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L. L. Goh · R. Koh · P. Loke · T. S. Sim

Thermostable malate synthase of *Streptomyces thermovulgaris*

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Abstract The gene, encoding malate synthase (MS), *aceB*, was cloned from the thermophilic bacterium *Streptomyces thermovulgaris* by homology-based PCR. The 1,626-bp cloned fragment encodes a protein consisting of 541 amino acids. *S. thermovulgaris* malate synthase (stMS) gene was over-expressed in *Escherichia coli* using a glutathione-S transferase (GST) fusion vector (pGEX-6P-1), purified by affinity chromatography, and subsequently cleaved from its GST fusion partner. The purified stMS was characterized and compared to a mesophilic malate synthase (scMS) from *Streptomyces coelicolor*. stMS exhibited higher temperature optima (40–60 °C) than those of scMS (28–37 °C). It was more thermostable and very resistant to the chemical denaturant urea. Amino acid sequence comparison of stMS with four mesophilic streptomycete MSs indicated that they share 70.9–91.4% amino acid identities, with stMS possessing slightly more charged residues (~31%) than its mesophilic counterparts (~28–29%). Seven charged residues (E85, R187, R209, H239, H364, R382 and K520) that were unique to stMS may be selectively and strategically placed to support its peculiar characteristics.

Keywords Malate synthase · *Streptomyces thermovulgaris* · *Streptomyces coelicolor* · Thermoactivity · Thermostablility

L. L. Goh · R. Koh · T. S. Sim (✉)
Department of Microbiology, Faculty of Medicine,
National University of Singapore, 5 Science Drive 2,
MD4A, 117597, Singapore
E-mail: micsimts@nus.edu.sg
Tel.: +65-68743280
Fax: +65-67766872

P. Loke
The Burnet Institute, Commercial Road, 3004
Melbourne, Victoria, Australia

Introduction

Malate synthase (EC 4.1.3.2) catalyzes the formation of malate from the aldol-condensation of glyoxylate and acetyl-CoA [15] in a reaction with Mg^{2+} as a cofactor. It is an essential enzyme of the glyoxylate cycle, providing a metabolic pathway, together with isocitrate lyase (EC 4.1.3.1), by which organisms can synthesize carbohydrates from two-carbon-unit compounds such as acetate and fatty acid [7].

Glyoxylate cycle enzymes are widely distributed in all three domains of living organisms, Eukarya, Bacteria, and Archaea. They appear to be rather diverse amongst most life forms and may have several important physiological implications. In microorganisms, the glyoxylate cycle mainly operates when the principal carbon source is a two-carbon compound. More recently, the implication of this pathway in the persistence of pathogens such as *Mycobacterium tuberculosis* and *Candida albicans* under stress conditions has been pointed out [13, 12]. In streptomycetes, the existence of the glyoxylate cycle has been suggested, although the physiological function is less known [1, 5, 9, 10].

Although MSs has been studied in many species, only two thermostable MSs from thermophilic *Bacillus* and *Sulfolobus acidocaldarius* [18, 20] have been reported so far. However, the full sequences of MS from these organisms have not been described. It is envisaged that the clarification of the molecular basis of protein stability will aid in the rational design of enzymes that can work efficiently at high temperatures. A comparison of the gene structure and function relationship of MSs from thermophilic and mesophilic streptomycetes was carried out in this study. Accordingly, *aceB* from *Streptomyces thermovulgaris* was cloned, sequenced, and expressed in *Escherichia coli* to provide the source of thermostable enzymes. The unique differences in the amino acid residues of stMS relative to the other mesophilic enzymes may contribute to its thermal characteristics, and warrant further investigation.

Materials and methods

Cloning and sequencing of stMS

S. thermovulgaris JCM 4240 was grown in tryptone soya broth (Oxoid) at 45 °C and its genomic DNA was isolated using the method of Hopwood et al. [4]. A pair of consensus primers was designed based on the 5′ and 3′ sequences of MS ORFs from streptomycetes and used to amplify *aceB* of *S. thermovulgaris*. To enable subsequent subcloning into the GST fusion vector, the restriction enzyme sites for *Bam*HI and *Eco*RI (italics) were incorporated into the forward (5′ GTGA*GGATCC*ATGTCCGC ACCAGCGCCGTC 3′) and reverse primers (5′ *GAATTC*TCA GCCCVTSAGCTGCTCGTACGC 3′) (S = G + C; V = C + A + G), respectively. PCR cloning was done using the touchdown amplification profile of eight cycles of 95 °C (30 s), 64–60 °C (30 s), 72 °C (4 min), followed by 27 cycles of 95 °C (30 s), 64 °C (30 s), 72 °C (4 min) and a final extension step at 72 °C for 5 min. A negative control omitting genomic DNA was included.

