transport into whole cells and into isolated membrane vesicles of *Ps. fluorescens* UK1.

METHODS

Media. The basal medium in which methionine was the only source of carbon and nitrogen contained (g/l): KH_2PO_4 , 1.35; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.26; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.003; methionine, 1.49. The solution was adjusted to pH 7.0 with KOH; 2 % (w/v) agar was added as required.

Bacteria. The strain UK1 of *Pseudomonas fluorescens* was isolated from heavily polluted sea water, and had been exposed for 2 h at 30 °C to a solution (100 µg/ml) of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in the basal medium. The bacteria were then collected by centrifugation at 5000 *g* for 15 min, washed with 0.9 % (w/v) NaCl and cultured overnight in the basal medium containing nutrient broth (0.5 %, w/v). They were next cultured for 3 days on the methionine agar medium, then several further times in the basal medium. The stock cultures produced in that way were stored on methionine agar slants at 4 °C.

Chemicals. [^{14}C]DL-Methionine (specific activity 7.91 mCi/mmol) and [^{35}S]L-methionine (specific activity 96 Ci/mmol) were purchased from New England Nuclear, Boston, Massachusetts, U.S.A. DL-Ethionine, lysozyme and DNase II were obtained from Sigma. S-methyl-L-cysteine and S-ethyl-L-cysteine were from California Corporation for Biochemical Research, Los Angeles, U.S.A.

Membrane preparations. The bacteria, cultured on methionine or methionine + nutrient broth (at 30 °C), were harvested in mid-exponential phase by centrifugation at 5000 *g* for 15 min. One portion of the cells was washed with 0.05 M-potassium phosphate buffer (pH 6.6) and assayed for transport activity. The remaining portion, having been washed twice with 10 mM-tris-HCl buffer (pH 8.0), was converted to membrane vesicles; 8 mg washed cells, 0.5 mg lysozyme, 3 mg trypsin and 4 mg DNase/ml of suspension were used for this purpose (Kaback, 1971). The isolated vesicles were suspended (1.2 to 1.6 mg protein/ml) in 0.1 M-potassium phosphate buffer (pH 6.6).

Measurement of methionine transport. The reaction mixtures (Mäntsälä, 1974), of 0.45, 0.9 or 1.8 ml, contained 0.03 M-potassium phosphate buffer (pH 6.6), 1 mM-MgSO₄, 0.01 mM-[^{14}C]methionine (specific activity 15.6 mCi/mmol) and either 0.4 mg dry wt bacteria/ml, or membrane vesicles equivalent to 0.35 mg protein/ml. The solutions were usually 20 mM with respect to the electron donors.

Enzyme and protein assays. Succinate dehydrogenase (EC. 1.3.99.1) was measured spectrophotometrically (Veeger, der Vartanian & Zeylemaker, 1969), as was L-malate dehydrogenase (EC. 1.1.2.4) (Arrigoni & Singer, 1962). Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

Identification of radioactive compounds taken up by whole cells and membrane vesicles. Whole cells or isolated membrane vesicles (0.8 mg protein) were exposed to 60 µM-[^{14}C]methionine (in 0.5 ml) and to 10.5 µM-[^{35}S]methionine in the presence of chloramphenicol. At 5 min intervals, 0.1 ml portions were filtered (Millipore, 0.45 µm pore-size). The filters were washed with 10 ml of 0.1 M-NaCl, extracted with 6 ml water at 80 °C and the extracts lyophilized. The residues that dissolved in 50 µl water were chromatographed on Whatman No. 1 paper with *n*-butanol-acetic acid-water (25:4:10, by vol.). The papers were autoradiographed and the radioactive spots assayed in a scintillation spectrometer (Wallac, Decem-NTL³¹⁴).

RESULTS

Effect of growth substrates and some metabolites of methionine degradation on methionine accumulation

Figure 1 demonstrates that the uptake of [^{14}C]methionine continued linearly for 2 min in cells adapted to utilize methionine. Because 2-oxobutyrate and propionate, two of the end products of methionine degradation, hindered methionine uptake, we suggest that this is controlled by end-product inhibition. Succinate, on the other hand, only slightly decreased methionine accumulation. Bacteria grown with Casamino acids, nutrient broth or glucose and then incubated for 2 h under carbon starvation accumulated much less [^{14}C]methionine.

