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Elucidation of enzymatic function encoded by *folD* and *fic* genes from dimethylglycine operon

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Analysis of the dimethylglycine oxidase encoding operon from *Arthrobacter globiformis* showed the presence of genes encoding putative 5-formiminotetrahydrofolate cyclodeaminase (*fic*) and 5,10-methylenetetrahydrofolate dehydrogenase/5,10-methenyltetrahydrofolate cyclohydrolase (*folD*). Both genes were cloned and expressed in *Escherichia coli* cells. *Fic* and *folD* genes were found to encode a monofunctional 5,10-methenyl-THF cyclohydrolase and a monofunctional NADP-dependent 5,10-methylene-THF dehydrogenase, respectively.

Key words: dimethylglycine oxidase operon, 5,10-methylenetetrahydrofolate dehydrogenase, 5,10-methenyltetrahydrofolate cyclohydrolase, *Arthrobacter globiformis*

INTRODUCTION

Glycine betaine is accumulated as a compatible solute in many bacteria [1–4]. This compound can be used by microorganisms as a carbon and nitrogen source [5, 6]. In the first catabolic step betaine is converted by betaine–homocysteine transmethylase to dimethylglycine [7]. Dimethylglycine dehydrogenase (or oxidase) further converts dimethylglycine into sarcosine, and sarcosine dehydrogenase oxidises sarcosine into glycine [8–10]. Corynebacterial sarcosine oxidase, as well as mammalian sarcosine dehydrogenase, enzymes that exhibit many similarities with dimethylglycine dehydrogenase, have been shown to bind tetrahydrofolate (THF) and in the presence of THF form 5,10-methylene THF as a second product of sarcosine oxidation [11, 12]. 5,10-methylene-THF and 10-formyl-THF are important intermediates in cellular pools of one-carbon units for catabolic and anabolic processes such as purine and thymidine biosynthesis. Interconversion between these forms is catalyzed by two enzymes – 5,10-methylene-THF dehydrogenase and 5,10-methenyl-THF cyclohydrolase. The first enzyme participates in the following reaction [13]:

5,10-methylene-THF + $NAD(P)^+$ ↔ 5,10-methenyl-THF^+ + NAD(P)H.

NAD(P)-dependent activity has been for the first time detected in a vertebrate liver preparation [14]. Later such monofunctional dehydrogenase was purified from *Clostridium cylindrosporum* [15]. It has been found that 5,10-methylene-THF dehydrogenase from some organisms functions as a bifunctional protein exhibiting activity of 5,10-methenyl-THF cyclohydrolase:

5,10-methenyl-THF^+ + H_2O ↔ 10-formyl-THF + + H^+.

The bifunctional form was isolated from *Clostridium thermoaceticum* [16] and *Escherichia coli* [17]. In eukaryotes [18, 19] the 5,10-methylene-THF dehydrogenase is the part of a trifunctional enzyme, called C1-THF synthase, that also exhibits 10-formyl-THF synthetase activity:

$HCOO^-$ + $MgATP^{2-}$ + THF ↔ 10-formyl THF + $MgADP^-$ + HPO_4^{2-}.

Folates are supposedly involved in the catabolism of glycine betaine, since the genes involved in C1-folate pathways are localized in sarcosine oxidase or dimethylglycine oxidase operons [20, 21]. The latter operon, cloned from *Arthrobacter globiformis*, contains *folD* and *fic* genes localized downstream of the dimethylglycine oxidase gene (*dmg*). Judging by the sequence similarity at the amino acid level, those genes encode putative 5,10-methylene-THF dehydrogenase/5,10-methenyl-THF cyclohydrolase and 5-formimino-THF cyclohydrolase (or 5,10-methenyl-THF cyclohydrolase), respectively [21].

The aim of this work was to clone the *folD* and *fic* genes, express them and elucidate the catalytic properties of proteins encoded by these genes.

MATERIALS AND METHODS

Cell strains, plasmids and growth conditions. *E. coli* DH5α and C41(DE3) were used for plasmid

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transformation and recombinant protein expression. Plasmid pEH1 harboring the entire *dmg* operon was described earlier [21]. Plasmids pTZ18R and pET15b were used for cloning experiments. Recombinant *E. coli* strains were grown on nutrient agar (Oxoid) containing ampicillin (50 μg/ml) at 37 °C or in a TB medium (g/l: tryptone 12, yeast extract 24, glycerol 4) containing 17 mM KH_2PO_4, 72 mM K_2HPO_4) and ampicillin (50 μg/ml) on a rotary shaker at 30 °C.

DNA manipulations. Restriction endonucleases, the linear DNA fragments isolation kit, T4 DNA ligase, Taq polimerase were purchased from MBI Fermentas (Lithuania) and used according to the manufacturer's instructions. Competent cells were prepared according to the method of Hanahan [22]. Standard recombinant DNA techniques were employed for transformation and isolation of plasmid DNA [23]. Electrophoresis of DNA fragments was carried out in 1% agarose gels in TAE buffer (pH8.3).

