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# Crystallization and improvement of crystal quality for X-ray diffraction of maltooligosyl trehalose synthase by reductive methylation of lysine residues

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Maltooligosyl trehalose synthase, one of the two enzymes in the coupled trehalose biosynthesis system in *Sulfolobus acidocaldarius*, has been purified and crystallized. The chemical modification of this enzyme by reductive methylation of lysine residues significantly improved the crystal quality for X-ray diffraction experiments. The crystals of the modified enzyme belong to orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a$ = 56.70, $b$ = 140.1, $c$ = 205.2 Å measured at cryo-temperature, and are found to contain two enzyme molecules per asymmetric unit.

## 1. Introduction

Trehalose ($\alpha$,$\alpha$-trehalose), a non-reducing disaccharide containing an $\alpha$,$\alpha$-1,1-glucosidic linkage, is known to be widely distributed in yeast, fungi and plants. It has about 45% of the sweetness of sucrose and is gaining significant attention as a food additive and ingredient. The biosynthesis of trehalose in *Escherichia coli* (Giaever *et al.*, 1988) and *Saccharomyces cerevisiae* (Cabib & Leloir, 1958) has been demonstrated to occur *via* an enzymatic system of trehalose-6-phosphate synthase/phosphatase. Other enzymatic syntheses have been reported: by the reverse reaction of trehalose phosphorylase from *Euglena gracilis* (Marehal & Belocopitow, 1972) or trehalose from *Lobospaera* sp. (Nakano *et al.*, 1994). However, none of these systems are well suited for application to mass production of trehalose. Recently, two efficient enzymes which catalyse trehalose biosynthesis have been found in two different bacterial sources, the mesophilic *Arthrobacter* (Nakada *et al.*, 1995*a*,*b*) and an archaebacterium, the thermophilic *Sulfolobus acidocaldarius* (Nakada, Ikagami, Chaen *et al.*, 1996; Nakada, Ikagami, Mitsuzumi *et al.*, 1996). The two-enzyme system consists of maltooligosyl trehalose synthase (MTSase) and maltooligosyl trehalose trehalohydrolase (MTHase), which catalyse the following reactions in a coupled manner.

$$\text{maltooligosaccharide} \xleftrightarrow{\text{MTSase}} \text{maltooligosyl trehalose} \xrightarrow{\text{MTHase}} \text{trehalose} + \text{maltooligosaccharide}.$$

The first reaction is an unusual intramolecular transglucosylation which converts the $\alpha$-1,4-glucosidic linkage to an $\alpha$,$\alpha$-1,1-glucosidic linkage at the reducing end of the maltooligosaccharide, and the second is a hydrolytic reaction at the penultimate $\alpha$-1,4 linkage of the reducing end, resulting in the release of trehalose. This paper presents the crystallization of the *Sulfolobus* MTSase, which was cloned and overexpressed in *E. coli* (Maruta *et al.*, 1996). We report a significant improvement in the crystal quality for X-ray diffraction experiments of this enzyme by the reductive methylation of its lysine residues (Means & Feeney, 1968).

## 2. Materials and methods

### 2.1. Production and purification of intact MTSase

*E. coli* strain BMH71-18 harbouring an MTSase overexpression plasmid pKST9 was cultured for 24 h at 310 K in a medium containing 2% maltose, 4% soy peptone, 2% yeast extract, 0.1% $Na_2HPO_4$ and 100 mg ml$^{-1}$ ampicillin, pH 7.0. The MTSase protein was extracted from *E. coli* cells by sonication and purified by the method described previously (Nakada, Ikagami, Matsuzumi *et al.*, 1996). Briefly, the crude enzyme extract was adsorbed onto a DEAE-Toyopearl 650S column (400 ml) equilibrated with 10 m*M* Tris–HCl buffer (pH 8.0) and eluted with a NaCl gradient of 0–0.2 *M*. The peak fractions were assayed for activity towards maltopentaose by the Somogyi–Nelson method, esentially as described by Somogyi (1952). Fractions containing active enzyme were pooled and adjusted to 1 *M* ammonium sulfate. After adsorption on a butyl-Toyopearl column (100 ml), the enzyme was eluted with an ammonium sulfate gradient of 1–0.5 *M*. Fractions containing active enzyme were assayed as above, pooled, dialyzed against 10 m*M* Tris–HCl (pH 8.0), applied onto a Q-Sepharose FF column and eluted with an NaCl gradient of 0–

0.2 *M*. The peak fractions after the final step gave a single band on SDS–PAGE. The protein solution was also checked for suitability for crystallization by using a dynamic light-scattering instrument, DynaPro-801 TC (Protein Solutions, Inc.) at 1.5 mg ml$^{-1}$ protein concentration in the same buffer at 278 K, resulting in a monodisperse profile.

### 2.2. Crystallization of MTSase

The purified MTSase was crystallized at 277 K by the hanging-drop vapour-diffusion method using a protein concentration of 30 mg ml$^{-1}$ in 0.1 *M* Tris–HCl (pH 8.5) with 0.2 *M* $MgCl_2$ and 20%(*w*/*v*) PEG 2000 as precipitant by mixing 5 µl of protein and 5 µl of precipitant solutions. The crystals typically grew to a size of 0.04 × 0.04 × 0.1 mm within a month. These crystals, however, diffracted X-rays only poorly as described below, and no significant improvement was obtained despite many trials with a large variety of crystallization conditions.

