Biochimica et Biophysica Acta, 735 (1983) 337–340
Elsevier

BBA 71892

TRANSPORT OF 2-METHYL-4-AMINO-5-HYDROXYMETHYLPYRIMIDINE BY *SALMONELLA TYPHIMURIUM*

EDWARD BELLION and TIMOTHY D. LASH

Department of Chemistry, University of Texas at Arlington, Box 19065, Arlington, TX 76019 (U.S.A.)

(Received March 21st, 1983)

Key words: Active transport; Pyrimidine; Thiamine; (S. typhimurium)

The transport of 2-methyl-4-amino-5-hydroxymethylpyrimidine (MAHMP) by *Salmonella typhimurium* was studied using synthetic [*methyl*-$^3H_3$]MAHMP. It was found that an active transport system existed for MAHMP, having K_m of 0.07 μM and V_{max} 45 nmol · min^{-1} · (g dry wt. cells)$^{-1}$, that required glucose as a source of energy and was pH and temperature dependent. Uptake was inhibited by cyanide, azide, *N*-ethylmaleimide, 2,4-dinitrophenol and carbonyl cyanide *m*-chlorophenylhydrazone. Uptake was also weakly inhibited by oxythiamine, but not by thiamine, 2-methyl-4-amino-5-aminomethylpyrimidine, or 4-amino-5-hydroxymethylpyrimidine, indicating that the transport system is specific for MAHMP.

Introduction

During our investigations into the conversion of aminoimidazole ribotide into 2-methyl-4-amino-5-hydroxymethylpyrimidine (MAHMP, the pyrimidine moiety of thiamine), in *Salmonella typhimurium*, we needed information regarding the uptake of thiamine, its separate moieties and possible precursors, into the cells. Although information on thiamine and 4-methyl-5-hydroxyethylthiazole (MHET) uptake was available for *Escherichia coli* [1–4], no data were available for MAHMP, no doubt because the labelled material is not commercially available. We undertook our own study of uptake of thiamine and MHET in *S. typhimurium* and found the results to be essentially similar to those found with *E. coli* [5]. We also synthesized tritium-labelled MAHMP and studied its uptake in *S. typhimurium*. This paper presents the results of the latter study.

Materials and Methods

The organism and growth conditions were as previously described [5].

Assay of [^{3}H]MAHMP uptake. These were performed with 10 ml cell suspensions to which the radioactive MAHMP, specific activity 12 μCi/μmol, was added at various concentrations. Sampling, filtering and counting was done as previously described [5]. Counting efficiency for tritium was 60% and counts were corrected to 100% value.

Chemicals. 2-[$^3H_3$]Methyl-4-amino-5-hydroxymethylpyrimidine was synthesized using an adaptation of the method of Neal [6]. [2-^{3}H]Acetonitrile, specific activity 2.6 Ci/mmol, was obtained from New England Nuclear. 12 mCi of this was diluted with 1 mmol unlabelled acetonitrile, taken up in 3 ml ethanol that had been saturated with dry HCl gas, and allowed to stand for two days at room temperature, The alcohol and excess hydrogen

Abbreviations: MAHMP, 2-methyl-4-amino-5-hydroxymethylpyrimidine; AHMP, 4-amino-5-hydroxymethylpyrimidine; MAAMP, 2-methyl-4-amino-5-aminomethylpyrimidine; MAMMP, 2-methyl-4-amino-5-methoxymethylpyrimidine; MHET, 4-methyl-5-hydroxyethylthiazole. (For structural formulae see Fig. 1. in Ref. 5.)