Fig. 1. Time course of L-methionine transport in the whole cells of *Ps. fluorescens* UK1. The reaction mixture contained, in 1.8 ml of 1 mM-MgSO₄, 0.01 mM-[¹⁴C]methionine (0.79 mCi/mmol) and 0.8 mg dry wt of bacteria. The bacteria were grown on the basal medium containing methionine (○), 0.5 % (w/v) nutrient broth (△), 0.5 % (w/v) Casamino acids (●), or 10 mM-glucose (▲). They were then starved for 2 h in the presence of 0.05 M-phosphate buffer (pH 6.6), 1 mM-MgSO₄ and 2 mM-(NH₄)₂SO₄. Citrate (■), 2-oxoglutarate (□), succinate (■), 2-oxobutyrate (●) and propionate (●) were added at 2 min (see arrow); final concentration 10 mM.

Fig. 2. The effect of sodium azide on the time course of methionine transport. Experimental conditions were as in Fig. 1. The arrow indicates when azide was added. Control (○); 2 mM-azide (●); 5 mM-azide (●); 10 mM-azide (●); 10 mM-azide from time zero (△).

Fig. 3. Conversion of methionine to other metabolites in the whole cells and in the membrane vesicles of *Ps. fluorescens* UK1. (a) The bacteria (2.2 mg dry wt) and (b) the membrane vesicles (0.8 mg protein) were put with 0.06 mM- $[^{14}\text{C}]$ methionine in 0.5 ml of 0.03 M-phosphate buffer (pH 6.6) and chloramphenicol (100 μg). Ascorbate (20 mM)+PMS (0.1 mM) was the electron donor system in (b). In (c) the reaction mixture was chromatographed without washing. In (d) $[^{35}\text{S}]$ methionine was used. In (e) $[^{14}\text{C}]$ methionine was incubated with 20 mM-ascorbate and 0.1 mM-PMS for 10 min in the absence of the vesicles. In (f) membrane vesicles were subsequently added to such a mixture.

Effect of azide on methionine transport

The presence of NaN_3 inhibited methionine transport (Fig. 2). Some concentrative accumulation appeared to occur even in the presence of 10 mM-azide, which also caused the efflux of the accumulated compounds.

Identification of the accumulated materials

Methionine was partly converted to the corresponding α -keto acid, to methionine sulphoxide and to several other compounds when the bacteria were used (Fig. 3). However, some methionine sulphoxide formed spontaneously during the assays (Fig. 3e). Methionine was recovered from the membrane vesicles both as methionine itself and as the sulphoxide (Fig. 3b). Methionine sulphoxide was apparently partly converted to methionine again after addition of membrane vesicles (Fig. 3f).

Fig. 4. The reciprocal of the initial velocity (v) of methionine uptake as a function of the reciprocal of the methionine concentration. The amount of DL-methionine absorbed in 2 min was assayed (see Fig. 1). The methionine concentration varied up to $200\ \mu\text{M}$.

Table 1. *The effect of some methionine analogues, sulphur compounds and amino acids on methionine uptake by the bacteria*

The concentration of DL-methionine was $20\ \mu\text{M}$; other experimental conditions were as in Fig. 1.

Analogue (2 mM)	Methionine absorbed (nmol/min/mg dry wt)
No additions	2.10
DL-Ethionine	0.80
α -Methyl methionine	1.15
S-methyl-L-cysteine	1.25
S-ethyl-L-cysteine	1.40
No additions	2.60
N-formyl-L-methionine	1.70
L-Methionine sulfoxide	1.75
DL-Homocysteine	1.60
L-Cysteine	1.80
N-acetyl-DL-methionine	2.65
DL-Allocystationine	2.40
L-Cysteic acid	2.75
L-Cystine	2.55
L-Glutathione	2.30
DL-Homocysteic acid	2.20
DL-Homocysteine thiolactone	2.60
3-Mercapto propionic acid	2.25
L-Lysine	2.40
L-Aspartic acid	2.60

