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The unique nucleotide specificity of the sucrose synthase from *Thermosynechococcus elongatus*

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ABSTRACT

Sucrose synthase catalyzes the reversible conversion of sucrose and UDP into fructose and UDP-glucose. In filamentous cyanobacteria, the sucrose cleavage direction plays a key physiological function in carbon metabolism, nitrogen fixation, and stress tolerance. In unicellular strains, the function of sucrose synthase has not been elucidated. We report a detailed biochemical characterization of sucrose synthase from *Thermosynechococcus elongatus* after the gene was artificially synthesized for optimal expression in *Escherichia coli*. The homogeneous recombinant sucrose synthase was highly specific for ADP as substrate, constituting the first one with this unique characteristic, and strongly suggesting an interaction between sucrose and glycogen metabolism.

1. Introduction

Starch and sucrose (Suc) are the primary photosynthetic end products in most plants [1]. Suc plays a key role for transporting newly fixed carbon to heterotrophic tissues, is a major storage compound and acts as a signalling molecule to regulate many metabolic and developmental processes. Under physiological and stress conditions, Suc is important as a storage reserve in many species and serves as compatible solute in response to abiotic stress [2–5]. Its metabolism has been largely studied in plants; whereas the role of Suc in cyanobacteria is still not fully understood. In filamentous diazotrophic cyanobacteria, such as strains from the genus *Anabaena*, research has shown that Suc is involved in nitrogen fixation [6] and the biosynthesis of polysaccharides [7]. These cyanobacteria can differentiate a vegetative cell into a heterocyst for spatial separation of two incompatible processes: oxygen-dependent photosynthesis and oxygen-sensitive nitrogen fixation. Therefore, export of carbon from vegetative cells is needed to support nitrogen fixation in the heterocysts. Conversely, in nitrogen-fixing unicellular cyanobacteria, the incompatibility between oxygenic photosynthesis and nitrogen fixation is solved by temporal separation of both processes through circadian control of gene expression. Thus, Suc could be accumulated as temporary carbon storage during the day and used to fix nitrogen at night [8,9].

In plants Suc synthesis occurs through the combined action of Suc-phosphate synthase and Suc-phosphatase. Suc is converted to glucose (Glc) and fructose (Fru) by invertases or, alternatively, into UDP-Glc and Fru in a reaction catalyzed by Suc synthase (EC 2.4.1.13), a retaining glycosyl transferase with a GT-B fold [10,11]. The reaction catalyzed by Suc synthase is freely reversible *in vitro*; however, the physiological direction of the reaction seems to be towards Suc cleavage. UDP-Glc produced by Suc synthase is used for cell-wall biosynthesis and respiration, after its conversion to Glc-6P by UDP-Glc pyrophosphorylase and phosphoglucomutase [2,4]. Cyanobacteria metabolize Suc by a similar set of enzymes but they are capable of utilizing not only UDP-Glc but also ADP-Glc as substrates for Suc synthesis [3]. Regarding cyanobacterial Suc synthase, it has been purified and characterized from both recombinant and the original source from *Anabaena* sp. PCC 7119, 7120, and *Anabaena variabilis*, with similar properties [7,12,13]. Recently, Kolman et al. [14] showed the presence of Suc synthase in three unicellular cyanobacteria strains (*Microcystis aeruginosa*

Abbreviations: ADP-Glc, ADP-glucose; Fru, fructose; Glc, glucose; Suc, sucrose; UDP-Glc, UDP-glucose

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PCC 7806, *Gloebacter violaceus* PCC 7421, and *Thermosynechococcus elongatus* BP-1). Following functional characterization of Suc synthase encoding genes (*susA*) by heterologous expression in *Escherichia coli*, the authors proved an increase in their transcript levels after a salt treatment or hypoxic stress [14]. However, in this study these enzymes were not studied in detail.

