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Note

Mechanistic study of the intramolecular conversion of maltose to trehalose by *Thermus caldophilus* GK24 trehalose synthase

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Abstract

This paper questions what types of molecular transformation are involved in the conversion of maltose to trehalose by trehalose synthase from *Thermus caldophilus* GK24. The reverse reaction pathway has been examined with the aid of α,α-(2,4,6,6′,2′,4′,6″,6‴-$^{2}H_{8}$)trehalose (**1**). The mass data of the isolated reaction products clearly indicate that deuterated glucose is confined only to substrate molecules, and thus the reversible enzymatic conversion of trehalose into maltose proceeds through an intramolecular pathway.

Keywords: Trehalose; Trehalose synthase; *Thermus caldophilus*; Intramolecular conversion; Reaction mechanism

α,α-Trehalose (α-D-glucopyranosyl α-D-glucopyranoside) is a natural cell-protecting agent, as well as an energy reservoir in many organisms.[1,2] The wide occurrence of biosynthetic routes to trehalose in microorganisms,[3–5] yeast,[6] fungi,[7] insects,[8] and plants,[9] indicates that this disaccharide is an essential component for maintaining cell viability. Lately, a unique type of trehalose synthase (TS, maltose α-D-glucosyltransferase, E.C. 5.4.99.16), which converts maltose into trehalose in the absence of coenzyme, has been identified[10–12] in *Thermus* strains and other microorganisms. The thermostable TS from *Thermus caldophilus* GK24, which is of sufficiently high molecular weight (110 kDa) to accommodate two-step reactions, readily converts maltose into α,α-trehalose and a small amount of α,β-trehalose (∼3%) in a reversible reaction, together with a small proportion of irreversibly formed glucose (up to 2%, Scheme 1). This facile process is suited to the production of trehalose on an industrial scale.

Of concern mechanistically is the question of how a single enzyme accommodates both hydrolysis of maltose and formation of trehalose, as well as what types of molecular transformation are involved. A radiometric assay[13] indicated that the conversion of maltose into trehalose does not involve intermolecular transglucosylation, as no participation of [^{14}C]glucose was observed when it was introduced into the reaction mixture. For the present study, the reverse reaction pathway has been examined with the aid of α,α-(2,4,6,6′,2′,4′,6″,6‴-$^{2}H_{8}$)trehalose (**1**). The synthesis of trehalose from maltose must entail two distinct steps: cleavage of the (1→4)-α-D-glucosyl linkage of maltose and the formation of the (1↔1)-α-D-glucosyl linkages of α,α-trehalose, respectively. Furthermore, mechanistically, both intra and intermolecular pathways must be considered. The use of deuterated α,α-trehalose (**1**) was expected to furnish information about each of these pathways, depending on whether or not it was incorporated into the reaction products.

To examine the reaction pathway possibilities, a mixture of trehalose (5 mg) and deuterated α,α-trehalose (**1**, 3 mg), in 20 mM potassium phosphate buffer (pH 6.3, 3 mL), was subjected to the TS reaction for 12 h at 80 °C for conversion into maltose and deuterated

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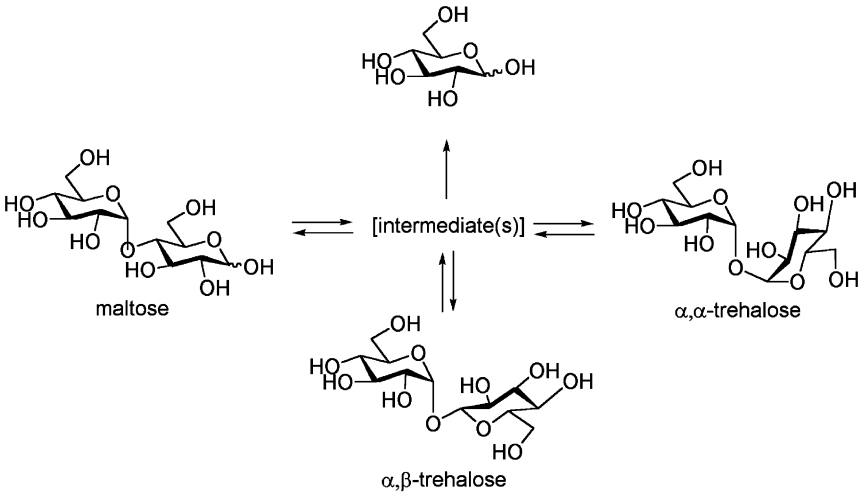


Scheme 1. Overview of trehalose synthesis from maltose by trehalose synthase.

maltose. Following equilibration, all reaction components corresponding to peaks a (deuterated α,α-trehalose, **1**), b (trehalose), c (2,4,6,6′-$^2H_4$)glucose), d (glucose), e (2,4,6,6′,2′,4′,6″,6‴-$^2H_8$)maltose, **2**), and f (maltose) in the chromatogram (Fig. 1) were isolated by high performance anion-exchange chromatography (HPAEC). It is noteworthy that the deuterated molecules (**1**, deuterated glucose, and **2**) migrated slightly faster on the column than their unlabeled forms.

