Characterization of a Bifunctional Enzyme Fusion of Trehalose-6-Phosphate Synthetase and Trehalose-6-Phosphate Phosphatase of *Escherichia coli*

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To test the effect of the physical proximity of two enzymes catalyzing sequential reactions, a bifunctional fusion enzyme, TPSP, was constructed by fusing the *Escherichia coli* genes for trehalose-6-phosphate (T6P) synthetase (TPS) and trehalose-6-phosphate phosphatase (TPP). TPSP catalyzes the sequential reaction in which T6P is formed and then dephosphorylated, leading to the synthesis of trehalose. The fused chimeric gene was overexpressed in *E. coli* and purified to near homogeneity; its molecular weight was 88,300, as expected. The \( K_m \) values of the TPSP fusion enzyme for the sequential overall reaction from UDP-glucose and glucose 6-phosphate to trehalose were smaller than those of an equimolar mixture of TPS and TPP (TPS/TPP). However, the \( k_{cat} \) values of TPSP were similar to those of TPS/TPP, resulting in a 3.5- to 4.0-fold increase in the catalytic efficiency (\( k_{cat}/K_m \)). The \( K_m \) and \( k_{cat} \) values of TPSP and TPP for the phosphatase reaction from T6P to trehalose were quite similar. This suggests that the increased catalytic efficiency results from the proximity of TPS and TPP in the TPSP fusion enzyme. The thermal stability of the TPSP fusion enzyme was quite similar to that of the TPS/TPP mixture, suggesting that the structure of each enzyme moiety in TPSP is unperturbed by intramolecular constraint. These results clearly demonstrate that the bifunctional fusion enzyme TPSP catalyzing sequential reactions has kinetic advantages over a mixture of both enzymes (TPS and TPP). These results are also supported by the in vivo accumulation of up to 0.48 mg of trehalose per g of cells after isopropyl-\( \beta \)-D-thiogalactopyranoside treatment of cells harboring the construct encoding TPSP.

The nonreducing disaccharide trehalose [\( \alpha,\beta-D\)-glucopyranosyl-(1→1)-\( \alpha,\beta-D\)-glucopyranose] has high water-holding activities, which maintain the fluidity of membranes under dry conditions (25). It also stabilizes enzymes, foods, cosmetics, and pharmaceuticals at high temperatures (8, 9, 38). Due to its desirable physical and chemical characteristics, commercial production of trehalose is anticipated. *Escherichia coli* synthesizes trehalose when exposed to high osmolarity (12, 20, 39, 41). In *E. coli*, trehalose is synthesized by two separate enzymes, trehalose-6-phosphate (T6P) synthetase (TPS) and trehalose-6-phosphate phosphatase (TPP), encoded by the genes *otsA* and *otsB*, respectively (15, 21). This is different from *Saccharomyces cerevisiae*, in which trehalose is synthesized by a large multisubunit complex with the catalytic activities of both TPS and TPP (6, 31, 43).

Overexpression of TPS and TPP might be one way to produce trehalose. We assumed that the physical proximity of two enzymes catalyzing sequential reactions might increase the reaction rate by facilitating transfer of the reaction intermediate when they are present in a complex. A variety of techniques have been applied to better understand the proximity effect of enzymes catalyzing sequential reactions, including cross-linking and coimmobilization (23, 32, 34). In many of these cases, the organized enzyme systems exhibited different kinetic or catalytic properties compared with their free counterparts in bulk solution. An attractive alternative approach to these systems is to fuse two enzymes by ligating their structural genes using recombinant DNA techniques. This procedure mimics the evolution of naturally occurring bifunctional enzymes, which might have evolved from smaller proteins through gene fusion (22). It also imitates naturally occurring multienzyme systems, such as the pyruvate dehydrogenase complex and fatty acyl coenzyme A synthase complex (18, 37). Fusion enzymes sometimes demonstrate their superiority over a mixture of individual native enzymes catalyzing the same multistep sequential reaction, but their effects have not been demonstrated definitively in kinetic parameters governing the reactions (5).