T. elongatus is a rod-shaped unicellular cyanobacterium that inhabits hot springs and has an optimum growth temperature of 55 °C [15]. In 2002, the complete genome sequence of *T. elongatus* strain BP-1 was published [16] and, based on a 16S rRNA phylogenetic analysis, has been located on a branch very close to the origin of cyanobacteria [17]. *T. elongatus* is an obligate photoautotrophic organism and has been used largely as a model organism for the study of photosynthesis. To better understand Suc metabolism in unicellular cyanobacteria, we have synthesized the sequence encoding *T. elongatus* Suc synthase and characterized the recombinant protein. In this work we show for the first time a Suc synthase that prefers ADP rather than UDP, which has important implications in the carbohydrate metabolism of these organisms.

2. Materials and methods

2.1. Chemicals, enzymes and bacterial strains

Restriction enzymes were purchased from New England Biolabs (Ipswich, MA, USA). For cloning procedures, *E. coli* NEB Turbo cells (New England Biolabs) were used. Proteins were expressed using *E. coli* Tuner (DE3) cells (Merck, Darmstadt, Germany). Substrates and coupled enzymes used for Suc synthase activity assays were from Sigma–Aldrich (Saint Louis, MO, USA). All the other reagents were of the highest quality available.

2.2. Synthesis and sub-cloning of the Suc synthase encoding sequence from T. elongatus

The *T. elongatus* Suc synthase encoding sequence, based in the report of Nakamura et al. [16] (GenBank ID: BAC08600), was synthesized de novo using oligonucleotides designed by reverse transcription of the amino acid sequence with optimized codon usage for *E. coli*, as previously described [18]. We added a His_6-tag at the C-terminus to facilitate protein purification by immobilized metal ion affinity chromatography (IMAC). The full gene was cloned into the StrataClone vector (Agilent Technologies, Santa Clara, CA, USA) and sequenced (CRC DNA Sequencing Facility, University of Chicago, Chicago, IL, USA). Finally, it was subcloned into the pET24a vector (Merck) between *Nde*I and *Sac*I sites, and the resulting construction was used for expressing the recombinant protein in *E. coli* Tuner (DE3) cells.

2.3. Protein expression and purification

Recombinant *T. elongatus* Suc synthase was expressed in *E. coli* cells grown in a 2.8 l flask containing 1 l of Luria–Bertani (LB) medium with 50 μg/ml kanamycin, at 37 °C and 250 rpm until $OD_{600\ nm}$ ~0.6, and induced for 16 h at 25 °C with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside. All purification steps were performed at 4 °C. Cells were harvested by centrifugation at 5000×*g* for 10 min, resuspended with *Buffer C* [20 mM Tris–HCl pH 8.0, 200 mM NaCl, 10 mM imidazole, 10% (v/v) glycerol] and disrupted by sonication. The resulting suspension was centrifuged twice at 30000×*g* for 15 min and the supernatant (crude extract) was loaded onto a 1 ml His-Trap column (GE Healthcare, Piscataway, NJ, USA) previously equilibrated with *Buffer C*. The recombinant protein was eluted with a linear gradient from 10 to 200 mM imidazole in *Buffer C* (40 ml), and fractions containing Suc synthase activity were pooled and concentrated to 2 ml. The sample was then loaded onto a Superdex 200 16/60 column (GE Healthcare) equilibrated with 50 mM HEPES-NaOH pH 8.0 and 300 mM NaCl. Fractions containing Suc synthase activity were pooled, concentrated, supplemented with 10% (v/v) glycerol and stored at −80 °C. Under these conditions the enzyme was stable for at least 3 months.

2.4. Protein methods

Denaturing protein electrophoresis was conducted as described by Laemmli [19]. Prestained molecular mass markers were from Bio-Rad. Protein concentration of the purified enzyme was determined by absorbance at 280 nm with a NanoDrop 1000 (Thermo Scientific, Wilmington, DE, USA) using an extinction coefficient of 1.273 ml mg^{-1} cm^{-1}, which was determined from the amino acid sequence by using the ProtParam server (http://web.expasy.org/protparam/) [20].