Fig. 5. Accumulation of methionine in isolated membrane vesicles from *Ps. fluorescens* UK1. Concentration of the added electron donors was 20 mM (PMS was 0.1 mM and FAD was 0.01 mM). The reaction mixture (see Fig. 1) contained 0.32 mg of membrane protein. (a) Ascorbate-PMS (○), ascorbate (●), no added electron donor (●). (b) Malate + FAD (△), malate (▲). (c) Succinate (□), DL-lactate (■). (d) 2-Oxoglutarate (□), fumarate (■).

Table 2. The effect of various compounds on methionine transport by *Ps. fluorescens* UK1 membrane vesicles

Experimental conditions were as in Fig. 6.	
Addition (20 mM)	Methionine absorbed (nmol/min/mg protein)
None	0.065
NADH	0.105
ATP	0.080
Glucose-6-P	0.060
Glucose-1-P	0.080
Glucose	0.070
Acetate	0.030
Pyruvate	0.065
Citrate	0.075
2-Oxobutyrate	0.100
Choline	0.205
Ethanolamine	0.150

Kinetics of methionine transport

A Lineweaver-Burk plot of methionine transport is shown in Fig. 4. One process exhibits saturation kinetics at methionine concentrations below 20 μ M. A second system may represent simple diffusion, since (i) the extrapolated $v_{\max}$ (Fig. 4, inset) appears to be very large

Fig. 6. Effect of malonate and azide on methionine uptake when succinate and malate were used as electron donors. Membrane vesicles were incubated (see Fig. 1) in the presence of: 0.01 mM- $[^{14}\text{C}]$ methionine, 20 mM-succinate and 20 mM-malonate (●); 0.01 mM- $[^{14}\text{C}]$ methionine, 10 mM-succinate, 10 mM-malate, 20 mM-malonate and 10 mM-azide (○); and 0.01 mM- $[^{14}\text{C}]$ methionine, 20 mM-malate, 10 mM-azide and 10 μM -FAD (Δ). The arrows show when the compounds were added.

Table 3. The effect of malonate and azide on the activities of succinate, L-malate and D-lactate dehydrogenases in membrane vesicles prepared from *Ps. fluorescens* UK1 grown with methionine

Inhibitor	Enzyme activity (nmol/min/mg protein)		
	Succinate dehydrogenase	L-Malate dehydrogenase	D-Lactate dehydrogenase
None	0.092	0.122	0.016
Malonate (20 mM)	0.034	0.116	—
Azide (10 mM)	0.068	0.102	—

and (ii) the addition of azide (Fig. 2) and cyanide (10 mM) failed to stop it. Among the compounds listed in Table 1, DL-ethionine, α -methyl-DL-methionine, S-methyl-L-cysteine and S-ethyl-L-cysteine were the strongest inhibitors. Their effects were competitive; apparent K_i values were 0.10, 0.20, 0.35 and 0.40 mM, respectively, when the methionine concentration was below 20 μM .

Accumulation of methionine in the membrane vesicles

As shown in Fig. 5, accumulation was enhanced by addition of 20 mM ascorbate with 0.1 mM phenazine methosulphate (PMS), the maximum levels being reached in 4 to 6 min. Several other compounds also served as electron donors. The most effective were FAD + malate, malate, succinate and 2-oxoglutarate (Fig. 5), and NADH, choline and ethanolamine (Table 2). Figure 6 demonstrates that malonate (20 mM) inhibited methionine

accumulation when succinate was the electron donor, but was less effective when succinate and malate were used together. When azide (10 mM) was added to the reaction mixture, methionine accumulation was completely inhibited even in the presence of FAD. Succinate dehydrogenase was inhibited by malonate (20 mM) and both succinate and L-malate dehydrogenases were partly inhibited by azide (10 mM) (Table 3). D-Lactate dehydrogenase was almost absent from the membrane vesicles prepared from the bacteria grown in the basal medium.

