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Engineering Recombinant *Streptomyces coelicolor* Malate Synthase with Improved Thermal Properties by Directed Mutagenesis

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Abstract *Streptomyces thermovulgaris* malate synthase (stMS) is known to be more thermostable and thermoactive than *S. coelicolor* malate synthase (scMS). Therefore, based on the amino acid sequence of stMS, 3 scMS mutants, namely P186R, T8PL9P, and T8PL9PP186R, were created by site-directed mutagenesis in an attempt to engineer a more thermoactive and thermostable enzyme. An enzymatic analysis of the wild-type and mutant MS revealed that P186R and T8PL9PP186R were more thermoactive than the wild-type scMS and T8PL9P. Furthermore, all 3 mutants exhibited a greater thermostability than scMS, thereby suggesting that both R186 and P8P9 can cause increased thermostability in scMS.

Key words: *Streptomyces thermovulgaris*, malate synthase, thermostability, thermoactivity

Thermostability is one of the most fascinating problems in protein science. Why two enzymes catalyzing the same reaction can differ so greatly in their thermostability remains a largely unresolved issue. For example, it is still not fully understood why *Taq* DNA polymerase, unlike its mesophilic *E. coli* counterpart, is capable of withstanding temperatures above 90°C. Although there are several proposed rule-of-thumbs regarding the various factors that affect thermostability and thermoactivity, a general rational method is still lacking. Frequently cited factors include electrostatic interactions (salt bridges), hydrogen bonds, hydrophobic interactions, packing of the protein, binding to metal cations, and the amino acid composition.

Understanding the role of these different factors in enhancing the thermostability of proteins is important both from an academic and industrial viewpoint, as it would aid in clarifying the mechanism of protein stability and designing proteins with greater stability. Engineered proteins showing either higher or lower thermostabilities than wild-types can also serve as good models in elucidating the basis of thermostability.

One promising method for uncovering the rules of thermostabilization involves sequence comparison between homologous proteins exhibiting different thermostabilities. For instance, Das and Gerstein [2] conducted a comprehensive genome comparison of 12 sets of genomes ranging from mesophilic eubacteria to thermophilic archaeons, and noted an increase in the number of charged residues and salt bridges in the genomes from thermophilic organisms. Amino acid substitution patterns and protein properties yielding improved thermostable variants may also provide some insight into protein thermostabilization.

Streptomyces thermovulgaris produces a more thermostable form of malate synthase A compared to *S. coelicolor* [3]. Malate synthase (MS) catalyzes the condensation of glyoxylate and acetyl-CoA to yield L-malate, and is one of two key enzymes involved in the glyoxylate cycle. This cycle is an inducible, anaplerotic metabolic pathway that facilitates the biogenesis of carbohydrates from 2-carbon compounds such as acetate, ethanol, or fatty acids [4]. There are two known forms of malate synthase: malate synthase A (encoded by *ace*B) and malate synthase G (encoded by *gln*C). These two genes share a homology of about 20%. To date, only the crystal structure of *E. coli* malate synthase G is known. Thus, without the crystal structure of malate synthase A, it is difficult to predict the amino acid residues involved in functional properties. Accordingly, the current study conducted a comparative analysis of the primary sequence identities of a thermophile against 33 mesophiles to assist in defining the probable amino acid residues contributing to MS thermostability, then the predictions were confirmed by an experimental analysis. The growth temperatures for *S. coelicolor* and *S. thermovulgaris* are 28°C and 45°C, respectively, while the optimum temperature for scMS is 37°C, and that for stMS

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is 55°C. Based on a sequence alignment, two sites in scMS, P186R and T8PL9P, were proposed for site-directed mutagenesis to engineer a more thermostable enzyme. As such, three scMS mutants, P186R, T8PL9P, and T8PL9PP186R, were generated, and enzymatic analyses showed that all three mutants exhibited improved thermal properties.

Materials and Methods

Multiple Sequence Alignment of MS Isozymes

The MS amino acid sequences were obtained from the GenBank database and aligned using the CLUSTAL W Multiple Sequence Alignment Program (version 1.7) [10].

