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Expression and purification of active, stabilized trimethyllysine hydroxylase

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ABSTRACT

Trimethyllysine hydroxylase (TMLH) catalyses the first step in carnitine biosynthesis – the conversion of N6,N6,N6-trimethyl-L-lysine to 3-hydroxy-N6,N6,N6-trimethyl-L-lysine. By changing carnitine availability it is possible to optimise cardiac energy metabolism, that is beneficial under certain ischemic conditions. Previous efforts have been devoted towards the inhibition of gamma-butyrobetaine dioxygenase, which catalyses the last step in carnitine biosynthesis. However, the effects of TMLH activity regulation are currently unexplored. To facilitate the development of specific ligands of TMLH, large quantities of recombinant protein are necessary for downstream binding and structural studies. Here, we describe an efficient system for expressing and purifying active and stable TMLH as a maltose-binding protein fusion in *Escherichia coli*.

Introduction

Carnitine is an important molecule in long-chain fatty acid transport across the inner membrane of mitochondria, where metabolic energy is produced by beta-oxidation. By changing the amount of available carnitine it is possible to optimise both glucose and, particularly, fatty acid utilization [1], that is beneficial under ischemic (low oxygen) conditions and improves the survival of cardiac muscle [2–4]. The two sources for bioavailable carnitine in humans are diet and biosynthesis. In organisms, carnitine is made through four successive reactions ([5], Fig. 1) from N6,N6,N6-trimethyl-L-lysine (TML),[1] which is a degradation product of proteins, particularly histones. TML is first hydroxylated by trimethyllysine hydroxylase (TMLH). The resulting 3-hydroxy-N6,N6,N6-trimethyl-L-lysine (HTML) is further converted to trimethylaminobutyraldehyde (TMABA) by hydroxytrimethyllysine aldolase (HTMLA). Subsequently, trimethylaminobutyraldehyde is oxidised to gamma-butyrobetaine (GBB) by trimethylaminobutyraldehyde dehydrogenase (TMABADH). Finally, GBB is hydroxylated by butyrobetaine dioxygenase (BBOX) to produce carnitine. In principle, inhibition of any of the mentioned steps would reduce the amount of bioavailable carnitine. The true identity of HTMLA has not been established, but the corresponding enzymatic activity has been demonstrated for both serine hydroxymethyltransferase and L-threonine aldolase [6,7], which are both involved in other important biochemical pathways in organisms; consequently, their inhibition might have undesirable effects. In humans, TMABADH appears to be the same enzyme as aldehyde dehydrogenase 9 [5]. Because the human genome encodes at least 19 different aldehyde dehydrogenases, it is probable that their activities are somewhat overlapping; therefore, specific inhibition of TMABADH might have little physiological effect. This leaves two viable steps of interaction – hydroxylation of TML and hydroxylation of GBB. Both reactions are carried out by evolutionarily related dioxygenases TMLH and BBOX, which display approximately 26% amino acid identity. Currently, one BBOX inhibitor – mildronate (3-(1,1,1-trimethylhydrazin-1-ium-2-yl)propanoate) is available in the market, and recently we have described several more efficient BBOX inhibitors [8]. However, the effects of TMLH inhibition are so far completely unexplored, and there is considerable interest towards the production of specific TMLH inhibitors. Therefore, there is a fundamental need for purified TMLH

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[1] *Abbreviations used:* TML, N6,N6,N6-trimethyl-L-lysine; TMLH, trimethyllysine hydroxylase; HTML, 3-hydroxy-N6,N6,N6-trimethyl-L-lysine; HTMLA, hydroxytrimethyllysine aldolase; TMABA, trimethylaminobutyraldehyde; TMABADH, trimethylaminobutyraldehyde dehydrogenase; GBB, gamma-butyrobetaine; BBOX, butyrobetaine dioxygenase; MBP, maltose-binding protein; MBP–TMLH, fusion protein of MBP and TMLH; PAAG, polyacrylamide gel; CBB, Coomassie Brilliant Blue; MTS, mitochondrial targeting sequence.

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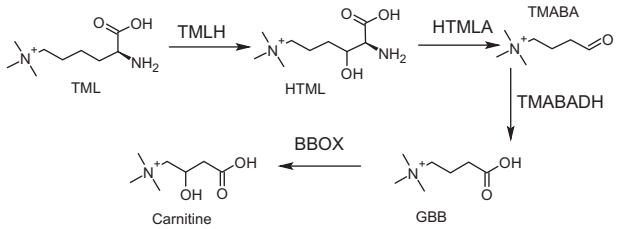

Fig. 1. Biosynthesis of carnitine.

enzyme for downstream inhibition and structural studies. Previously, we established an efficient BBOX expression system in *Saccharomyces cerevisiae* [8]. However, a similar strategy failed for TMLH and did not result in detectable amounts of produced protein. Here, we describe efficient production of TMLH as a fusion with maltose-binding protein (MBP) in the presence of the molecular chaperonins GroES/EL in a bacterial system.