Effect of temperature on methionine transport in whole cells and membrane vesicles

The initial rate of uptake of methionine was greatest at 42 to 45 °C in both cases. Using the vesicles, an activation energy of about 75 kJ/mol was estimated from the temperature-dependence of the rate.

DISCUSSION

Many transport systems for amino acids are constitutive (Cohen & Monod, 1957; Britten & McClure, 1962; Kepes & Cohen, 1962); however, especially in pseudomonads, some are inducible (Rosenfeld & Feigelson, 1969; Gryder & Adams, 1970; Miller & Rodwell, 1971 *a, b*), as we found the strain UK1 to be with respect to methionine uptake. It seems relevant that Kay & Gronlund (1969*a*) found that, when cultures of *Ps. aeruginosa* were maintained in the stationary growth phase for 3 h in a glucose minimal medium, all the intracellular and extracellular amino acids were utilized, except for methionine which accumulated outside the cells.

Membrane vesicles from *E. coli* ML308-225 convert accumulated methionine to an unknown compound (Kaback & Milner, 1970), which may be either the corresponding α -keto acid or possibly methionine sulphoxide formed in the presence of ascorbate-PMS. In *Ps. fluorescens* UK1 methionine is obviously transported by the vesicles without deamination. If 2-oxomethionine had been demethylated, the 2-oxobutyrate formed would be labelled and at least three radioactive spots would be detected on the autoradiograms. These were not observed. Consequently demethylation may be a cytoplasmic process.

Ethionine is a competitive inhibitor of methionine in several micro-organisms (Gits & Grenson, 1967; Piperno & Oxender, 1968; Ayling & Bridgeland, 1972). In *Ps. fluorescens* UK1, too, ethionine inhibited methionine accumulation competitively. On the other hand, although methionine sulphoxide had no inhibitory effect in *Salmonella typhimurium* (Ayling & Bridgeland, 1972), it did in *Ps. fluorescens*.

For most organisms studied the artificial system, ascorbate-PMS, has been found to be the most effective electron donor. However, a wide variety of oxidizable compounds such as lactate (Kaback & Milner, 1970; Matin & Konings, 1973), succinate, DL- α -hydroxybutyrate (Lombardi & Kaback, 1972), malate, pyruvate, oxalacetate, glucose (Stinnett, Guyman & Eagon, 1973), NADH (Sprott & MacLeod, 1972) and α -glycerol-P (Short, White & Kaback, 1972) act as electron donors. Succinate, malate and 2-oxoglutarate were evidently electron donors in *Ps. fluorescens* UK1. This depended on the way the vesicles were prepared; when ultrasonic disintegration or an X-press were used, only ascorbate-PMS accelerated methionine transport (unpublished observations).

REFERENCES

- ARRIGONI, O. & SINGER, T. P. (1962). Limitations of the phenazine methosulphate assay for succinic and related dehydrogenases. *Nature, London* **193**, 1256-1258.
- AYLING, P. D. & BRIDGELAND, E. S. (1972). Methionine transport in wild-type and transport-defective mutants of *Salmonella typhimurium*. *Journal of General Microbiology* **73**, 127-141.