Subcloning of *folD* gene. For incorporation of *folD* into the expression vector pET15b, the gene was amplified by PCR using pEH1 as a DNA template. The reaction was carried out using the following primers: FOLPAG 5'-ACGGGAAAGGATCC TCTCATGACGGCATC-3' and FOLXHO 5'-ACCA GGCTCGAGCGCGGTCCAGTC-3'. The sense primer was designed with *Pag*I restriction site so that the amplified gene started with the ATG start codon and the antisense primer was designed with a *Xho*I restriction site. The PCR product (981 bp) was digested with *Pag*I and *Xho*I and ligated into the pET15b expression vector previously digested with *Xho*I and *Nco*I restriction enzymes. The resulting expression plasmid was named pFOL and maitained in *E. coli* DH5α. pFOL was then used to transform *E. coli* C41(DE3) for protein expression.

Subcloning of *fic* gene. DNA fragment containing *fic* gene was prepared by digestion of pEH1 plasmid with *Mbi*I and *Pst*I restriction enzymes, separation by electrophoresis and recovering of the 0.85 kb DNA fragment. The obtained fragment was inserted into pTZ18R, linearized with *Sma*I and *Pst*I restriction enzymes. The construct thus obtained was designated as pMP5 and used to transform *E. coli* DH5α.

Preparation of cell-free extract. Bacterial strains grown in TB media were harvested, washed with 0.9% NaCl, resuspended in 50 mM Tris-HCl buffer pH 7.5 and disrupted by sonication. Cell debris was removed by centrifugation at 14000 *g* for 10 min. The resulting cell-free extract was used to assay the enzymes.

Assay of 5,10-methenyl-THF cyclohydrolase activity. The assay was performed in 1 cm cuvettes in a total volume of 1 ml at 30 °C. 5,10-Methenyl-THF cyclohydrolase activity was determined by measuring the decrease in absorbance at 356 nm ($\varepsilon = 24.9\ mM^{-1}\ cm^{-1}$). The standard assay contained 2 M potassium maleate buffer pH 7.0, 0.1 M potassium hydroxide, 0.4 M 2-mercaptoetanol and 5,10-methenyl-THF in 0.12 N HCl (0.0073–0.0293 mM). The reaction was started by the addition of protein. The rate of spontaneous hydrolysis of 5,10-methenyl-THF was considered. One unit is defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of 5,10-methenyl-THF per minute [24].

Assay of 5,10-methylene-THF dehydrogenase activity. The assay was performed in 1 cm cuvettes in a total volume of 1 ml at 30 °C. The enzyme was assayed in the direction of NADPH and 5,10-methenyl-THF formation. Both products absorb at 356 nm, so the combined molar absorption coefficient $\varepsilon = 29.7\ mM^{-1}\ cm^{-1}$ was used for calculations. The standard assay mixture contained 1 M potassium maleate, pH 7.0, 12 mM NADP and 3.75 mM 5,10-methylen-THF. The reaction was started by the addition of protein. One unit of enzyme is defined as the amount that catalyzes the formation of 1 μmol NADPH and 5,10-methenyl THF per minute [24].

5,10-methenyl-THF and 5,10-methylene-THF were synthesised from tetrahydrofolate and 5-formyltetrahydrofolic acid respectively, as described previously [24, 25].

RESULTS AND DISCUSSION

Expression of the *fic* and *folD* genes. The *fic* and *folD* genes were subcloned from pEH1 into pTZ18R and pET15b vectors resulting in the pMP5 and pFOL plasmids, respectively, by the procedures described in Materials and Methods. Cell-free extracts of IPTG-induced *E. coli* (pMP5) and *E. coli* (pFOL) cells were subjected to SDS-PAGE to detect *fic* and *folD* gene products. In both cases a good expression of the recombinant protein was achieved according to the analysis (Fig. 1). The cell-free extract of *E. coli* DH5α harboring the recombinant plasmid pMP5 formed an additional protein band on SDS-PAGE in comparison with *E. coli* (pTZ18R) (Figure 1, lanes 1 and 2). The determined molecular mass of this band is 22 kDa, which is in agreement with the molecular mass calculated using the known sequence of the *fic* gene. In the cell-free extract of *E.coli* (pFOL) an additional protein was found. The cell-free extract of *E. coli* (pET15b) served as a control. The molecular mass (31 kDa) of this protein determined by SDS-PAGE is in fair agreement with the molecular mass calculated using the nucleotide sequence data of *folD* gene (Figure, lanes 4 and 5).