Here we describe the enzymatic properties of the recombinant fusion enzyme TPSP, which contains TPS and TPP. The catalytic efficiency of TPSP was 3.5 to 4.0 times higher than that of a mixture of individual enzymes, showing the kinetic advantage of the fusion enzyme. We also examined the internal trehalose accumulation of cells harboring the construct encoding the recombinant fusion protein after IPTG (isopropyl-\( \beta \)-D-thiogalactopyranoside) treatment.

**MATERIALS AND METHODS**

**Bacterial strains.** All the plasmids constructed were propagated in *E. coli* MC1061. Plasmid pRSETB, based on the T7 RNA polymerase-driven pET system (24), was used as an expression vector and purchased from InVitrogen. For protein overexpression, plasmids were transformed in *E. coli* BL21(DE3) (pLysS) (hereafter called BL21) as described by Studier et al. (40) and obtained from InVitrogen.

**Reagents and enzymes.** The restriction endonucleases, Klenow fragment, alkali phosphate, and T4 DNA ligase were purchased from New England Biolabs and Boehringer Mannheim. The Taq DNA polymerase and deoxynucleoside triphosphates were also from Boehringer Mannheim.
tic acid (NTA) agarose resin was from Qiagen. Trehalose, T6P, UDP-glucose (UDPG), glucose, and glucose 6-phosphate (G6P) were from Sigma Chemical Co., and the silica gel thin-layer chromatography (TLC) plate was from Aldrich.

**Expression and purification of recombinant enzymes.** To express the recombinant enzymes, transformed *E. coli* BL21. The TPS gene was obtained from TPS gene (15, 21), respectively. EcoRI and HindIII restriction enzyme sites (underlined) were introduced into pRTPS and PTPP, respectively. The TPP gene was amplified in the same manner with the primers PTPP (5′-CCGGATCCATGATGACGTTA-3′) and PTPP (5′-CTACAGCAATGTGCAAAAAG-3′), which contain the translation initiation and termination codons (bold) of the *E. coli* TPS gene (15, 23), respectively. EcoRI and HindIII restriction enzyme sites (underlined) were introduced into PTPP and PTPP, respectively. The amplified DNA was ligated into EcoRI- and HindIII-digested pRSET-B (pRTPS for TPP) and Smal- and HindIII-digested pRSET-C (pRTPP for TPP) and transformed into *E. coli* BL21.

**Expression and purification of recombinant enzymes.** To express the recombinant enzymes, transformed *E. coli* BL21 were inoculated into 2 liters of Luria-Bertani (LB) (590 ml) medium containing ampicillin (50 μg/ml) and grown at 37°C. When the A590 reached 0.6, IPTG was added to a final concentration of 0.5 mM. Incubation was continued for another 3 h at 37°C. One milliliter of the culture was taken and centrifuged for 15 min at 12,000 × g, and the supernatant was analyzed for trehalose by TLC and HPLC.

**Expression and purification of bifunctional fusion enzyme TPSP.** To test the effect of the physical proximity of two enzymes catalyzing sequential reactions, the *E. coli* genes encoding TPS and TPP were fused, and the recombinant fusion enzyme (TPSP) was expressed. Recombination was carried out so that the C terminus of TPS was fused with the N terminus of TPP in frame, as shown in Fig. 1A. The linker was designed to facilitate the recombinant DNA construction without deleting any amino acids of either protein moiety. This resulted in the insertion of 8 amino acids, LGSRSAAE, during construction. The expression vector also has a hexahistidine tag (His tag) at the N terminus of the recombinant enzyme that can be utilized for rapid purification by Ni2+–NTA chromatography (17, 19, 24). Recombinant TPS and TPP were also expressed and purified separately for comparison.