- BRITTEN, R. J. & MCCLURE, F. T. (1962). The amino acid pool in *Escherichia coli*. *Bacteriological Reviews* **26**, 292–335.
- COHEN, G. N. & MONOD, J. (1957). Bacterial permeases. *Bacteriological Reviews* **21**, 169–194.
- GITS, J. J. & GRENSON, M. (1967). Multiplicity of the amino acid permeases in *Saccharomyces cerevisiae*. III. Evidence for a specific methionine-transporting system. *Biochimica et biophysica acta* **135**, 507–516.
- GRYDER, R. M. & ADAMS, E. (1970). Properties of the inducible hydroxyproline transport system of *Pseudomonas putida*. *Journal of Bacteriology* **101**, 948–958.
- HUNTER, D. R. & SEGEL, I. H. (1973). Control of the general amino acid permease of *Penicillium chrysogenum* by transinhibition and turnover. *Archives of Biochemistry and Biophysics* **154**, 387–399.
- KABACK, H. R. (1971). Bacterial membranes. In *Methods in Enzymology*, vol. 22, pp. 99–120. Edited by W. B. Jacoby. New York and London: Academic Press.
- KABACK, H. R. & MILNER, L. S. (1970). Relationship of a membrane-bound D-(–)-lactic dehydrogenase to amino acid transport in isolated bacterial membrane preparations. *Proceedings of the National Academy of Sciences of the United States of America* **66**, 1008–1015.
- KADNER, R. J. (1974). Transport systems for L-methionine in *Escherichia coli*. *Journal of Bacteriology* **117**, 232–241.
- KAY, W. W. & GRONLUND, A. F. (1969*a*). Amino acid transport in *Pseudomonas aeruginosa*. *Journal of Bacteriology* **97**, 273–281.
- KAY, W. W. & GRONLUND, A. F. (1969*b*). Amino acid pool formation in *Pseudomonas aeruginosa*. *Journal of Bacteriology* **100**, 282–291.
- KEPES, A. & COHEN, G. N. (1962). Permeation. In *The Bacteria*, vol. 4, pp. 179–221. Edited by J. C. Gunsalus. New York: Academic Press.
- LOMBARDI, F. J. & KABACK, H. R. (1972). Mechanisms of active transport in isolated bacterial membrane vesicles. VIII. The transport of amino acids by membranes prepared from *Escherichia coli*. *Journal of Biological Chemistry* **247**, 7844–7857.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.
- MÄNTSÄLÄ, P. (1974). Correlations between pantothenate uptake, phospholipid synthesis and pantothenate-binding protein formation in *Pseudomonas fluorescens* P-2. *Acta chemica scandinavica* **28**, 78–84.
- MATIN, A. & KONINGS, W. N. (1973). Transport of lactate and succinate by membrane vesicles of *Escherichia coli*, *Bacillus subtilis* and a *Pseudomonas* species. *European Journal of Biochemistry* **34**, 58–67.
- MILLER, D. L. & RODWELL, V. W. (1971*a*). Metabolism of basic amino acids in *Pseudomonas putida*. Properties of the inducible lysine transport system. *Journal of Biological Chemistry* **246**, 1765–1771.
- MILLER, D. L. & RODWELL, V. W. (1971*b*). Metabolism of basic amino acids in *Pseudomonas putida*. Intermediates in L-arginine catabolism. *Journal of Biological Chemistry* **246**, 5053–5058.
- PIPERNO, J. R. & OXENDER, D. L. (1968). Amino acid transport systems in *Escherichia coli* K-12. *Journal of Biological Chemistry* **243**, 5914–5920.
- ROSENFELD, H. & FEIGELSON, P. (1969). Synergistic and product induction of the enzymes of tryptophan metabolism in *Pseudomonas acidovorans*. *Journal of Bacteriology* **97**, 697–704.
- SEGAL, W. & STARKEY, R. L. (1969). Microbial decomposition of methionine and identity of the resulting sulfur products. *Journal of Bacteriology* **98**, 908–913.
- SHORT, S. A., WHITE, D. C. & KABACK, H. R. (1972). Active transport in isolated bacterial membrane vesicles. V. The transport of amino acids by membrane vesicles prepared from *Staphylococcus aureus*. *Journal of Biological Chemistry* **247**, 298–304.
- SPROTT, G. D. & MACLEOD, R. A. (1972). Na⁺-dependent amino acid transport in isolated membrane vesicles of a marine pseudomonad energized by electron donors. *Biochemical and Biophysical Research Communications* **47**, 838–845.
- STINNETT, J. D., GUYMAN, L. F. & EAGON, R. G. (1973). A novel technique for the preparation of transport-active membrane vesicles from *Pseudomonas aeruginosa*: observations of gluconate transport. *Biochemical and Biophysical Research Communications* **52**, 284–290.
- VEEGER, C., DER VARTANIAN, D. V. & ZEYLEMAKER, W. P. (1969). Succinate dehydrogenase. In *Methods in Enzymology*, vol. 13, pp. 81–90. Edited by J. M. Lowenstein. New York and London: Academic Press.