BIOSYNTHESIS OF L-METHIONINE IN *Corynebacterium glutamicum* X300

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Enzymes leading to the methylation of homocysteine to produce L-methionine in the mutant *Corynebacterium glutamicum* X300 were investigated in this present study. S-adenosyl methionine served as a methyl group donor to homocysteine to form L-methionine. The enzymatic pathway examined in this present study was cobalamine-independent pathway. No methylcobalamin homocysteine transmethylase activity was detected in this microorganism.

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Introduction

Trials for microbial production of L-methionine were initiated in 1970s in Japan using *Corynebacterium glutamicum*.[1,2] Cystathione, an intermediate of biosynthesis of L-methionine in microorganism follw two alternative pathways, namely: (1) trans-sulfuration pathway and (2) direct sulfhydration pathway.[3,4] Several reports are available on the microbial synthesis of L-methionine.[3,5,6] In our present investigation, we are intended to investigate the enzymatic pathway for L-methionine production in the mutant *Corynebacterium glutamicumX300.*

Materials and methods

Selection of microorganism: A regulatory mutant *Corynebacterium glutamicum X1* (accumulated only 0.6 mg mL^{-1} L-methionine) developed in our laboratory from its parent strain *Corynebacterium glutamicum* (basically a L-glutamic acid producing bacterium which does not accumulate L-methionine) which was isolated from North Bengal soil was subjected for mutational study.[7]

Optimum cultural conditions: Volume of medium, 25 ml; initial pH, 7.0; shaker's speed, 150 rpm; age of inoculum, 48 h; optimum cell density, $4.0x10^8$ cells mL^{-1}; temperature, 28 °C and period of incubation, 72 h.[8]

Composition of basal salt medium for L-methionine fermentation: L-methionine production was carried out using the following basal salt medium (per litre): glucose, 60 g; $(NH_4)_2SO_4$, 1.5 g; K_2HPO_4, 1.4 g; $MgSO_4 \cdot 7H_2O$, 0.9 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; biotin, 60 µg.[9]

Composition of synthetic medium (per liter): glucose, 100 g; $(NH_4)_2SO_4$, 8.0 g (in terms of nitrogen); K_2HPO_4, 2.2 g; $MgSO_4.7H_2O$, 1.5 g; $FeSO_4.7H_2O$, 0.03 g; KH_2PO_4, 2.0 g; $ZnSO_4.7H_2O$, 1.6 mg; $CaCO_3$, 1.5 g; $Na_2MoO_4.2H_2O$, 5.0 mg; $MnSO_4.4H_2O$, 2.5 mg; biotin, 80 mg and thiamine-HCl, 70 µg.[10]

Analysis of L-methionine: Descending paper chromatography was employed for detection of L-methionine in culture broth and was run for 18 hours on Whatman No.1 Chromatographic paper. Solvent system used includes n-butanol: acetic acid: water (2:1:1). The spot was visualized by spraying with a solution of 0.2 % ninhydrin in acetone and quantitative estimation of L-methionine in the suspension was done using colorimetric method.[11]

Preparation of cell free extract for enzymatic assay: Freshly harvested cells of *Corynebacterium glutamicum* X300 was suspended in 20 mM potassium phosphate buffer (pH 8.0) containing 2-mercaptoethanol (7 mM) and was ruptured by two passages through a Fresnch pressure cell using a pressure of 7800 lb in^{-2} (53.8 MN m^{-2}) as described by French and Milner (1955).[12] The crude extract was then centrifuged at 30,000 rpm for 15 minutes and the supernatant was used as a source of enzymes. The protein content was spectrophotometrically determined by the method as described by Layne.[13] The estimation of cobalamin and enzymes involved in L-methionine biosynthesis and S-adenosylmethionine as described by Salem *et al*.[14]

Statistical analysis: All the data were expressed as mean ± SEM, where *n*=6.

All the chemicals used in this study were analytical grade (AR) grade and obtained from E mark. Borosil glass goods and triple distilled water used throughout the study.

