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The Effect of Cysteine-43 Mutation on Thermostability and Kinetic Properties of Citrate Synthase from *Thermoplasma acidophilum*

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In this study, we have substituted serine-43 by cysteine in the recombinant citrate synthase from a moderately thermophilic Archaeon *Thermoplasma acidophilum,* for site-specific attachment of labels and have investigated the effects of this mutation on the biochemical properties and thermal stability of the enzyme. Both wild-type and the mutant enzymes were purified to homogenity using affinity chromatography on Matrex Gel Red A. The mutant *Thermoplasma* citrate synthase is very similar to wild-type citrate synthase in its substrate and co-factor specificities, pH profile and thermal stability. The mutation, however, has decreased the enzyme activity. The newly introduced reactive sulphydryl group could be easily modified by DTNB and labelled with 4-chloro-7-sulphobenzofuran, without loss of any activity. © 1996 Academic Press, Inc.

Analysis of structure-function relationships of enzymes from thermophiles is expected to provide insights into the molecular mechanism which nature employs to maintain such extreme thermostability. Specific interactions that may be related to thermostability may be sought through comparisons between homologous proteins from mesophiles and thermophiles. In order to investigate the molecular basis of thermal stability, we have chosen the central metabolic enzyme citrate synthase from thermoacidophilic Archaeon *Thermoplasma acidophilum,* as a model protein. Citrate synthase (EC 4.1.3.7) catalyzes the condensation of acetyl coenzymeA (acetyl-CoA) and oxaloacetate (OAA) to form citrate and free coenzymeA (CoA), and thus effects the entry of carbon into the citric acid cycle. This central role in cell metabolism is demonstrated by the presence of the enzyme throughout the three phylogenetic domains of living organisms (1, 2).

The citrate synthase purified from *Tp. acidophilum* was shown to be a dimer (subunit Mr:43,000) (3). The gene encoding for the enzyme was cloned, sequenced and expressed in *E. coli* (4,5). Recently the crystal structure of the *Tp. acidophilum* citrate synthase has been resolved to 2.5°A and compared with the equivalent structure of the pig heart enzyme (6). To achieve a detailed understanding of the structural features that confer thermotsability to *Thermoplasma* citrate synthase a number of site-specific mutagenesis experiments have been carried out in our laboratory. Among these, mutagenesis was used for one specific reason to introduce a cysteine residue to a selected location in the recombinant *Thermoplasma* citrate synthase. As the native enzyme does not bare any cysteine residue the mutational substitutions provided the functional -SH groups in the protein, that could be modified easily.

Analysis of 3D structure of *Tp. acidophilum* recombinant citrate synthase allowed the establishment of the precise locations for the residue exchanges. A site-directed mutant of the enzyme was prepared, with a single Serine→ Cysteine substitution at position 43. This substitution is

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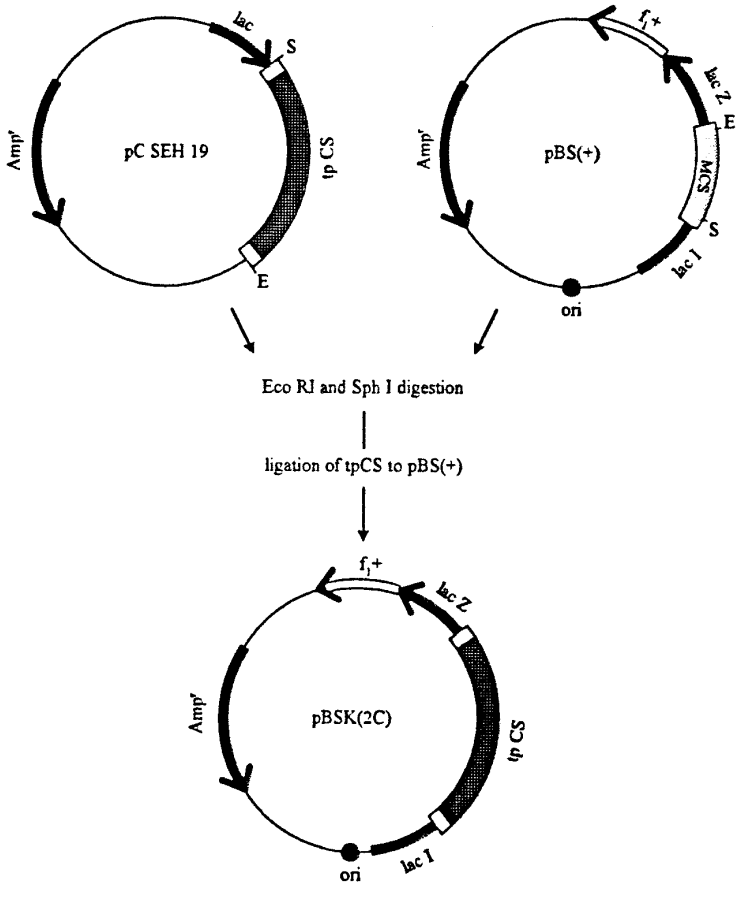