Site-Directed Mutagenesis and DNA Sequencing

The pGEX-6P-1 vector harboring the wild-type scMS gene was used as the template for the site-directed mutagenesis (SDM). *In vitro* site-specific mutations of the cloned scMS gene were created by a PCR mutagenesis strategy using designed mutagenic primers and a Quikchange™ SDM kit (Stratagene, La Jolla, CA, U.S.A.), according to the supplier's instructions. The mutagenic primers used to generate the mutations are 5'GTCGACGG CCGCC**G**GGTCCCCGGCGCC3' and 5'GGCGCCGGGGACC**C**GGCGGCCGTCGAC3' for P186R, and 5'CCAGCGCCGTCCC**C**GCC**G**GCCATCGTCGAC3' and 5'GTCGACGATGGC**C**GGC**G**GGGACGGCGCTGG3' for T8PL9P. The mutagenic codons for the mutagenic primers are shown in bold and underlined. A triple mutant was constructed using the primers for the double mutation and the template from the single mutation. To confirm the presence of the desired mutations, the putative mutant genes were sequenced in their entirety using an ABI PRISM™ Big Dye™ terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA, U.S.A.) and analyzed using an ABI PRISM 377 DNA sequencer.

Heterologous Expression and Purification of GST-MS Enzymes

Overnight recombinant *E. coli* cells were inoculated into 100 ml of Luria Bertani medium containing ampicillin ($100\,\mu g ml^{-1}$) and incubated at 37°C with agitation at 220 rpm until the optical density at 600 nm reached 0.8–1.0. At this point, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. Expression was carried out at ambient temperatures (22°C–24°C) with agitation at 200 rpm for 15 h. The cells were harvested by centrifugation, washed, resuspended in a PBS buffer (0.14 M NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3), and lysed using an MSE Soniprep (Crawley, U.K.) with a 1/8 inch (3 mm) probe. Cell debris was removed by centrifugation and the resulting cell-free extract (soluble fraction) was purified via affinity chromatography. The glutathione S-transferase (GST) MS fusion protein was purified using Glutathione Sepharose 4B (Amersham Pharmacia, Piscataway, NJ, U.S.A.). The fusion protein from the cell-free extract was allowed to bind to the sepharose beads for 1 h at 4°C. The beads were then washed three times with PBS buffer to remove unbound and unwanted cellular proteins. The scMS protein was cleaved from the GST fusion partner using an appropriate amount of PreScission protease at 4°C for 12 h. The purified protein was then separated from the GST (bound to the sepharose beads) by centrifugation.

Protein Determination and Enzymatic Assays

The protein concentrations were determined using a protein assay reagent (Bio-Rad Hercules, CA, U.S.A.). A 5 ml of protein assay reagent was added to 50 μl of purified protein. The solution was briefly mixed and allowed to stand for 5 min. Thereafter, the absorbance was measured at 595 nm. The reading was checked against a standard curve to obtain the protein concentration. The enzymatic assay was based on Ornston and Ornston [7]. The assay mixture consisted of 10 μM $MgCl_2$, 0.2 μM acetyl-CoA, 10 μM glyoxylate, and a 100 μM Tris-HCl buffer (pH 8) in a total volume of 0.9 ml. Color formation was initiated by the addition of 0.1 ml of 10 mM 5,5'-dithio-bis-(2-nitrobenzoic acid) (DNB), and the absorbance measured at 412 nm after 10 minutes. An increment of 4.53 absorbance units corresponded to the release of 1 μM of CoA per 3 ml of reaction mixture. The thermoactivities and thermostabilities of wild-type and mutant MSs were determined as described previously [3]. For thermoactivity analysis, the enzyme assay was performed at different temperatures, ranging from 20°C to 55°C. The assay mixtures were incubated at the indicated temperatures for 5 min prior to the initiation of the reaction. For thermostability analysis, the purified enzymes were incubated at 28°C and 35°C for 10 min. Aliquots were then taken and assayed for their residual activities at their optimum temperature, i.e., 35°C for scMS, P186R, T8PL9P, and T8PL9PP186R, and 55°C for stMS.