Electron-spray mass spectra (Fig. 2) of the components corresponding to peaks a and b (Fig. 1), showed the presence of the parent ion of **1** (deuterated trehalose, *m*/*z*, 348.8) and of trehalose (*m*/*z*, 340.9). However, no peak due to a ($^2H_4$)trehalose, or a ($^2H_4$)maltose adduct (*m*/*z*, 344.8) (data not shown) was detected. This clearly indicated that deuterated glucose was confined only to substrate molecules, and thus the reversible enzymatic conversion of trehalose into maltose proceeds through an intramolecular pathway (Scheme 2). Furthermore,

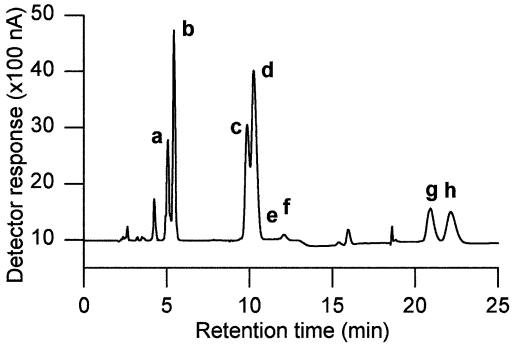


Fig. 1. HPAEC–PAD chromatogram of TS reaction products. The chromatogram was obtained from HPAEC–PAD with CarboPac PA1 with the reaction products, after TS reaction with trehalose and deuterated α,α-trehalose (**1**) was completed. Peaks: a, deuterated α,α-trehalose; b, α,α-trehalose; c, deuterated D-glucose; d, D-glucose; e, deuterated α,β-trehalose; f, α,β-trehalose; g, deuterated maltose; and h, maltose.

the yield of the glucose was not 2 molar equivalents of that of the trehalose, which agrees with the observation that maltose is not intermolecularly converted into trehalose by TS.

We propose that in the TS reaction, a molecule of maltose, located initially in the catalytic pockets of the enzyme, undergoes normal cleavage of the glucosidic bond via a carbocation intermediate generated by involvement of basic amino acid residues. This cleavage, in fact, bears close resemblance to the amylolytic hydrolysis of a glycoside with retention of configuration. Then the two enzyme-bound glucopyranose residues are rearranged as the enzyme undergoes a change in conformation, whereby the anomeric hydroxyl group of one residue is suitably oriented for nucleophilic attack on the anomeric carbon of the other residue. Finally, a displacement involving a second carbocation intermediate gives rise to the 1,1-α,α-disaccharide structure of the trehalose product. The formation of some α,β-trehalose as a minor side-product indicates the existence of another TS conformation, which accommodates steric strain of the substrate in catalytic pocket. Alternatively the β configuration may represent some relaxation in the enzyme structure at the elevated temperature of the reaction.[10] In addition, the formation of glucose as another minor product suggests the involvement of a water molecule acting as a nucleophile within the catalytic pocket to induce hydrolysis prior to formation of the glucosidic linkage.[10]

For the conversion of trehalose into maltose, the same mechanistic pathway applies in reverse. However, the higher yield (up to 80%) of trehalose formed at 40 °C, indicates that the transformation of trehalose into maltose requires higher activation energy than for the formation of trehalose from maltose.

In summary, the action of TS entails two sequential steps, namely, cleavage of the α-(1→4)-linkage in maltose and the formation of the (1↔1)-linkage in the