chloride was removed on a rotary evaporator to yield the imino ether. To this was added a saturated solution of ammonia in dry ethanol (3 ml) and the mixture was allowed to stand for 2 h. Acetamidine hydrochloride was recovered as a colorless crystalline solid after removal of the ethanol and excess ammonia, and was dissolved in 20 ml anhydrous ethanol and treated with 1 ml of sodium ethoxide (made from 30 mg sodium in 1 ml ethanol). The filtered solution was immediately added to a solution of 122 mg ethoxymethylenemalononitrile in 1 ml of ethanol. The resultant pale yellow precipitate of 2-methyl-4-amino-5-cyanopyrimidine was filtered and washed with cold ethanol. 80 mg of this material was dissoved in 20 ml anhydrous ethanol containing a little dry hydrogen chloride. The mixture was shaken overnight with 50 mg of 5% palladium on charcoal under hydrogen at 48 lb · inch^2 at room temperature. After removal of the catalyst by filtration the solution was evaporated under reduced pressure. This yielded a pale yellow oil that was taken up in 1 M HCl and treated dropwise with sodium nitrite solution (280 mg in 1 ml H_2O) at 90°C. After the addition was complete, the pH was brought to 8.5 and the mixture evaporated to dryness. The residue was extracted by Soxhlet with chloroform overnight, and the chloroform removed by rotary evaporation. The residue of 2-[$^3H_3$]methyl-4-amino-5-hydroxymethylpyrimidine was purified by thin-layer chromatography on silica gel with 1-propanol/0.2 N ammonium hydroxide (3 : 1, v/v) as solvent. Bands containing MAHMP were scraped off and the MAHMP was eluted with methanol. Evaporation of the solvent yielded the pure compound that co-chromatographed with authentic MAHMP.

4-Amino-5-hydroxymethylpyrimidine (AHMP). This was prepared by catalytic reduction of 4-amino-5-cyanopyrimidine, synthesized by the method of Baddiley et al. [7] using 4 g of 4-amino-5-cyanopyrimidine suspended in 100 ml 5% Pd-C in hydrogen at 55 lb · inch^2, followed by hydrolysis of the amine with nitrous acid as described by Neal [6].

Other chemicals were obtained as previously described [5].

Results

Uptake of [Me-$^3H_3$]MAHMP and energy requirement

The time-course of MAHMP uptake in *Salmonella typhimurium* is shown in Fig. 1. A marked dependence on the presence of glucose is clearly apparent. A slight inhibition of uptake was observed if 4-methyl-5-hydroxyethylthiazole was included in the assay.

Kinetics and Substrate dependence

Fig. 2 shows a Lineweaver-Burk plot of the uptake rate as a function of MAHMP concentration. This analysis revealed a K_m of 0.07 μM with a V_{max} of 45 nmol · min^{-1} · (g dry weight cells)$^{-1}$.

Effect of thiamine and oxythiamine

Fig. 3 shows that uptake is not inhibited by the presence of thiamine but that uptake is markedly reduced by oxythiamine.

Effect of other pyrimidines on uptake

Both 2-methyl-4-amino-5-aminomethylpyrimidine (MAAMP) and 4-amino-5-hydroxymethylpyrimidine (AHMP) have been proposed as intermediates in MAHMP biosynthesis [8,9]. However, neither compound is able to affect significantly the uptake of [*Me*-$^3H_3$]MAHMP after a 5-min preincubation although a similar preincubation with unlabelled MAHMP produced a drastic drop in the subsequent uptake rate (Fig. 4).

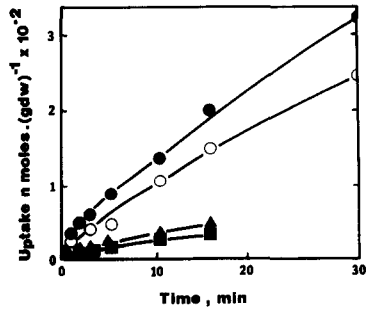

Fig. 1. Time-course of uptake of [$^3H_3$]MAHMP by *S. typhimurium*. Symbols: ●, MAHMP + 0.4% glucose; ○, MAHMP + 0.4% glucose and 0.1 mM MHET; ▲, MAHMP + 0.1 mM MHET; ■, MAHMP only. Concentration of MAHMP was 0.1 mM.

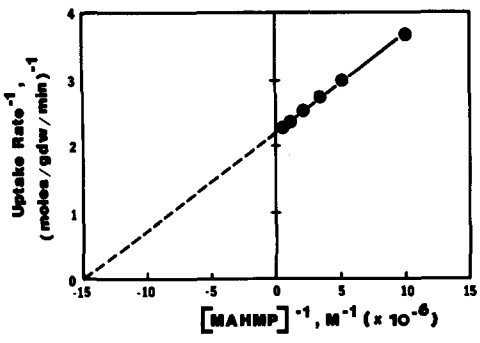

Fig. 2. Double reciprocal plot of rate of uptake of [$^3H_3$]MAHMP as a function of MAHMP concentration.