Results

Sequence Alignment

Presently, stMS is the only thermophilic MS with a known sequence (GenBank accession no. AF489515). It is anticipated that unique differences in the amino acid residues in stMS relative to other mesophilic isozymes may account for the differential thermal properties observed. Particularly, the current study was interested to find out the possible thermostabilizing role of arginine and proline in stMS, since both amino acids have already been implicated

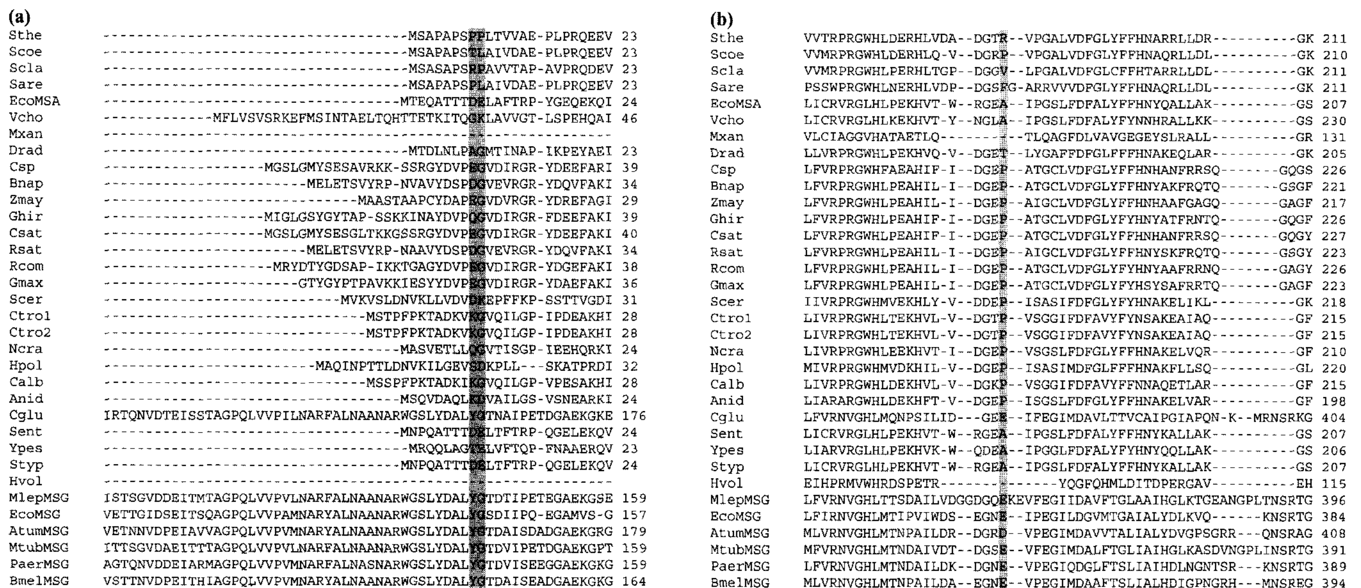

Fig. 1. Multiple sequence alignment of 34 known malate synthases. Only the amino acid sequences in the mutation regions are shown. The unique residues, P8P9 and R187, for the site-directed mutagenesis in *S. coelicolor* MS are shaded. The ascession numbers are indicated in brackets.

Sthe, *Streptomyces thermovulgaris*; Scoe, *S. coelicolor* (AF206498); Scla, *S. clavuligerus* (AAC83648); Sare, *S. arenae* (P77947); EcoMSA, *Escherichia coli* malate synthase A (M36854); Vcho, *Vibrio cholerae* (AAF93899); Mxan, *Myxococcus xanthus* (U81372); Drad, *Deinococcus radiodurans* (AAF12480); Csp, *Cucurbita* species (X56948); Bnap, *Brassica napus* (J04468); Zmay, *Zea mays* (L35914); Ghir, *Gossypium hirsutum* (X52305); Csat, *Cucumis sativus* (X15425); Rsat, *Raphanus sativus* (X78852); Rcom, *Ricinus communis* (X52806); Gmax, *Glycine max* (L01629); Scer, *Saccharomyces cerevisiae* DAL17 (X64407); Ctro1, *Candida tropicalis* CTPMS1 (D13415); Ctro2, *C. tropicalis* CTMPS2 (D13416); Ncra, *Neurospora crassa* (X56672); Hpol, *Hansenula polymorphae* (P21360); Calb, *Candida albicans* (AAF34695); Anid, *Aspergillus nidulans* (X56671); Cglu, *Corynebacterium glutamicum* (CAA55243); Sent, *Salmonella enterica* subsp. *enterica* serovar Typhi (NP_458503); Ypes, *Yersinia pestis* (NP_407176); Styp, *Salmonella typhimurium* LT2 (AAL23007); Hvol, *Haloferax volcanii* (CAC48389); MlepMSG, *Mycobacterium leprae* malate synthase G (CAA15459); EcoMSG, *E. coli* malate synthase (GAAC76012); Atum, *Agrobacterium tumefaciens* str. C58 malate synthase G (AAL41078); Mtub, *Mycobacterium tuberculosis* H37Rv malate synthase G (NP_216353); Paer, *Pseudomonas aeruginosa* malate synthase G (NP_249173); Bmel, *Brucella melitensis* malate synthase G (AAL51561).