### 2.3. Chemical modification of MTSase

Since the crystals of the MTSase were of insufficient size and diffraction properties, the enzyme was subjected to reductive methylation of lysine residues as described for hen egg-white lysozyme (Rypniewski *et al.*, 1993), essentially according to the method of Means & Feeney (1968). Briefly, the protein solution (10 mg ml$^{-1}$) was dialyzed against 0.2 *M* sodium borate pH 8.5. To 1 ml of this solution, 30 ml of 1 *M* formaldehyde was added, followed by addition of 6 ml 1 *M* sodium borohydride and then by a further 3 ml after 10 min. This modification reaction was repeated six times at 20 min intervals, during which the reaction solution was left on ice. Finally after 30 min of stirring, 6 ml of 1 *M* sodium borohydride was added to completely reduce the unreacted formaldehyde. The reaction mixture was stirred for 1 h and 0.5 g of finely ground ammonium sulfate was then added to stop the reaction and precipitate the protein. The modified enzyme was recovered by centrifugation, dissolved in a minimal amount of 5 m*M* Tris–HCl buffer (pH 7.5) and dialysed against the same buffer for crystallization experiments.

### 2.4. Crystallization of the reductively methylated MTSase

The crystallization was performed under the same conditions as for the unmodified enzyme. The crystals grew to a typical size of 0.15 × 0.15 × 1 mm, and in some cases to 0.2 × 0.2 × 1 mm or larger, within two weeks. For cryo-temperature data collection, the crystals were subjected to flash freezing in liquid nitrogen after soaking in 10% glycerol for 30 min and in 20% glycerol for another 30 min.

### 2.5. Activity measurement

The reverse reaction of the enzyme catalysis, in which the product maltotriosyl trehalose is converted to maltopentaose and detected by its reducing power, was used for activity measurement. The procedure for the activity assay is essentially the same as described by Nakada, Ikagami, Mitsuzumi *et al.* (1996): 0.01 ml of enzyme solution was added to 0.25 ml reaction mixture containing 1% maltotriosyl trehalose in 20 m*M* sodium acetate (pH 5.5) kept at 333 K. After stirring for 20 min, 0.25 ml of Somogyi reagent is added to stop the reaction. The amount of maltopentaose liberated was measured by the Somogyi–Nelson method. The activity assay was applied to both intact and modified enzymes.

## 3. Results and discussion

The crystals from intact MTSase only diffracted to 3.5 Å resolution on a rotating anode X-ray generator operated at 40 kV and 100 mA and the crystals showed high mosaicity. On the other hand, crystals of reductively methylated MTSase diffracted up to 2.8 Å resolution under the same experimental conditions and to 2.4 Å resolution using the synchrotron SPring-8 BL41XU beamline at cryo-temperature (Kamiya *et al.*, 1995). The results of the activity measurement showed that the specific activity decreased from 1.35 × 10$^{-6}$ to 7.5 × 10$^{-8}$ mol s$^{-1}$ mg$^{-1}$ for unmodified and modified enzymes, respectively.

Reductive methylation of lysine $\varepsilon$-amino groups in protein has been used for radioactive labelling of proteins (Rice & Means, 1971; Ottesen & Svensson, 1971) and was shown to be accompanied by minimal changes in the gross physical properties (Means & Feeney, 1968). The structures of reductively methylated lysine proteins have been determined for myosin subfragment 1 (Rayment *et al.*, 1993) and hen egg-white lysozyme (Rypniewski *et al.*, 1993). We compared the structures of methylated-lysine hen egg-white lysozyme (PDB code 132l) and its intact enzyme structure (Oki *et al.*, 1999; PDB code 1bgi) with the least-squares method based on the main-chain atoms. The results showed a root-mean-square deviation of 0.88 Å for the main-chain atoms and 1.4 Å for all atoms. Large deviations occurred on the arginine side-chain atoms of Arg14, Arg45, Arg114 and Arg128, with a maximum deviation of 12.8 Å on the Arg128. Smaller deviations were seen for the other lysine side chains with a maximum deviation of 3.8 Å at Lys116. Thus, we may well expect that there will be only minor perturbations in the molecular structure of the present enzyme as well. The improvements in the crystal size and X-ray diffraction properties should be attributed to an improved ordering of the molecular packing in the crystal owing to the change in the surface properties of the protein.

The preliminary crystallographic data obtained under cryo-conditions for the modified MTSase crystal are orthorhombic, space group $P2_12_12_1$, with unit-cell dimensions $a$ = 56.70, $b$ = 140.1, $c$ = 205.2 Å. The asymmetric unit contains two molecules with a solvent content of 54%, but the possibility of one or three molecules per asymmetric unit cannot be disregarded, with corresponding solvent contents of 78 and 32%, respectively. The one full set of intensity data at 2.4 Å resolution has been collected at cryo-temperature at SPring-8 synchrotron radiation facility. The merging $R$ and the completeness of the data were 5.3 and 92%, respectively. Heavy-atom derivative searches are now under way.

The synchrotron radiation experiments were performed at the SPring-8 with the approval of the Japan Synchrotron Radiation Research Institute (JASRI) (proposal No. 1998 A0184-NL-np).

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