FIG. 1. Construction of phagemid pBSK(2C). E:EcoR I; S:Sph I

at particularly selected location of the enzyme, that is, it is on the surface and easily accessible for labelling.

This study aimed purification and characterisation of wild-type and cysteine-43 mutant *Thermoplasma* citrate synthase enzymes. The effect of amino acid replacement on the kinetic characteristics and thermal resistance was investigated. Thus, eligibility of cysteine-43 mutant enzyme for specific labelling and for analysis by spectroscopy was discussed.

MATERIALS AND METHODS

Recombinant phagemid pBSK(2C) which was the source of wild-type *Thermoplasma* citrate synthase gene in this study was constructed by cloning the *Thermoplasma* citrate synthase gene from recombinant plasmid vector pCSEH19 (5). Citrate synthase gene was removed from above plasmid by double digestion with EcoRI and SphI, and the excised fragment was ligated to pBS(+) phagemid at the same sites (Figure 1). In pBSK(2C) the *Thermoplasma* citrate synthase is expressed under the control of Lac promoter/operator. High level of expression of the citrate synthase in pBSK(2C) was achieved in *E. coli* XL1-Blue strain (Δ(*mcrA*) 183Δ(*mcrCB-hsdS MR-mrr*) 173 *end A1 sup E44 thi-1 rec A1 gyrA96 relA1 lac* [F′ *proAB lacI*^{q} *ZΔM 15 Tn 10* (Tet^r)]), as well as production of single stranded DNA which was suitable for site-directed mutagenesis experiments.

Oligonucleotide-directed, site-specific mutagenesis was performed mainly according to the method of Kunkel *et al* (7) as previously described by Kocabıyık and Perlin (8). The mutagenic oligonucleotides (synthesized by Severn Biotech. Ltd., Worcs., UK) differed from the wild-type by one nucleotide, thus changing Serine-43 to Cys. Aliquots of the reactions were used to transform competent *E. coli* XL1-Blue cells using a one-step transformation procedure (9). Randomly selected transformants were analysed by DNA sequencing (10) using a T7 DNA polymerase kit

TABLE 1
Purification of *Thermoplasma* Recombinant Wild-Type and Cysteine-43 Mutant Citrate Synthases Using Affinity Chromatography

	Total Protein (mg)	Total Enzyme Activity (U)	Specific Activity (U/mg)	Yield (%)
Crude	7.30	54.40	7.45	100
Extract	(9.90)*	(9.6)	(0.97)	100
Heat	1.17	43.74	37.38	80.40
Treatment	(1.62)	(7.11)	(4.39)	(74.06)
(65°C, 15 min)				
Matrex Gel	0.50	25.70	51.40	47.20
Red A	(0.53)	(4.73)	(9.00)	(49.27)

* The values for the mutant enzyme were given in paranthesis.