2.5. Enzyme assays

Assay A, reverse direction, synthesis of Suc. ADP formation was continuously coupled with the production of pyruvate and further oxidation of NADH. Unless otherwise stated, the standard reaction mixture for the continuous coupled assay contained 50 mM HEPPS-NaOH pH 7.0, 10 mM $MgCl_2$, 0.3 mM phosphoenolpyruvate, 0.3 mM NADH, 1 mM ADP-Glc, 20 mM Fru, 2 U pyruvate kinase (PK), 2 U lactate dehydrogenase (LDH), 0.2 mg ml^{-1} BSA, and enzyme at an appropriate dilution, in a final volume of 200 μl. Alternatively, ADP-Glc was replaced by UDP-Glc and UDP production was coupled to NADH oxidation. Reactions were incubated at 37 °C in a 96-well microplate and oxidation of NADH was followed at 340 nm [21]. It has been reported that sugar-nucleotides are unstable at alkaline pH values in presence of $MgCl_2$ [22]. Therefore, our assays were performed at pH 7.0 and started by adding the NDP-Glc substrate. In addition, enzyme activity was determined at initial velocity, which minimizes any possible instability of the substrates.

Assay B, forward direction, cleavage of Suc. Unless otherwise stated, the standard reaction mixture contained 50 mM HEPPS-NaOH pH 7.0, 10 mM $MgCl_2$, 1 mM ADP, 200 mM Suc, and enzyme at an appropriate dilution, in a final volume of 50 μl. Alternatively, ADP was replaced by CDP, GDP, UDP, and TDP. Reactions were incubated at 37 °C for 10 min and stopped in a boiling water bath for 1 min. Fru production was discontinuously coupled to NAD^+ reduction by the addition of 1 mM ATP, 1 mM NAD^+, 0.5 U hexokinase, 0.5 U phosphoglucose isomerase, 0.5 U Glc-6P dehydrogenase, and 0.2 mg ml^{-1} BSA. Reduction of NAD^+ was determined at 340 nm [23]. All activity measurements were determined at initial velocities.

One unit of enzyme activity is defined as the amount of protein catalyzing the conversion of 1 μmol of product in 1 min under the specified conditions. The absorbance at 340 nm was followed either in an ELx808 microplate reader (BioTek, Winooski, VT, USA) or a Multiskan Ascent (Thermo Electron Corporation, Waltham, MA, USA).

2.6. Kinetic characterization

Data of enzyme activity were plotted versus substrate concentration and fitted to the Hill equation using the program Origin 8.0 (OriginLab Corporation). $S_{0.5}$ is defined as the concentration of substrate that produces 50% of the maximal velocity (V_{max}) and n_H is the Hill coefficient. Alternatively, data were fitted to a random bi–bi mechanism using the program previously reported by Ziegler et al. [24]. Kinetic parameters shown were the mean of two independent data sets reproducible within ±10%.

2.7. Stability and temperature dependence of Suc synthase activity

The activity of *T. elongatus* Suc synthase was determined in the Suc cleavage direction (*Assay B*) from 20 to 75 °C. Data of enzyme activity were used to calculate the activation energy (E_a) by Arrhenius plots [25]. Thermal stability of *T. elongatus* Suc synthase was analyzed by incubating the enzyme for 10 min over a temperature range of 45–72 °C in 50 mM HEPPS-NaOH pH 8.0 (control) and, alternatively, with the addition of substrates for Suc cleavage (1 mM ADP, 10 mM UDP, and 200 mM Suc). After incubation, aliquots were taken to determine the remaining activity in the direction of Suc cleavage (*Assay B*).