The amplified blunt-end product was cloned directly into the cloning vector pCR-BluntII-TOPO according to the manufacturer's instructions (Invitrogen) prior to transformation into *E. coli* TOP10. The recombinant plasmid construct was extracted using the Wizard *Plus* SV Minipreps DNA Purification System (Promega) and sequenced twice on both strands to ascertain its identity.

Expression and purification of recombinant stMS

Utilizing appropriate restriction enzyme digestions, *aceB* was excised from the recombinant pCR-BluntII-TOPO construct and gel-purified using the Qiagen MinElute gel extraction kit according to the manufacturer's instructions. The purified product was then subcloned into the similarly digested pGEX-6P-1 expression vector (Amersham Pharmacia Biotech), and transformed into *E. coli* BL21(DE3) (Novagen). The resultant plasmid was designated pstMS1 and resequenced to confirm its frame of insertion. Expression of the pstMS1 construct and preparation of cell-free extracts were done as previously reported [11]. Induction was carried out at 25 °C for 15 h with a final isopropyl-β-D-thiogalactopyranoside (IPTG) concentration of 1 mM. *aceB* of *S. coelicolor* previously cloned into pGEX-6P-1 was also expressed for subsequent comparative enzymatic analysis [11]. The soluble protein fractions were purified by affinity chromatography using Glutathione Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The MS proteins were subsequently cleaved from the GST fusion partner with an appropriate amount of PreScission Protease (Amersham Pharmacia Biotech) at 4 °C for approximately 16 h. The crude cell-extracts and purified proteins were analyzed by SDS-PAGE.

Enzymatic analyses and protein quantitation

Malate synthase activity assays were based on the method of Ornston and Ornston [15]. The reaction was initiated by the addition of purified enzyme into an assay mixture containing 10 μM $MgCl_2$, 0.2 μM acetyl-CoA, 10 μM glyoxylate, and 100 μM Tris-HCl buffer (pH 8), in a total volume of 0.9 ml. After 10 min, the reactions of stMS and scMS were terminated by adding 2 ml 4 M guanidine HCl and 6 M urea, respectively. Color development was facilitated by the addition of 0.1 ml 10 mM 5,5′-dithio-bis-(2-nitrobenzoic acid) (DNB), followed by absorbance determination at 412 nm after 10 min. An increment of 4.53 absorbance units at this wavelength corresponded to the release of 1 μmol CoA per 3-ml reaction mixture.

To determine the optimum temperature for enzyme activity, the MS assay was carried out at different temperatures, ranging from 4 to 75 °C. The assay mixtures were incubated at the indicated temperatures for 5 min prior to initiation of the reaction. At various time intervals, aliquots were taken and assayed for their residual activity.

Protein concentrations were determined using the Bradford reagent (Bio-Rad), with bovine serum albumin (Sigma) as the standard. All experiments were done in triplicate and repeated, with the average values taken.

Computational analyses

The G + C composition of the nucleotide sequence obtained was analyzed by the FramePlot program [6]. The protein sequences were determined using the software package accessed under the ExPASy molecular biology server on the Web. Protein secondary structure was predicted using the self-optimized prediction method (SOPM) [2]. Amino acid sequence alignment of MS was carried out using the ClustalW multiple sequence alignment program (version 1.7) [19]. The accession numbers of other sequences used in this study are as follows: *S. coelicolor*, AAG29597; *S. clavuligerus*, AAC83648; *S. griseus*, AF337556; *S. arenae*, U63518.

Results

Cloning, heterologous expression, and purification of stMS

stMS *aceB* was cloned by homology-based PCR. The entire sequence encoding stMS was determined for both strands and deposited in GenBank under the accession number AF489515. The gene consists of 1,626 bp and encodes a 541 amino acid protein with a calculated mass of 60.9 kDa. This value is comparable to the molecular mass (62–64 kDa) of most class A malate synthases, which are in the range of 60–65 kDa [14]. The overall G + C content of the coding region is 70.8% with 97.3% GC bias in the third codon position, which is in agreement with the G + C content of streptomycete genes.

S. thermovulgaris aceB was subcloned into pGEX-6P-1 for heterologous expression in *E. coli* BL21(DE3). Upon induction with IPTG, stMS protein was overexpressed in the soluble fraction by the recombinant construct pstMS1 (Fig. 1, lane 4). The mobility of the GST-stMS fusion protein was similar to that of GST-scMS. The expressed MS proteins were cleaved by PreScission protease from the GST fusion partner, and purified by affinity separation through a column of Glutathione Sepharose 4B. Figure 1 shows the purified MS proteins separated on a 8% SDS polyacrylamide gel, which revealed single bands with a molecular mass of approximately 60 kDa.