in enhancing thermostability. An initial amino acid alignment of stMS and scMS revealed 15 positions where arginine was found in stMS, yet not in scMS. In order to narrow down the target locations, stMS was aligned with 33 mesophilic malate synthase isozymes (Fig. 1). The results of the alignment showed that of the 15 locations found earlier, arginine was unique at only one position (position 187) in stMS (Fig. 1b), which corresponded to proline 186 in scMS, and was the only position where arginine was found in stMS, yet not in any of the 33 mesophilic MSs.

The alignment of stMS with scMS also revealed 5 positions where proline was found in stMS, yet not in scMS (data not shown). The alignment of stMS with the 33 mesophilic malate synthase isozymes revealed that none of the 5 prolines mentioned above were unique to stMS. However, it was noted that 2 of the 5 prolines formed a pair (Fig. 1a), and this proline pair at positions 8 and 9 was not found in any of the 33 mesophilic MSs, making it unique to stMS. The corresponding residues at positions 8 and 9 in scMS were threonine and leucine, respectively. Thus, based on sequence alignment, the proposed mutation sites were shown to be unique to thermophilic MS.

Heterologous Expression and Purification of Wild-Type and Mutant MSs

Mutations were introduced into scMS and confirmed by DNA sequencing (data not shown). Wild-type scMS, P186R, T8PL9P, and T8PL9PP186R mutant scMSs were expressed and purified as described in the previous section to yield the respective cell-free extracts. SDS-PAGE analysis revealed a distinct band of about 86 kDa, which corresponded to the molecular mass of the GST-MS fusion protein (Fig. 2). Similarly, high levels of soluble mutated GST-MS were also obtained. The wild-type and mutant GST-MS were purified using Glutathione-Sepharose 4B column chromatography and cleaved with PreScission protease. The purified cleaved MS corresponded to an expected size of about 60 kDa (Fig. 2). The concentrations of the purified cleaved proteins were about 1.5 mg/ml, as determined by Bradford assays.

Comparison of Wild-Type and Mutant MS Activities

Thermostability, as defined by Danson *et al.* [1], is the ability of an enzyme to withstand temperatures that would rapidly denature most proteins from mesophiles. On the other hand, an enzyme is considered thermoactive if it