(Sequenace Version 2.0, Stratagene, La Jolla, CA) to find out the mutants that possessed the desired nucleotide changes.

E. coli pBSK(2C) and pBSK(43) cells expressing the *Thermoplasma* wild-type and cysteine-43 mutant genes, respectively, were cultured overnight in 200 ml LB-broth, containing 50μg/ml ampicillin. The cells were harvested by centrifugation (15 min. at 4,500 rpm) and resuspended in a 10ml volume of 20mM Tris-HCl, 1mM EDTA buffer (pH 8.0). After lysis by sonication, the cell debris was removed by centrifugation (60 min. at 12,000 rpm) and the cell lysate was incubated at 65°C for 10 min. After removal of cell debris by centrifugation, the extract was then applied to a Matrex Gel Red A column (Amicon Corp., MA, USA)(volume: 10ml), equilibrated in the same buffer as that in which the extract was prepared. The chromatography was carried out as described by James *et al.* (11). Enzyme purity was assessed by SDS-PAGE (12).

Citrate synthase activity was assayed at 412 nm by the method of Srere *et al.* (13). The assay was performed at 55°C in 20mM Tris-HCl, 1mM EDTA (pH 8.0) buffer, containing 0.2mM OAA, 0.15mM acetyl-CoA and 0.2mM 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB). One unit (U) of enzyme activity is 1μmol CoASH produced per min.

The Michaelis constants were determined from double reciprocal plots of initial rates at OAA concentration was kept constant and Ac-CoA concentration varied and *vice versa.* For all concentrations of the substrates tested, the increase in the absorbance at 412 nm was recorded for 5 min. The initial velocity was expressed in units of Δ A_{412}nm min^{-1}.

The thermostability of the purified citrate synthase enzyme was assessed in 20mM Tris 1mM EDTA (pH 8.0) at an enzyme concentration of 1U/ml. Enzyme samples were incubated in water baths, at temperatures between 50°C to 90°C for 60 min. Aliquotes were removed at every 5 or 10 min. , and chilled rapidly on ice. The remaining activity was determined at 55°C using the standard assay procedure.

To test the effect of pH on the activity of the purified wild-type and mutant enzymes citrate synthase activities were measure in equal amounts of 20mM of various buffers, each containing 1mm EDTA. The enzyme activities were determined as described above.

Protein was assayed at 235nm and 280nm according to Witaker and Granum (14).

RESULTS AND DISCUSSION

Recently elucidated crystal structure of the recombinant *Thermoplasma* citrate synthase provided a basis for the rational design of site-directed mutagenesis experiments for engineering of this enzyme. In the present study, the targeted mutation was done to introduce a cysteine residue into recombinant *Thermoplasma* citrate synthase. Sulfhydryl group of cysteine is extremely useful for modifications of the proteins (15, 16), or for introduction of structural probes (15, 17), which allow structural analysis in biomolecules by a variety of spectroscopic tools.

The thermostability of *Thermoplasma* citrate synthase allowed a simple heat treatment to be used as an initial step in the purification of the enzyme and in the elimination of heat-labile *E. coli* proteins. The steps involved in the purification of the wild-type and mutant *Thermoplasma* citrate synthases are summarised in Table 1. The homogenity of the enzyme preparations was demonstrated by SDS-polyacrylamide gel electrophoresis. Citrate synthases from wild-type and mutant strains exhibited identical protein bands on SDS-PAGE corresponding

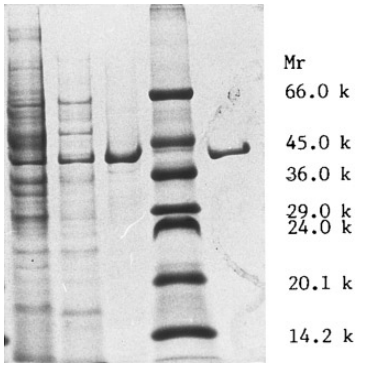

FIG. 2. SDS-PAGE of recombinant wild-type and mutant *Thermoplasma* citrate synthases, purified by Matrex Gel Red A chromatography. Wild-type homogenate; lane 1, before heat treatment; lane 2, after heat treatment; lane 3, after affinity chromatography. Lane 4, standard proteins; lane 5, cysteine-43 mutant protein after affinity chromatography.

to a relative molecular mass of 43,000 for the subunit (Figure 2). These results are in agreement with earlier observations of Smith *et al.* (3) and that of Southerland *et al.* (5) for wild-type and recombinant *Thermoplasma* enzymes, respectively.