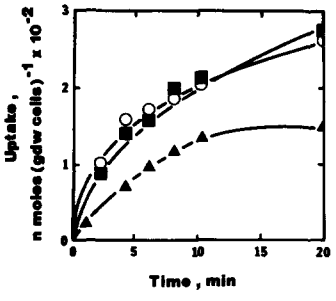

Fig. 3. Effects of thiamine and oxythiamine on [$^3H_3$]MAHMP uptake by *S. typhimurium*. Symbols: ○, no additions; ■, MAHMP + 0.1 mM thiamine; ▲, MAHMP + 0.1 mM oxythiamine.

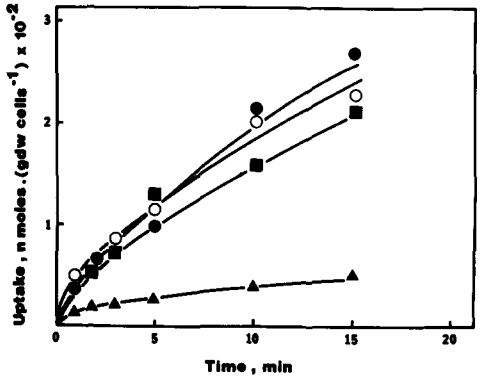

Fig. 4. Effects of various pyrimidine derivatives on [$^3H_3$]MAHMP uptake by *S. typhimurium*. Symbols: ●, no additions; ○, MAHMP+0.1 mM AHMP; ■, MAHMP+0.1 mM MAAMP; ▲, unlabelled MAHMP (0.1 mM) preincubated with cells for 5 min prior to addition of radioactive MAHMP. [$^3H_3$]MAHMP concentration was 0.1 mM.

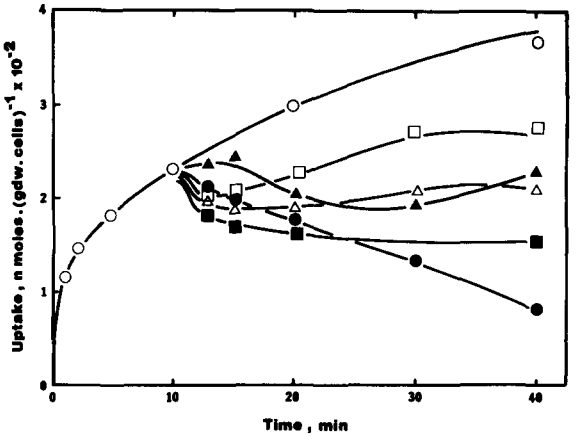

Fig. 5. Effects of inhibitors and uncouplers on [$^3H_3$]MAHMP uptake by *S. typhimurium*. Symbols: ○, no additions; □, sodium azide; △, sodium cyanide; ▲ 2,4-dinitrophenol; ■, *N*-ethylmaleimide; ●, carbonyl cyanide *m*-chlorophenylhydrazone. Test compounds were added after 10 min incubation to give final concentrations of 1 mM; [$^3H_3$]MAHMP concentration was 0.1 mM.

Effects of inhibitors and uncouplers

Fig. 5 shows the results obtained when various inhibitors and uncouplers were added to the assay mixtures after 10 min. In each case there was a sharp curtailment of uptake and moreover CCCP caused the efflux of MAHMP from the cells. These results, together with the dependence of uptake on the presence of glucose as an energy source are consistent with the presence of an active transport system for MAHMP in *S. typhimurium*.

Effects of temperature and pH

The pH dependence of MAHMP uptake showed a maximum at 6.8 but this was a broad rather than a sharply-defined optimum. No uptake was observed when the experiments were conducted at 4°C.