Results and Discussion

Blakley reported the methylation of homocysteine for L-methionine production in *Escherichia coli.*[15] Serine or glycine may serve as a methyl donor for L-methionine production.[16] Guest *et al*. and Whitfield *et al*. claimed that an Mg^{2+} (or Mn^{2+}) dependent transmethylase catalyze the

transfer of methyl group from a conjugated 5-methyl tetra hydrofolate to homocysteine.[17,18] Guest *et al*. also reported that $CH_3H_4PteGlu_3$ or 5-$CH_3H_4PteGlu_1$ may donate methyl group to homoserine only in presence of cobalamin in the medium.[17]. The cobalamin content of the mutant *Corynebacterium glutamicum X300* was measured by the method as described by Foster *et al.*[19] The mutant cell contained 827.3±1.618 ng of cobalamin g^{-1} dry cell weight. The methyl group donor for homocysteine was serine in the mutant when $H_4Pteglu_3$ was added as folate coenzyme. The comparatively low activity of $H_4Pteglu_1$ proved that at least one reaction in the methionine synthesis was specific for polyglutamate folate. The activities of 5,10-methylenetetrahydrofolate reductase and 5-methyltetrahydrofolate homocysteine transmethylase were extensively investigated in *Corynebacterium glutamicum X300* for the conversion of serine and homocysteine into L-methionine. The production of L-methionine was stimulated in this organism on addition of $H_4Pteglu_3$ or $H_4Pteglu_1$ (Table 1), suggesting thereby the common occurrence of 5,10-methylenetetrahydrofolate homocysteine transmethylase in the supernatant was indicated by improved L-methionine accumulation. But this activity requires a polyglutamate folate.

Table 1. 5,10-methylene tetrahydrofolate reductase and 5-methyltetrahydrofolate homocysteine transmethylase activity in *Corynebacterium glutamicum* X300

Source of folate	L-methionine (nmol)
$H_4Pteglu_1$	21.6±0.913
$H_4Pteglu_3$	43.2±0.883

Values were expressed as mean±SEM , where n=6. Each sample contained 4.8 mg of protein which were incubated in a vial containing serine and homocysteine as suggested by Salem *et al*.[14] Incubations were carried out at 28 °C for 60 min under H_2. The endogenous folates were removed by passing through the columns of Dowex 1 resin (Cl^- form)].

The transmethylase activity in extracts was examined by using 5-[^{14}C]methyltetrahydrofolate as a methyl group donor to homocysteine. 5-$CH_3H_4PteGlu_3$. Transmethylation from 5-$CH_3H_4PteGlu_1$ was not initiated by incubating under H_2 even though the addition of different cofactors like S-adenosylcobalamin and reductase system (H) was resulted.

Table 2. 5-$CH_3H_4PteGlu_3$-homocysteine transmethylase activity in *Corynebacterium glutamicum* X300

Nature of folate compound	L-methionine (nmol h^{-1} mg^{-1} of protein)
5-$^{14}CH_3H_4PteGlu_1$	21.4±1.136
5-$^{14}CH_3H_4PteGlu_3$	45.8±0.981

Values were expressed as mean± SEM , where *n*=6. Cell extracts (8 mg of protein) were incubated at 28 °C for 1 h in a mixture containing $MgSO_4.7H_2O$ (5 mM), DL-homocysteine (25 mM) and either 5-$^{14}CH_3H_4PteGlu_1$ or 5-$^{14}CH_3H_4PteGlu_3$ (2 mM; 0.7 µci µmol^{-1}), S-adenosylmethionine (10 µM), FAD (100 nmol), NAD (100 nmol), ethanol (100 nmol), $H_4PteGlu_3$ (10mM) and alcohol dehydrogenase 100 µg.

Extract was tested for the ability to use methylcobalamin as a methyl group donor to homocysteine. No methylcobalamin homocysteine transmethylase activity was detected in this microorganism. S-adenosylmethionine was also tested to examine its ability to donate methyl group to homocysteine for L-methionine biosynthesis in this mutant. Production was increased 16nmol h^{-1} mg^{-1} of protein with S-adenosylmethionine, suggesting thereby it was considered as a methyl group donor to homocysteine for L-methionine biosynthesis. Shapiro in *Aerobacter aerogenes*, Balish and Shapiro, Mardon and Balish in *Candida albicans*, Shapiro, Shipiro *et al*. and Botsford and Parks in *Saccharomyces cerevisiae* reported similar pattern of methyl group transfer.[1-4,8,20] Thus, synthesis of L-methionine occurs by transmethylation to homocysteine from a polyglutamate folate. 5,10-methylene tetrahydrofolate reductase transferred methyl group from either a conjugated folate or monoglutamate folate. From this present study, it can be tentatively concluded that the mutant used S-adenosylmethionine as a methyl group donor to homocysteine to form L-methionine. The enzymatic pathway examined in this present study was cobalamine-independent pathway in this mutant similar to *E.coli* as suggested by Woods *et al*.[9]

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