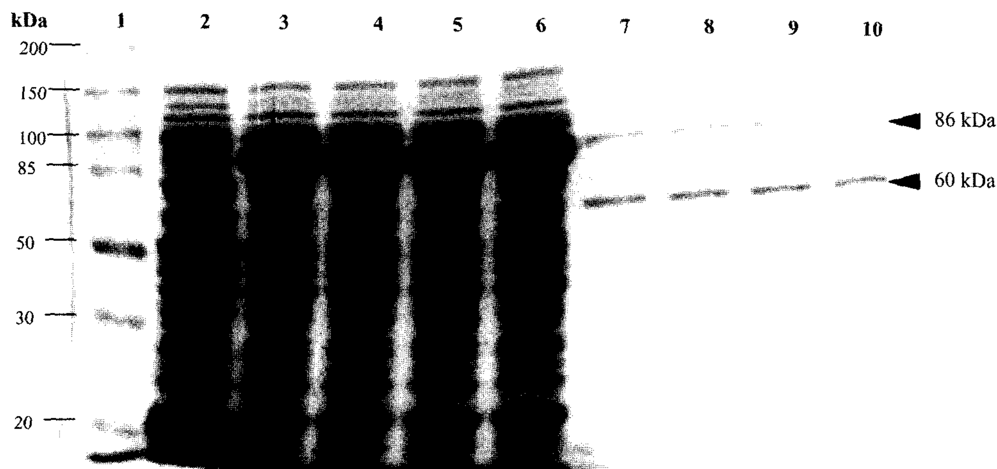

Fig. 2. SDS-PAGE analysis of purified wild-type and mutant malate synthases.
Lane 1, molecular weight marker. Lane 2, soluble fraction of *E. coli* BL21 (DE3)/pGEX-6p-1 vector. Lanes 3 to 6, soluble fractions of wild-type scMS, P186R, T8PL9P, and T8PL9PP186R, respectively. Lanes 7 to 10, purified wild-type scMS, P186R, T8PL9P, and T8PL9PP186R malate synthases, respectively. The purified, cleaved wild-type and mutant malate synthases corresponded to an expected size of 60 kDa. The 86 kDa band observed in the purified samples corresponded to GST-MS.

shows a temperature optima at or slightly above the growth temperature of the source organism. A comparison of the enzymatic activities of the wild-type scMS with that of the three mutated MSs showed that the activity of the wild-type scMS was higher than that of all three mutant MSs at 28°C. The activity of stMS at this temperature was low as expected, since the optimum temperature for this enzyme is 55°C. When the assay temperature was shifted to 45°C, the wild-type scMS lost more than 60% of its activity (Fig. 3). In contrast, both P186R and T8PL9PP186R retained at least 90% of their optimal activities. Both these mutant enzymes showed higher activities than the wild-type scMS. Thus, there was an increase of about 8°C in the working temperature of the enzyme by just a change of a single amino acid.

Thermostability assays were carried out where the enzymes were first incubated at various temperatures before being assayed for their residual activities. All three mutant enzymes showed some degree of improvement in thermostability as compared to the wild-type enzyme. After incubation for 10 min at 35°C, the wild-type scMS lost about 50% of its original activity (Fig. 4). Conversely, all three mutants retained between 90–95% of their original activities, losing most of their activities only when the incubation temperature was shifted to 40°C.

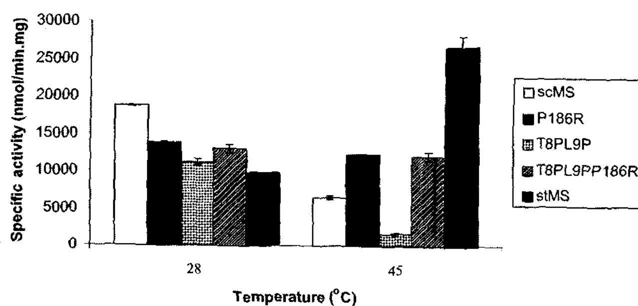

Fig. 3. Comparison of specific activities of purified wild-type scMS, three mutated malate synthases (P186R, T8PL9P, and T8PL9PP186R), and stMS at 28°C and 45°C.

DISCUSSION

Mutation Site P186R

Arginine is a common target for mutation in attempts to improve thermostability. A study by Kumar *et al.* [5] compared sequence and structural parameters in representatives of 18 nonredundant families of thermophilic and mesophilic proteins. It was found that thermophilic proteins tend to

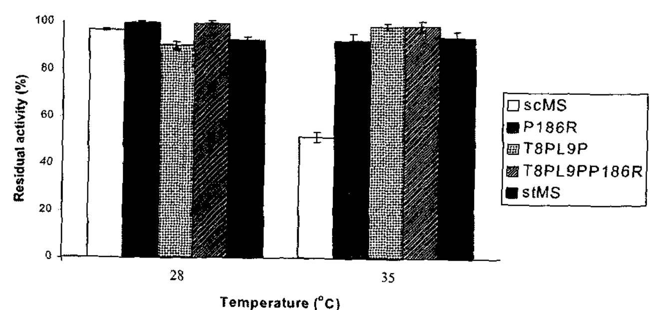

Fig. 4. Comparison of percentage residual activities of wild-type scMS, three mutated malate synthases (P186R, T8PL9P, and T8PL9PP186R), and stMS after 10 min incubation at 28°C and 35°C.
The residual activities for scMS and the three mutants were assayed at 35°C while that for stMS was assayed at 55°C.