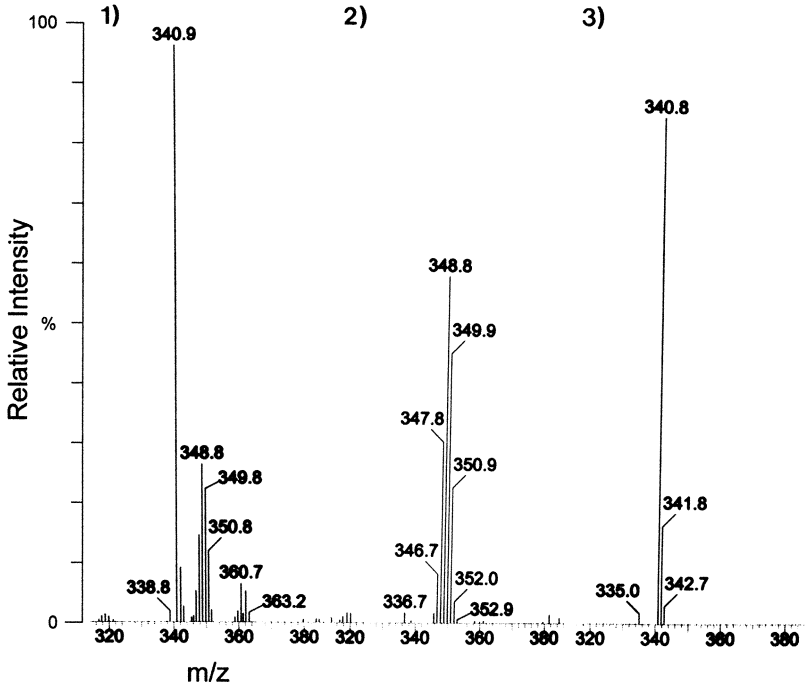


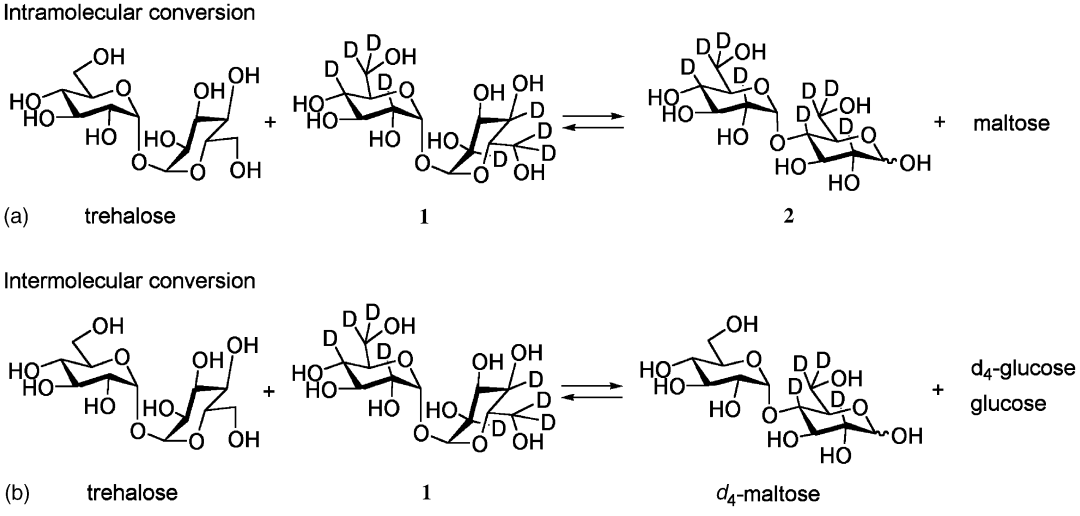
Fig. 2. Electron spray mass spectra of products of the trehalose synthase reaction; (1) mass spectrum of a mixture of trehalose and deuterated α,α-trehalose (**1**) before the TS reaction; (2) and (3) were mass spectra of the equilibrated products of the TS reaction, deuterated α,α-trehalose (**1**) and trehalose, respectively which were isolated by HPAED–PAD.

non-reducing disaccharide trehalose, with overall retention of configuration. To account for these events, the mechanistic pathway appears to require a conformational change in the enzyme that flips the D-glucose product at the active-site so that C-1–OH is in position to attach the C-1–Nuc group, making the 1,1′-α,α-acetal linkage. It is anticipated that further understanding of catalysis by the TS enzyme will benefit from structure–function studies on the reaction mechanism with enzyme analogs.

1. Experimental

1.1. Gene cloning and trehalose synthase preparation

For the production of recombinant TS from *Escherichia coli*, the TS gene was first identified by Southern analysis of *T. caldophilus* GK24 genomic DNA and plaque hybridization of its λ phage genomic library, using a synthetic DNA probe deduced from an N-terminal peptide sequence[14] of the TS from *T. caldophi-*



Scheme 2. TS reaction with trehalose and deuterated trehalose (**1**).

lus GK24. Then, the TS gene sequence[15] was determined and cloned into *E. coli* MV1184. *E. coli* cells harboring plasmid pTHS3 containing TS gene with a *tac* promoter were grown and induced by isopropyl 1-thio-β-D-galactopyranoside. Recombinant TS was purified by simple heat treatment of the induced *E. coli* cell extract, which was particularly effective for removing any *E. coli*-derived enzymes and glycosyl hydrolases, and then by DEAE-Sephacel column chromatography on FPLC.