Effects of temperature and chemical denaturant on enzyme activities and stabilities

Experiments to compare the enzymatic profiles of stMS with those of a mesophilic homologue, scMS, were carried out. First, the temperature optima were examined by comparing activity at various temperatures. Figure 2A shows that stMS was thermoactive within the

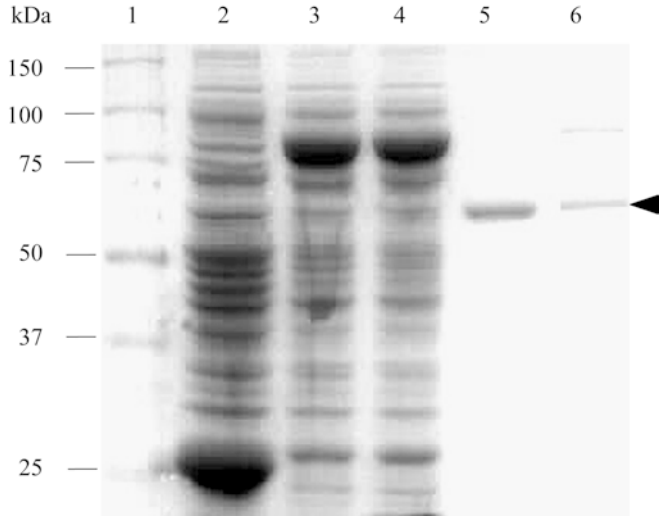

Fig. 1 SDS-PAGE analysis of the soluble and purified protein fractions of streptomycete malate synthase (MSs). *Lane 1* Molecular mass markers, *lane 2 Escherichia coli* BL21(DE3)/pGEX-6P-1 (non-recombinant), *lane 3* pGEX-scMS (fusion GST-*Streptomyces coelicolor* MS), *lane 4*, pGEX-stMS (fusion GST-*Streptomyces thermovulgaris* MS), *lane 5* purified scMS (MS from *S. coelicolor)*, *lane 6* purified stMS (MS from *S. thermovulgaris*). *Arrowhead* Position of the purified MS protein

range of 40–60 °C, with an apparent optimum temperature of 55 °C. Rapid reduction of stMS activity occurred at temperatures higher than 60 °C.

Next, the thermostabilities of both streptomycete MSs were determined by incubating stMS and scMS at 55 and 37 °C, respectively. Although stMS was progressively inactivated, it retained about 80% of its initial activity after a 20-min incubation at 55 °C. Thermal inactivation of scMS occurred more rapidly since more than 50% of its activity was lost after a 20-min incubation at 37 °C (Fig. 2B).

stMS is resistant to the chemical denaturant urea at high concentrations (up to 6 M, data not shown). Hence, a stronger denaturant, guanidine HCl, was used in the assay for stMS activity. Aliquots of stMS incubated with several different concentrations of guanidine HCl for 10 min were assayed for residual activity at 55 °C. As seen in Fig. 2C, over 50% of the activity was retained at 0.2 M guanidine HCl, and complete inactivation of stMS was achieved at 1.0 M denaturant.