contain higher proportions of arginine and tyrosine as compared to their mesophilic counterparts. As such, this raises the possibility of increased electrostatic interactions in thermophilic proteins, especially by the arginine residue, as it contains a guanidine group capable of forming salt bridges or hydrogen bonds. For example, in a study on CuZn superoxide dismutases (CuZnSOD) from various organisms, Rocha *et al.* [9] found that lysine at position 9 is conserved among vertebrates. The only known exception is in the enzyme from the swordfish, where it is replaced by an arginine. Site-directed mutagenesis was employed to replace the lysine residue at position 9 in human CuZnSOD with an arginine residue. The half-life for the wild-type CuZnSOD at 85°C was 17 min, whereas the half-life at that temperature for the mutant enzyme increased nearly six-fold to 98 min. Model-building suggests that the improved stability occurred because the arginine side chain formed at least one additional hydrogen bond with another polar group in the protein. Similar electrostatic interactions could also have contributed to the enhanced thermostability and thermoactivity exhibited by the P186R mutant in this study.

Mutation Site T8PL9P

Proline differs from all other amino acids because the side chain curls back to the preceding peptide-bond nitrogen and forms a five-member pyrrolidine ring. It is well-known that a pyrrolidine ring imposes rigid constraints on the conformation of the proline residue in a polypeptide chain, and this has been suggested to increase protein thermostability [11]. Several successful proline mutations have been documented. Recently, the thermostability of an industrially important enzyme involved in the production of ethanol, alpha-glucosidase from barley (*Hordeum vulgare*) was improved by replacing threonine at position 340 with a proline [6]. The basis for this substitution arose from the alignment of the amino acid sequences of the wild-type mesophilic barley α-glucosidase with that of three other published deduced amino acid sequences of thermophilic α-glucosidase isozymes. The alignment revealed conserved proline residues in the three thermostable α-glucosidases that were not found in the barley enzyme. Site-directed mutagenesis was applied to the recombinant barley α-glucosidase to create mutants with prolines at these conserved regions. One of these proteins, the T340P mutant, exhibited a 10°C increase in thermostability (T_{50}).

In another study, the thermostability of xylose isomerase from *Streptomyces diastaticus* was increased by replacing the glycine residue at position 138 with a proline [12]. The mutant exhibited about a 100% increase in thermostability as compared to the wild-type. Molecular modeling studies on the mutant substantiated the proposal that the introduction of proline residues reduced the flexibility of the polypeptide, thereby changing the rigidity and possibly the thermostability of the protein. This hypothesis is further supported by the current study, as the mutant T8PL9P showed that the double proline acquisition indeed altered the thermostability of malate synthase.

Both charged and hydrophobic residues have been implicated in the thermostabilization of proteins. For example, a cold-shock protein of mesophilic origin was converted to a highly thermostable form by the substitution of glutamic acid residues at positions 3 and 66 with arginine and leucine, respectively [8]. It was speculated that the increase in thermostability was due to the electrostatic contribution of arginine 3, and the hydrophobic interactions of leucine 66. Perhaps the increased thermoactivity and thermostability seen in the three mutants in the current study was due to similar electrostatic and hydrophobic interactions from arginine 186, and prolines 8 and 9, respectively. Since the mutation P186R was common to both mutants P186R and T8PL9PP186R, this suggests that arginine 187 in stMS was one of the amino acids contributing to the increased thermoactivity observed in stMS. Meanwhile, the loss of activity in the double mutant T8PL9P at 45°C suggests that the proline pair at positions 8 and 9 was probably not one of the crucial amino acids involved in the thermoactivity of stMS.

The improvement in thermostability observed in the three mutant enzymes suggests that both mutation sites P186R and T8PL9P likely contributed to the thermostability of scMS. However, the effects of both mutations were not cumulative, as the triple mutant T8PL9PP186R, similar to P186R and T8PL9P, lost about 85% of its activity at 40°C incubation and above (data not shown). The results of the current study also supports the notion that in the absence of a crystal structure, it is still possible to obtain some degree of thermostabilization via a sequence comparison of known themophilic and mesophilic homologous sequences.

Future work from this point will include the cloning of other thermophilic MSs and the elucidation of the corresponding amino acid sequences, since stMS is the only thermophilic malate synthase A with a known sequence. This will then allow sequence comparison and the uncovering of conserved residues within thermophilic MSs that may contribute to thermal stability. Information on the crystal structure of malate synthase A will also allow the mapping of these residues with respect to the tertiary conformation of the enzyme, thereby uncovering possible trends in the locations of charged and hydrophobic residues.

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