**Trehalose synthesis by the bifunctional fusion enzyme TPSP.** The trehalose synthesis activity of the purified bifunctional fusion enzyme TPSP was tested using a mixture of G6P and UDPG as the substrate. As shown in Fig. 2 (lane 9 in panel A and plate d in panel B), TPSP produced trehalose from this mixture. This demonstrated that TPSP was functional and catalyzed two sequential reactions, from G6P and UDPG to T6P and then to trehalose. This result is consistent with the activity of the individual recombinant enzymes catalyzing each reaction; the recombinant TPS catalyzed the synthesis of T6P from G6P and UDPG, as shown in Fig. 2A (lane 6), and the recombinant TPP catalyzed the hydrolysis of T6P to trehalose (Fig. 2A, lane 7). An equimolar mixture of these two enzymes produced trehalose from G6P and UDPG by catalyzing the sequential reactions (lane 8 in panel A and plate c in panel B of Fig. 2).

**Kinetic parameters for the bifunctional fusion enzyme TPSP.** The time course and reaction rate of trehalose synthesis from UDPG and G6P by the recombinant fusion enzyme TPSP were investigated. The amount of trehalose synthesized from UDPG and G6P increased with time. The rate of trehalose synthesis by TPSP was at least 65% faster under the standard assay conditions than that by TPS/TPP, the equimolar mixture of the individual enzymes TPS and TPP, as shown in Fig. 3. The catalytic activity was proportional to the amount of enzyme and was not affected by adding bovine serum albumin to the assay mixture as an inert protein (data not shown).

To characterize the enzymatic properties of the bifunctional fusion enzyme TPSP, the kinetic parameters shown in Table 1 were determined. These values were obtained from Lineeweaver-Burk plots, which were linear within the experimental error (Fig. 4). Since it was impossible to separate the two sequential reactions catalyzed by TPSP, the kinetic parameters for the overall synthesis of trehalose from G6P and UDPG were determined. The kinetic parameters for the overall synthesis of trehalose from G6P and UDPG were determined. The kinetic parameters for the overall synthesis of trehalose from G6P and UDPG were determined.
were determined. These results were compared with the results of a parallel assay with an equimolar mixture of TPS/TPP. The \( K_m \) values of TPSP for UDPG and G6P were 69 and 76%, respectively, lower than those of TPS/TPP. The \( k_{cat} \) values, the turnover number, of TPSP for UDPG and G6P were, however, similar to those of TPS/TPP. The catalytic efficiency of the bifunctional fusion enzyme, calculated as \( k_{cat}/K_m \), was 3.5 to 4.0 times greater than that of TPS/TPP.

The kinetic parameters for the TPP activity of TPSP and TPP were also measured at various concentrations of T6P. The \( K_m \) and \( k_{cat} \) values for T6P hydrolysis of TPSP were similar to those of TPP. The catalytic efficiency (\( k_{cat}/K_m \)) of TPSP for
changed at various temperatures between 10 and 60°C. As shown in Fig. 5A, the highest activity was observed at 30 to 40°C for both the TPSP fusion enzyme and the TPS/TPP mixture. Although the absolute activities of trehalose synthesis by TPSP and TPS/TPP were different, there was not much difference in their temperature dependence.

To determine differences in their stability against thermal denaturation, both the fusion enzyme TPSP and the equimolar mixture TPS/TPP were incubated for a specified time at 50°C. As shown in Fig. 5B, TPSP and TPS/TPP retained 11 and 3% of their original T6P hydrolysis activities after incubation for 30 min at 50°C (Fig. 5C). These results suggest that fusion of the two enzymes did not cause any significant structural perturbation.