3. Results and discussion

3.1. Expression and purification of T. elongatus Suc synthase

Even though Suc synthase from plants and filamentous cyanobacteria have been previously studied [12,23,26–30], there is no thorough kinetic characterization of the enzyme from unicellular cyanobacteria. To shed light on Suc metabolism in *T. elongatus*, we synthesized the sequence encoding *T. elongatus* Suc synthase with the addition of a His_6-tag at the C-terminus (Supplementary Table S1). Because the gene was synthesized using the *E. coli* codon usage, there was a very high level of expression (Fig. 1, lane 3). The purified recombinant enzyme migrated in SDS–PAGE as a single band of ~95 kDa (Fig. 1, lanes 4 and 5), in accordance with the molecular mass calculated from the primary sequence (94.8 kDa). Purified *T. elongatus* Suc synthase eluted from the Superdex 200 gel filtration column as a 341 kDa protein (data not shown).

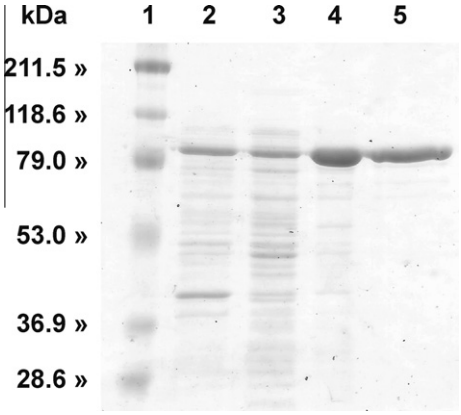

Fig. 1. Analysis of *T. elongatus* Suc synthase expression and purification by SDS–PAGE. The recombinant enzyme was highly expressed in the soluble fraction and purified by IMAC and gel filtration chromatography. Lane 1: prestained molecular mass markers; lane 2: insoluble fraction; lane 3: soluble fraction (crude extract); lane 4: IMAC fraction; lane 5: gel filtration fraction.

No significant changes in the migration pattern of the recombinant enzyme were observed when run in 50 mM HEPPS pH 7.0, 8.0, and 9.0, suggesting a tetrameric quaternary conformation within this pH range (data not shown), similar to what had been reported for the *Anabaena* sp. PCC 7119 enzyme [12].

3.2. Nucleotide specificity

It has been reported that the main nucleotide used by Suc synthase is UDP with a certain level of promiscuity [12,23,26–30] (see Supplementary Tables S2–S8). Porchia et al. [12] reported that the K_m for Fru was lower when using ADP-Glc rather than UDP-Glc for the *Anabaena* sp. PCC 7119 Suc synthase. We explored the specificity of *T. elongatus* Suc synthase for different nucleotides from pH 7.0–9.0. With CDP, GDP, TDP, and UDP the activity was higher at pH 7.0 (Fig. 2), in agreement with other cyanobacterial Suc synthases [12,14]. The activities with 1 and 10 mM ADP were similar at pH 7.0, suggesting that saturation had been already reached. Conversely, the activity for the other nucleotides was considerably higher at 10 mM, regardless of the pH (Fig. 2). At pH 7.0, and 10 mM concentration, the order of activity was CDP ~ GDP > UDP > ADP > TDP (Fig. 2). The calculated kinetic parameters of *T. elongatus* Suc synthase for different NDPs at pH 7.0 indicated a substrate preference for ADP (Table 1). In fact, at higher pH (9.0) the preference for ADP is striking: the activity with 10 mM ADP is 14- to 100-fold higher than with the other NDPs tested (Fig. 2). In agreement with Fig. 2, the smallest $S_{0.5}$ was for ADP, whereas those for CDP, GDP, TDP, and UDP were 5- to 8-fold higher (Table 1). Consequently, the highest $V_{max}/S_{0.5}$ ratio, analogous to the catalytic efficiency V_{max}/K_m for hyperbolic kinetics, was obtained with ADP (Table 1). To the best of our knowledge, this is the first report of a Suc synthase with a clear preference for ADP (see Supplementary Tables S2–S8).