Discussion

The results described in this paper indicate that *S. typhimurium* possesses an active transport system for MAHMP. It is dependent on the presence of an energy source, glucose, and shows saturation kinetics. Transport is curtailed by phosphorylation uncouplers and inhibitors, and by *N*-ethylmaleimide. It has previously been shown that this

organism possesses an active transport system for thiamine [5] as does *E. coli* [1,2]. The thiazole moiety (MHET), however, is taken up in *S. typhimurium* by a passive rather than by an active system. Thiazole uptake was rapid only in the presence of MAHMP and glucose and under these conditions data indicative of an active transport system were obtained [5]. It is now clear that the effects seen in those experiments were a function of the MAHMP uptake and it was the MAHMP uptake system that was perturbed. Without the transport of MAHMP, MHET is not itself actively transported. Moreover, the K_m value obtained for MAHMP uptake, 0.07 μM, correlates well with the observed concentration range (0.01 to 1.0 μM) for effective stimulation of MHET uptake by MAHMP in this organism [5].

In contrast to this, MAHMP uptake was found to be independent of the presence of MHET. Thiamine does not inhibit the uptake of MAHMP nor MHET, whereas oxythiamine slightly inhibits MAHMP uptake but not MHET uptake. Since MHET uptake is dependent upon simultaneous MAHMP uptake this finding was surprising. It could be that even in the presence of oxythiamine, sufficient MAHMP is still transported to allow MHET uptake. Additionally, uptake of neither moiety appears to be regulated directly by the levels of thiamine in the cell. The transport system for MAHMP appears to be very specific since no inhibition was observed with the structurally similar pyrimidines AHMP and MAAMP, nor with thiamine. This also shows that MAHMP is not transported by the thiamine system. Thus, *S. typhimurium* possesses independent active transport systems for thiamine and its pyrimidine moiety, but not for its thiazole moiety. The active transport of the latter is solely dependent on the presence and transport of MAHMP. Of course, the possibility also exists that MHET and MAHMP are co-transported, with MHET transport dependent on simultaneous MAHMP transport but not vice versa. The reason why there should exist an independent active transport system for one moiety and not for the other can perhaps be explained as follows. Newell and Tucker [10] showed that MHET synthesis is regulated by feedback, whereas MAHMP does not regulate its own synthesis. Purines can prevent MAHMP synthesis by exerting an effect on aminoimidazole ribotide biosynthesis [11,12] and if MAHMP synthesis is reduced in this manner, then MHET synthesis also would need to be slowed. Likewise for transport; there is an active transport system for MAHMP because its synthesis could be irregular. If it is taken up, it can be immediately used for thiamine synthesis since MHET can be then synthesized in like amounts. An independent active transport system for MHET is unnecessary, since there may not be a sufficient supply of MAHMP with which to react, and moreover, high levels of MHET could be toxic to cells.

Acknowledgements

This work was supported by grant GM 24804 from the National Institute of General Medical Sciences, Grant Y-488 from the Robert A. Welch Foundation, Houston, TX, and by the University of Texas at Arlington Organized Research Fund.

References

1 Kawasaki, T., Miyata, I., Esaki, K. and Nose, Y. (1969) Arch. Biochem. Biophys. 131, 223–230
2 Kawasaki, T., Miyata, I. and Nose, Y. (1969) Arch. Biochem. Biophys. 131, 231–237
3 Kawasaki, T. and Esaki, K. (1971) Arch. Biochem. Biophys. 142, 163–169
4 Yamasaki, H., Sanemori, H., Yamada, K. and Kawasaki, T. (1973) J. Bacteriol. 116, 1280–1286
5 Bellion, E., Lash, T.D. and McKellar, B.R. (1983) Biochim. Biophys. Acta 735, 331–336
6 Neal, R.A. (1968) J. Biol. Chem. 243, 4634–4640
7 Baddiley, J., Lythgoe, B. and Todd, A.R. (1943) J. Chem. Soc., 386
8 Diorio, A.F. and Lewin, L.M. (1968) J. Biol. Chem. 243, 4006–4012
9 White, R.H. (1981) Science 214, 797–798
10 Newell, P.C. and Tucker, R.G. (1966) Biochem. J. 100, 517–524
11 Newell, P.C. and Tucker, R.G. (1968) Biochem. J. 106, 279–287
12 Moyed, H.S. (1964) J. Bacteriol. 88, 1024–1029