**Table 1. Kinetic parameters of recombinant enzymes TPS, TPP, TPS/TPP, and TPSP with different substrates**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>UDGP</th>
<th>G6P</th>
<th>T6P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (mM)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$k_{cat}/K_m$ (mM$^{-1}$ s$^{-1}$)</td>
</tr>
<tr>
<td>TPS</td>
<td>8.6 ± 0.8</td>
<td>7.3 ± 0.8</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>TPP</td>
<td>8.6 ± 0.8</td>
<td>7.3 ± 0.8</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>TPSP</td>
<td>5.1 ± 0.6</td>
<td>3.6 ± 0.2</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>TPS/TPP</td>
<td>16.7 ± 1.6</td>
<td>3.5 ± 0.3</td>
<td>0.2 ± 0.03</td>
</tr>
</tbody>
</table>

a Data are the mean values and standard deviations of three independent experiments. Experiment conditions are described in the legend to Fig. 4.
b Parameters for overall sequential reaction from G6P and UDGP to trehalose.
no intrinsic differences in the structures of each enzyme moiety in the fusion enzyme and the individual enzymes, even though TPSP was slightly more stable than the TPS/TPP mixture. Still, some studies showed that the fusion enzyme was more stable against thermal denaturation than the individual enzymes were (16, 30).

To facilitate independent folding of each enzyme moiety in the fusion protein, any structural perturbation caused by intramolecular strain in the complex should be minimized and the linker should accommodate conformational flexibility. The effects of linker peptides in fusion proteins are variable (1, 10, 11). In a β-galactosidase/galactose dehydrogenase fusion enzyme, the specific activity of the galactose dehydrogenase moiety increased with a longer linker (3 versus 9 or 13 amino acids) and the sequential reaction was carried out more efficiently (7). Although a longer linker might release steric perturbation, the longer linker would be more accessible to degrading enzymes, resulting in lower stability (5). We tested a linker 17 amino

FIG. 4. Lineweaver-Burk plots for trehalose synthesis catalyzed by TPSP or TPS/TPP in the presence of UDPG and G6P: effect of UDPG and G6P concentrations on enzyme activity. (A) Double-reciprocal plot of the initial velocity at various concentrations of UDPG against 30 mM G6P. Reactions involving the TPSP fusion enzyme (●) and TPS/TPP mixture (□) are shown. (B) Double-reciprocal plot of initial velocity at various concentrations of G6P against 15 mM UDPG. (C) Double-reciprocal plot of the initial velocity at various concentrations of T6P. Each point represents the mean of three determinations. Linear least-squares analyses were used to determine the slopes and intercepts in the double-reciprocal plots. Ten picomoles of TPSP, TPS, or TPP was used, and the reaction velocity (v) is expressed as nanomoles of trehalose formed per minute.

FIG. 5. Trehalose synthesis activity of TPSP and TPS/TPP. (A) Temperature dependence. Ten picomoles of TPSP (●) or the TPS/TPP equimolar mixture (□) was incubated for 60 min at various temperatures between 10 and 60°C in the standard assay mixture. After boiling for 3 min at 100°C, trehalose was analyzed as described in Materials and Methods. (B) Heat stability. One hundred picomoles of TPSP (●) or the TPS/TPP equimolar mixture (□) was incubated for the indicated lengths of time at 50°C in a final volume of 100 µl of a reaction mixture containing 33 mM Tris-HCl (pH 7.4) and 2.5 mM MgCl₂. (C) Heat stability of T6P phosphatase activity. One hundred picomoles of TPSP (●) or TPP (□) was incubated for the indicated lengths of time at 50°C in a final volume of 100 µl of a reaction mixture containing 33 mM Tris-HCl (pH 7.4) and 2.5 mM MgCl₂. After quenching on ice for 5 min, 7.5 mM UDPG and 15 mM G6P (B) or 10 mM T6P (C) was added to the reaction mixture. The remaining activity is expressed as a percentage of the original activity.
acids long, \( \text{S(GGGGS)}_3 \text{V} \), but this resulted in fast degradation of TPSP (data not shown).

The fusion of structural genes in frame to produce a fusion enzyme catalyzing sequential reactions can increase the efficiency of the enzyme, as shown in this study. It is also advantageous to control the expression of two genes as a single unit and to purify the recombinant fusion protein in one process. Recombinant DNA technologies for creating and overexpressing a gene fusion can be used to improve the productivity of enzyme technology.

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REFERENCES