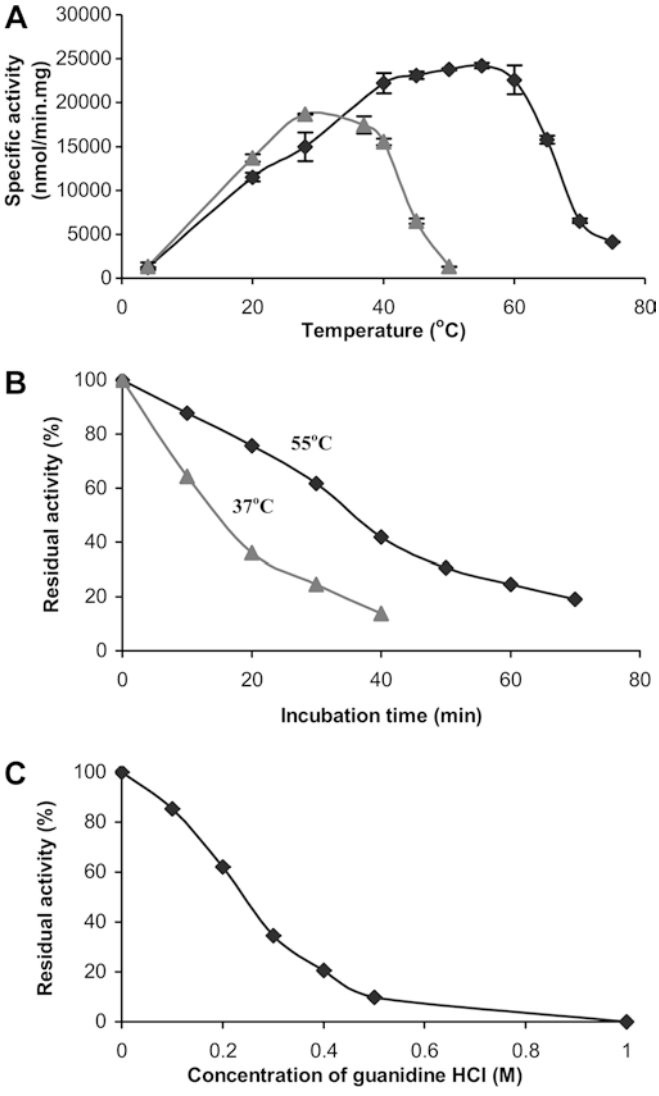

Fig. 2A–C Enzymatic analysis of streptomycete MSs. **A** Determination of optimum temperature. **B** Thermal denaturation of stMS and scMS at 55 and 37 °C, respectively. **C** Activity of stMS in the presence of guanidine HCl. ♦ stMS, △ scMS

Amino acid sequence comparison of stMS with those of MSs from mesophilic *Streptomyces* species

Amino acid sequence comparison of stMS with four mesophilic MSs from *S. coelicolor*, *Streptomyces clavuligerus*, *Streptomyces arenae*, and *Streptomyces griseus* [9, 1, 5, 10] revealed high sequence similarities (70.7–91.4% identity). Further computational analyses revealed that the thermophilic and mesophilic MSs show similar predicted secondary structures and hydrophobicity profiles (data not shown). The overall amino acid composition of stMS is also similar to those of the mesophilic enzymes. Despite the high homologies, it was noted that the frequency of the thermolabile amino acid residue methionine (1.3%) is lower in stMS than in the mesophilic MSs (1.5–1.7%). Furthermore, it is apparent that compared to its mesophilic counterparts stMS has the highest proportion of charged residues (30.9% vs 27.6–28.7%). Amino acid sequence alignment revealed seven charged residues (E85, R187, R209, H239, H364, R382 and K520) unique to the thermophilic stMS.

Discussion

In this study, the enzymatic characteristics and amino acid sequence analyses of MS from the thermophilic actinomycete *S. thermovulgaris* are reported. The high

activity exhibited by stMS supports the functionality of the cloned gene and suggests the likely existence of the glyoxylate pathway in *S. thermovulgaris*. Enzymatic analyses also revealed different thermal characteristics between stMS and its mesophilic counterpart, scMS. Apart from displaying higher temperature optima, a course of heat inactivation experiments showed that the resistance to thermal denaturation of stMS also surpassed that of scMS. Thus, stMS is not only more thermoactive but is also more thermostable than scMS. This supports the notion that the innate thermal properties of proteins from thermophilic microorganisms are greater than those of their counterparts from mesophilic organisms.

To explore the various factors that might contribute to the thermal properties of stMS, the amino acid sequences and secondary structures of stMS and four mesophilic streptomycete MSs were compared using computational analyses. The results indicated that the MSs share close sequence and structural similarities. However, some differences in the amino acid distribution were observed and these may correlate with the specialized properties of stMS. For example, in stMS, the thermolabile methionine residue has the lowest frequency of occurrence. This is in good agreement with the study by Russell et al. [17] which showed that the lower occurrence of thermolabile residues appears to stabilize some thermophilic proteins. Also, there appears to be a tendency towards a higher content of charged residues in stMS, seven of which (E85, R187, R209, H239, H364, R382, and K520) were unique to the thermophilic enzyme. These charged residues may be involved in the formation of salt bridges and electrostatic interactions, which have been found to increase the stability of some thermophilic proteins [8, 21]. Mutagenic studies are needed to determine whether these charged residues are strategically located to support the enzyme's thermoactivity and thermostability.

The predicted secondary structures and hydrophobicity profiles of the thermophilic and mesophilic MSs were compared, since it has been suggested that thermophilic proteins have a higher content of helical regions and are substantially more hydrophobic [16, 3]. In this study, no obvious trend that could contribute to the thermal properties of stMS was observed due to the similar secondary conformations and hydrophobicity profiles of all the MSs.

In conclusion, MS from *S. thermovulgaris* was cloned and characterized. This enzyme was closely related to other streptomycete MSs in terms of sequence and function, but was different in its resistance to thermal and chemical denaturation. This is probably due to the unique differences in the amino acid residues of stMS relative to the other four mesophilic enzymes analyzed in this study. If so, this may serve as a model to study the bonding network of thermophilic MS.

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