Materials and methods

Design of constructs

For protein expression, we used the TMLH-a isoform (NCBI Reference Sequence: NP_060666.1), which lacks the N-terminal mitochondrial targeting sequence (MTS) and hydrophobic sequences. The TMLH-a gene with bacterially optimised codons was synthesised at GenScript. As a first step, the MBP-encoding gene *MalE* along with a N-terminally located 6xHis tag and C-terminally located rTEV protease cleavage site were cloned into the pETDuet-1 vector (Novagen) between the *Nco*I and *Bsp*TI restriction sites, resulting in the pETDt_MalE vector. Then, the TMLH-a gene was PCR-amplified using oligonucleotide primers Fw 5′-CTAGGCCTTCACCATACAGCCTCCAAGTC-3′ and Rv 5′-TTAGA TCTTAAGCCTGAAGCCCCAAG-3′, cleaved by *Eco*147I/*Bsp*TI (sites underlined) and cloned into pETDt_MalE using the same restriction sites (Fig. 2).

Expression conditions

The MBP–TMLH fusion gene was expressed in *Escherichia coli* strain BL21-AI (Invitrogen) following the manufacturer's protocol with minor modifications. Briefly, the cells were cultivated at 37 °C on a shaker at 200 rpm in 2-L Erlenmeyer flasks containing 400 mL of rich 2xTY medium until an OD_{590} of 0.6–0.8. For induction, 0.1 mM IPTG and 0.1% arabinose (w/v) were added, and cultivation was continued for another 4 h. Cells were then harvested by low-speed centrifugation and stored at −20 °C until use. Co-expression with molecular chaperonins was performed in BL21-AI cells harbouring a resident, ColEI replicon-compatible plasmid encoding the GroES/EL genes and conferring kanamycin resistance. The utilised antibiotic concentrations were 50 μg/mL and 30 μg/mL for ampicillin and kanamycin, respectively.

Protein purification

The cells were resuspended in lysis buffer LB (20 mM Tris–HCl, pH 8.0, 200 mM NaCl), at a proportion of 5 mL of buffer per 1 g of wet cells, and disrupted with a French press (3 cycles, 20,000 psi). The soluble fraction was separated by centrifugation for 30 min at 15,500×*g*, manually (with syringe) passed through a 1-mL HisTrap™ FF crude or MBPTrap™ HP column (GE Healthcare) and eluted with 2 mL of LB containing either 300 mM imidazole or 10 mM maltose, respectively. The eluted proteins were loaded on a Superdex™ 200 10/300 GL column connected to an ÄKTA chromatography system (Amersham Biosciences). The column was pre-equilibrated with LB and run at 0.5 mL/min. Peak fractions containing the MBP–TMLH protein were either used directly for activity assays or cleaved with rTEV protease in the presence of 1 mM DTT overnight at +4 °C. MBP and TMLH were separated on a Superdex™ 200 pg HiLoad 16/600 column in LB at 1 mL/min. All purification steps were monitored by sodium dodecyl sulphate polyacrylamide gel electrophoresis, with a 4% stacking and 15% separating polyacrylamide gel (PAAG), according to standard protocols. To visualise protein bands, the gels were stained with Coomassie Brilliant Blue (CBB) G-250.

TMLH activity determination

TMLH activity was assayed by measuring the formation of HTML from TML. The reaction mixture (final volume of 0.2 mL) contained the following: 20 mM potassium phosphate pH 7.0, 20 mM potassium chloride, 3 mM 2-oxoglutarate, 0.25 mM ferrous ammonium sulphate, 10 mM sodium ascorbate, 0.16 mg of catalase, 50–2000 μM TML and 0.5 μg of enzyme. The reaction was initiated by the addition of TML, and the mixture was incubated at 37 °C for 30 min. The reaction was stopped with 0.8 mL of ice-cold acetonitrile:methanol (1:3). Then, the mixture was spun at 20,000×*g* for 10 min at 4 °C. The supernatant was decanted and used for HTML measurements. The K_m and V_{max} values were determined by nonlinear regression fitting using a model derived from the Michaelis–Menten equation and the GraphPad Prism 3.0 software.

Determination of the HTML and TML concentrations

Determination of the HTML and TML was performed by ultra-performance liquid chromatography–tandem mass spectrometry (UPLC/MS/MS) in positive ion electrospray mode. Twenty microlitres of supernatant were transferred into 1.5-mL chromatographic glass vials, 50 μL of borate buffer (0.1 M, pH 8.1) were added, samples were briefly vortexed and 40 μL of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC, 3 mg/mL in acetonitrile) were added. The vials were tightly closed, vortexed and left for 1 min at room temperature. Then, the vials were put into the heat block for 10 min at 55 °C to complete the reaction. After heating, the vials were allowed to cool to room temperature, 700 μL of

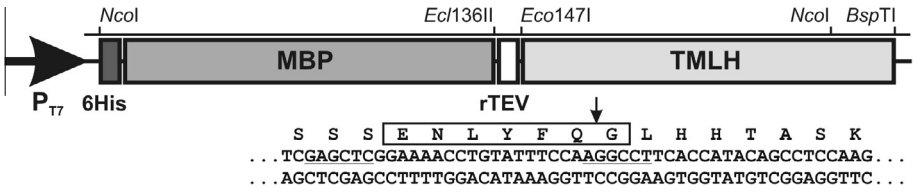

Fig. 2. Design of MBP–TMLH fusion construct. Schematic presentation of MBP–TMLH expression cassette within plasmid pETDt_MalE_TMLH. More detailed picture of MBP–TMLH fusion region is depicted below. rTEV amino acid recognition sequence is boxed and its cleavage site indicated by arrow. Unique restriction sites *Ecl*136II and *Eco*147I are underlined.