1.2. Deuterium exchange reaction for deuterated α,α-trehalose

Disaccharide **1** (deuterated trehalose) was prepared by a Raney nickel-catalysed exchange reaction.[16] A solution of α,α-trehalose (3 g) in ($^2H_2$)O was heated under gentle reflux for 2 days. The product **1** (deuterated trehalose) recovered by filtration was directly used for the enzyme reaction and NMR analysis. ^1H and ^{13}C NMR spectra showed that H-2, H-4, H-6, and H-6′ of both D-glucose moieties of the trehalose had been completely exchanged with deuterium, giving **1** (deuterated trehalose), in which H-3 and H-5 were partly deuterated, as in Fig. 3a. Deuterated trehalose was purified by HPAEC, using a pulsed amperometric detector (PAD), on a Carbopac PA1 preparative column (9 × 250 mm).

1.3. TS reaction with deuterated α,α-trehalose

To examine the reaction pathway possibilities, a mixture of trehalose (5 mg) and **1** (deuterated trehalose, 3 mg), in 20 mM potassium phosphate buffer (pH 6.3) (3 mL), was subjected to the TS reaction for 12 h at 80 °C for conversion into maltose and deuterated maltose. Following equilibration, all reaction components corresponding to peaks a (deuterated trehalose, **1**), b (trehalose), c (deuterated glucose), d (glucose), e (deuterated α,β-trehalose), f (α,β-trehalose), g (deuterated maltose, **2**), and h (maltose) in the chromatogram (Fig. 1) were isolated by HPAEC with PAD, on a Carbopac PA1 preparative column (9 × 250 mm). It is noteworthy that the deuteriated molecules (**1**, deuterated glucose, and **2**) migrated slightly faster on the column than their unlabeled forms.

1.4. NMR and mass spectra

Three hundred megahertz ^1H and 75 MHz ^{13}C NMR spectra were recorded at 25 °C with a Varian instrument (UNITY300). Chemical shifts (δ) in ^1H and ^{13}C NMR spectra (Fig. 3b) are reported with reference to Me_4Si, taking δ 4.8 and 50.4 as the chemical shifts of HOD and MeOD, respectively, for D_2O solutions.

Mass spectra were recorded with an Finnigan spectrometer (Navigator) at an ionization source energy of 17–70 eV, 200 °C and 60 μA.

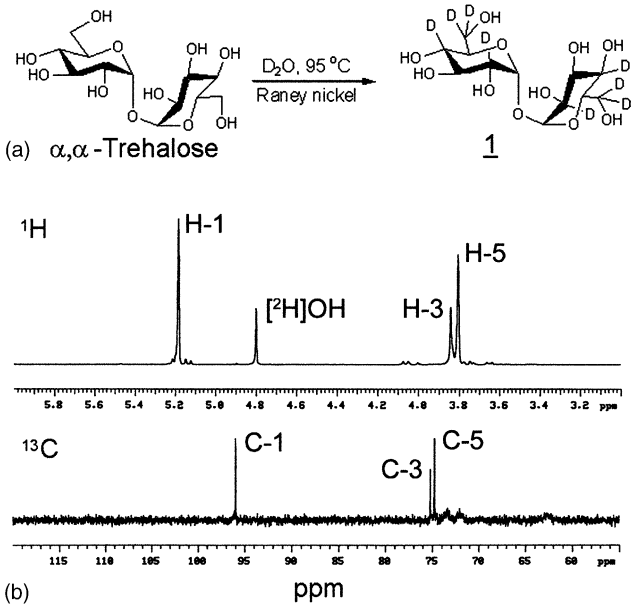


Fig. 3. Deuterium exchange reaction of α,α-trehalose; (a) the exchange reaction of trehalose in deuterium oxide catalyzed by Raney nickel at 95 °C for 2 days gave (2,4,6,6′,2′,4′,6″,6‴-$^2H_8$)trehalose (**1**); (b) 300 MHz ^1H and 75 MHz ^{13}C NMR spectra of deuterated α,α-trehalose (**1**) in deuterium oxide. (Chemical shifts of deuterated α,α-trehalose (**1**) in ^1H and ^{13}C NMR spectra are δ 5.19 (H-1), 3.85 (H-3), 3.81 (H-5), and δ 96.4 (C-1), 75.2 (C-3), 74.9 (C-5), respectively.)

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