Wild-type and cysteine-43 mutant citrate synthases were very stable at 50°C, 60°C and 70°C with no detectable loss in their activities, even after 60 min. The half-lives of irreversible inactivation ($t_{1/2}$), for wild-type and mutant citrate synthases at 80°C were 23 min and 21 min, respectively. The activities of both enzymes decreased remarkably at 90°C after 5 min. At an enzyme concentration of 1U/ml, wild-type and mutant *Thermoplasma* citrate synthases were active at 70°C after 60 min, but their activities reduced by 40% under the same conditions when the enzyme concentration was 0.1 U/ml. These results indicate that, enzyme concentration is critical in the studies investigating the thermal inactivation of *Thermoplasma* citrate synthase. Thermostability also has been shown to be concentration dependent for native alcohol dehydrogenase from *Sulfolobus solfataricus* (18).

Evaluation of initial rates from double-reciprocal plots yielded K_m (acetyl-CoA)= 10.6 and 10.8 μM and K_m (oxaloacetate)= 10.0 and 10.2 μM for wild-type and mutant enzymes, respectively. The V_{max} for OAA was calculated as 3.16 μmoles/lt for both enzymes. The V_{max} for Ac-CoA of wild-type and of mutant enzymes were 2.9 μmoles/lt and 3.31 μmoles/lt, respectively. When the citrate synthase activities were measured in different buffer systems at different pHs, both enzymes have exhibited similar pH profiles and the highest activities were obtained at pH 8.0 (Figure 3).

The cysteine-43 mutant citrate synthase is similar to wild-type recombinant citrate synthase, in its substrate and co-factor specificities, its molecular size and thermal stability. Although the mutation did not have any effect on thermostability of the *Thermoplasma* citrate synthase, the overall enzyme activity has decreased as evidenced by the reduced specific activity of the mutant enzyme (Table 1). Michaelis constants of the wild-type and mutant enzymes were quite similar, which means that mutation has no effect on substrate binding. Instead, there might be a possible influence of the mutation on the catalytic site which affects the reaction, or mutation might lead to misalignment of the substrates in the active-site although their binding affinities did not change. The change in the V_{max} (for Ac-CoA) as a result of the mutation, supports this notion.

The results reported in this study revealed that, cysteine-43 mutation did not alter the thermal resistance characteristics of the wild-type *Thermoplasma* citrate synthase. Our preliminary results showed that newly introduced sulphydryl group in the enzyme could easily be modified

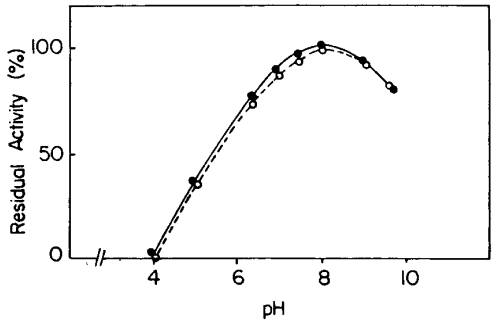

FIG. 3. Effect of pH on citrate synthase activity. Wild-type enzyme: (–●–●–); cysteine-43 mutant enzyme: (—○—○—).

by DTNB and be labelled with fluorescent label, 4-chloro-7-sulphobenzofuran, with no loss in the activity. Thus, cysteine-43 residue has proved to be accessible for specific attachment of labels to map the structure and local dynamic processes by spectroscopic means.

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