3.3. ADP-Glc is a better substrate than UDP-Glc

The distinctive nucleotide specificity observed led us to analyze the preference for ADP-Glc or UDP-Glc of recombinant *T. elongatus* Suc synthase in the Suc synthesis direction at pH 7.0 (Table 2). The

Table 1
Kinetic parameters of *T. elongatus* Suc synthase in the direction of Suc cleavage. Reactions were performed at pH 7.0 using *Assay B* with varying NDP concentrations and 200 mM Suc, as described under Section 2.

Substrate	V_{max} (U mg^{-1})	$S_{0.5}$ (mM)	n_H	$V_{max}/S_{0.5}$ (U mg^{-1} mM^{-1})
ADP	1.28 ± 0.06	0.18 ± 0.02	1.1 ± 0.1	7.1
CDP	3.8 ± 0.2	0.9 ± 0.1	1.7 ± 0.4	4.2
GDP	3.9 ± 0.3	1.1 ± 0.2	1.5 ± 0.4	3.5
TDP	1.41 ± 0.05	1.4 ± 0.1	2.0 ± 0.3	1.0
UDP	2.2 ± 0.2	1.3 ± 0.3	1.1 ± 0.2	1.7

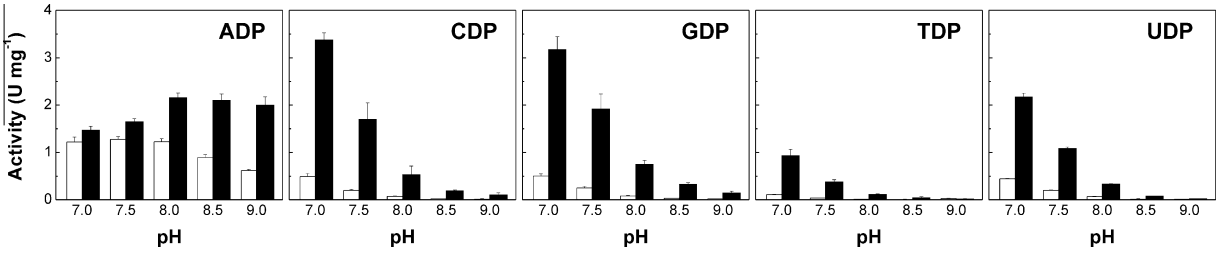

Fig. 2. Activity of *T. elongatus* Suc synthase with different NDPs. The activity of the enzyme was determined in the Suc cleavage direction (*Assay B*) using 1 mM (white bars) and 10 mM (black bars) NDPs and 200 mM Suc. Values are the mean of three independent measurements ± standard error. Assays were performed at pH values ranging from 7.0 to 9.0, as described under Section 2.

Table 2
Kinetic parameters of *T. elongatus* Suc synthase in the direction of Suc synthesis. Reactions were performed at pH 7.0 using *Assay A* with both ADP-Glc and UDP-Glc, as described under Section 2, to determine the specific activity (V_{max}), K_m, and the catalytic efficiency per active site (k_{cat}/K_m).

Substrate	V_{max} (U mg^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
ADP-Glc	1.1	0.033	70094
Fru$_{(ADP-Glc)}$		5.6	406
UDP-Glc	2.9	1.7	2673
Fru$_{(UDP-Glc)}$		12	380