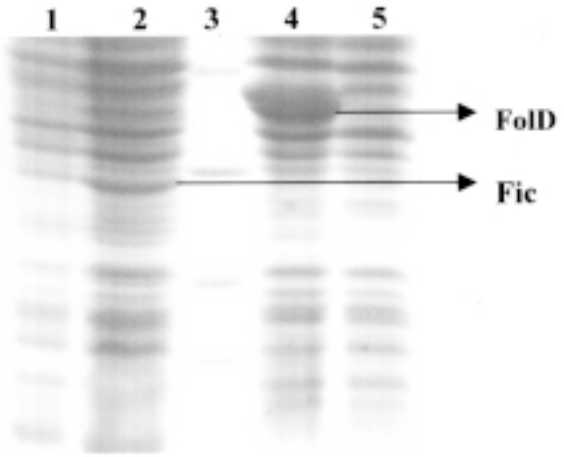

Figure. SDS-PAGE (14%) analysis of cell-free extracts. The cell-free extracts: 1 – *E. coli* (pTZ18R), 2 – *E. coli* (pMP5), 4 – *E. coli* (pFOL), 5 – *E. coli* (pET15b). 3 – molecular mass markers (35.0 kDa, 25.0 kDa, 18.4 kDa, 14.4 kDa). Recombinant proteins are marked by arrow

Elucidation of *folD* and *fic* gene functions. According to the BLASTA and FASTA analysis, the product of *fic* gene was most homologous to eukaryotic 5-formimino-THF cyclohydrolase [21] and the FchA protein from *Methylobacterium extorquens* AM1 [26], which exibits 5,10-methenyl-THF cyclohydrolase activity. The *folD* gene localized in the dimethylglycine oxidase operon from *Arthrobacter globiformis* showed a high homology to genes encoding a bifunctional 5,10-methylene-THF dehydrogenase/cyclohydrolase [21]. To elucidate the function of both proteins, the enzymatic activities of recombinant Fic and FolD proteins were measured in appropriate cell-free extracts. The cell-free extract of *E. coli* (pMP5) was found to catalyze the hydrolysis of 5,10-methenyl-THF to 5-formyl-THF with a specific activity of 0.018–0.159 U/mg of protein. No activity was found in the control cell-free extract of *E. coli* containing an empty pTZ18R vector. Furthermore, none of additional assays of folate-dependent activities related to Fic protein showed a positive result. Up to the recent time a similar monofunctional 5,10-methenyl-THF cyclohydrolase has been found only in the methylotrophic proteobacterium *Methylobacterium extorquens* AM1 [29].

A 5,10-methylen-THF dehydrogenase activity was detected in the *E. coli* (pFOL) cells. We found that the control cells produced this enzyme, but the specific activity (1.23 U/mg of protein) was approximately forty times lower as compared to the recombinant strain containing the *folD* gene (46.4 U/mg of protein). Moreover, the recombinant dehydrogenase did not exhibit 5,10-methenyl-THF cyclohydrolase activity. It could be concluded that this enzyme is a monofunctional enzyme. Dehydrogenase encoded by *folD* gene was specific for NADP instead of NAD. Most of prokaryotic 5,10-methylene-THF dehydrogenases as well as the mitochondrial enzyme (for example, from *Clostridium thermoaceticum*, *Escherichia coli*, *Photobacterium phosphoreum* [27]) are bifunctional and catalyze the cyclohydrolytic cleavage of 5,10-methenyl-THF, too. Among the enzymes that have been purified to homogeneity, monofunctional 5,10-methylene-THF dehydrogenases were from *Clostridium cylindrosporum* [15], *Peptostreptococus productus* [28], both specific for NADP, and from *C. formicoaceticum* [24], *A. woodii* [29], both NAD-dependent. It should be stressed that in contrast to *Arthrobacter globiformis* all these microorganisms are anaerobes.

The results of this study allow the following conclusions: *i*) the *fic* gene localized in the dimethylglycine oxidase operon from *Arthrobacter globiformis* encodes a monofunctional 5,10-methenyl-THF cyclohydrolase, *ii*) the *folD* gene localized in the same operon encodes a monofunctional and NADP-dependent 5,10-methylene-THF dehydrogenase.

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DIMETILGLICINO OPERONO *FOLD* IR *FIC* GENŲ KODUOJAMŲ BALTYMŲ FUNKCIJŲ TYRIMAS

S a n t r a u k a

Buvo tirtos dviejų dimetilglicino oksidazės operono iš *Arthrobacter globiformis* genų (*fic* ir *folD*) koduojamų baltymų katalitinės savybės. Tam tikslui šie genai buvo klonuoti atskirose plazmidėse. Nustatyta, kad rekombinantinėmis plazmidėmis transformuotos *Escherichia coli* ląstelės sintetino baltymus, kurių molekulinė masė atitiko teoriškai apskaičiuotą. Analizuojant fermentinį aktyvumą ląstelių ekstraktuose pastebėta, kad *fic* geno produktas pasižymi 5,10-meteniltetrahidrofolato ciklohidrolaziniu aktyvumu, o *folD* genas koduoja 5,10-metilentetrahidrofolato dehidrogenazę.