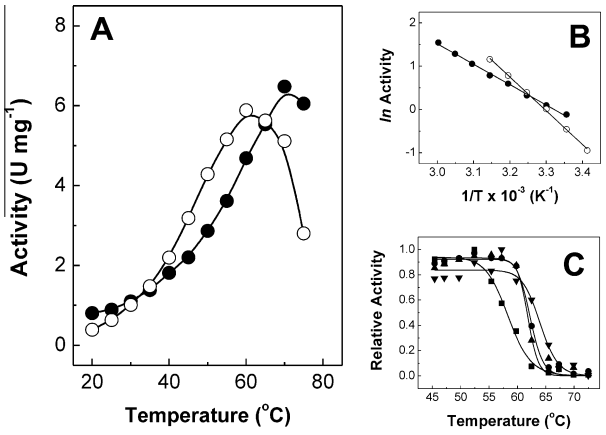

Fig. 3. Activity and stability of *T. elongatus* Suc synthase at different temperatures. Activity of the recombinant enzyme was assayed in the Suc cleavage direction (*Assay B*) at temperatures ranging from 20 to 75 °C. (A) Enzyme activity was assayed using 10 mM UDP (○) or 1 mM ADP (●). (B) Arrhenius plots for determining the E_a using data from (A) with UDP (○) and ADP (●). (C) Activity of *T. elongatus* Suc synthase after 10 min incubation at the stated temperatures in absence of substrates (■) or in presence of 1 mM ADP (●), 10 mM UDP (▲), and 200 mM Suc (▼). Enzyme activity assays were performed using the standard conditions, as described under Section 2.

V_{max} with UDP-Glc was 2.6-fold higher than with ADP-Glc, whereas the K_m for ADP-Glc was 51-fold lower than for UDP-Glc (Table 2). Remarkably, the K_m of *T. elongatus* Suc synthase for ADP-Glc was in the μM range, which was significantly lower than that observed for other Suc synthase forms [12,23,26–30]. Interestingly, the K_m for Fru was smaller when using ADP-Glc instead of UDP-Glc (Table 2), which is in good agreement to results obtained with the enzyme from *Anabaena* [12] (Table S2). The catalytic efficiency of *T. elongatus* Suc synthase was 26-fold higher with ADP-Glc than with UDP-Glc (Table 2). Conversely, no significant differences were observed in the catalytic efficiency for Fru when the co-substrate was either ADP-Glc or UDP-Glc (Table 2). A distinctive physiological consequence of the unique usage of adenine nucleotides by *T. elongatus* Suc synthase is that it could directly link both Suc and glycogen metabolisms [1,31].

3.4. Temperature dependence of T. elongatus Suc synthase activity and stability

Since *T. elongatus* is a thermophilic organism, we analyzed the activity of *T. elongatus* Suc synthase between 20 and 75 °C (Fig. 3A). In the direction of Suc degradation the activity reached a maximum at 60 °C with UDP and 70 °C with ADP (Fig. 3A). The maximal activity in both cases was ~6 U mg^{-1}. Interestingly, the E_a for the reaction with UDP was higher than with ADP (64.8 and 39.2 kJ mol^{-1}, respectively, Fig. 3B), which agrees with the preference of the enzyme for the latter (Table 1). The enzyme remained stable after 10 min incubation up to 50 °C, but it sharply decayed beyond 55 °C (Fig. 3C). Addition of 1 mM ADP, 10 mM UDP, or 200 mM Suc enhanced thermal stability by almost 5 °C, indicating that *T. elongatus* Suc synthase could bind separately both substrates (Fig. 3C).

In conclusion, our data showed that *T. elongatus* Suc synthase is highly specific for ADP and ADP-Glc, which constitutes the first report of a Suc synthase with this distinctive specificity. To analyze the nucleotide binding pocket of *T. elongatus* Suc synthase, we built two different models of the C-terminal domain using the crystal structure of *Arabidopsis thaliana* Suc synthase as template [11] (see Supplementary data). Fig. S1 shows that UDP and ADP interact differentially with residues close to the active site of the enzyme. We hypothesize that H-bonds established by UDP and ADP could lock the structure in different conformations, being the one with ADP more favorable for catalysis. A similar result was obtained when the C-terminal domain of the *Anabaena* sp. PCC 7119 Suc synthase was modeled with UDP and ADP (data not shown). However, biochemical data previously reported for the *Anabaena* enzyme showed similar K_m values for both UDP and ADP (Table S2) [12]. Sequence identity between Suc synthases from *T. elongatus* and *Anabaena* is 72%; therefore, other residues important for substrate binding and/or catalysis could be responsible for the observed differences in the kinetic parameters. Experiments to test our hypothesis are currently under way.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.11.011.

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