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First evidence of sucrose biosynthesis by single cyanobacterial bimodular proteins

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ABSTRACT

The net synthesis of sucrose (Suc) is catalysed by the sequential action of Suc-phosphate synthase (SPS) and Suc-phosphate phosphatase (SPP). SPS and SPP from *Anabaena* sp. PCC 7120 (7120-SPS and 7120-SPP) define minimal catalytic units. Bidomainal SPSs, where both units are fused, occur in plants and cyanobacteria, but they display only SPS activity. Using recombinant proteins that have fused 7120-SPS and 7120-SPP, we demonstrated that they are bifunctional chimeras and that the arrangement 7120-SPS/SPP is the most efficient to catalyse the sequential reactions to yield Suc. Moreover, we present the first evidence of a bidomainal SPS present in the cyanobacterium *Synechococcus elongatus* PCC 7942 with both, SPS and SPP activity.

1. Introduction

Studies in cyanobacteria and the availability of complete genome sequences have significantly increased our knowledge of the structure of proteins involved in sucrose (Suc) metabolism, their origin and further evolution. Enzymes involved in Suc biosynthesis are proteins with modular architectures that might have arisen from functional domains shuffled during evolution [1,2]. It has been reported that biosynthetic pathways are evolutionarily more modular than catabolic ones [3] and that in some cases one primordial multienzyme may have catalysed the whole sequence of reactions of an anabolic pathway [4]. Those enzymes may have given origin to more specific and efficient catalysts leading to extant pathways [4–6]. Thus, modularity can be exploited by both evolution and protein engineering through the study of domain recombination to generate functional chimeras [7].

In plants and cyanobacteria, the net synthesis of Suc is catalysed by a two-step pathway. From UDP-Glc (or ADP-Glc) and fructose-6P (Fru-6P), Suc-phosphate synthase (SPS, EC 2.4.1.14) produces Suc-6P, which in turn, is dephosphorylated by the specific Suc-phosphate phosphatase (SPP, EC 3.1.3.24) [2,8–9]. Studies in *Anabaena* sp. PCC 7120 (also known as *Nostoc* sp. PCC 7120) showed that 7120-SPS and 7120-SPP define two independent minimal catalytic units: a functional glucosyltransferase (GTD) and a phosphohydrolase (PHD), respectively [1]. Also, in *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 (two unicellular cyanobacteria) and in plants, it has been reported the presence of bidomainal SPSs (containing a functional GTD fused to a non-functional PHD) in addition to independent SPP proteins that are catalytic PHD. The presence of an inactive PDH in bidomainal SPS proteins was usually associated with the existence of a separate SPP protein [2,10–12].

The crystallization of SPS and SPP proteins contributed to accurately predict protein functionality. Crystallographic analyses using as models *Synechocystis* sp. PCC 6803 SPP (6803-SPP) [13] and SPS from *Halothermothrix orenii* [14] revealed the essential amino acids for their activities. Thus, *H. orenii* SPS consists of two domains, the A-domain: Ile^{7}-Gly^{229} and Tyr^{443}-Arg^{462}, and the B-domain: Val^{230}-Arg^{442} that form a deep substrate binding cleft at the interface. Both Fru-6P and Suc-6P bind to the A-domain while

Abbreviations: GTD, glucosyltransferase domain; PHD, phosphohydrolase domain; SPS, sucrose-phosphate synthase; SPP, sucrose-phosphate phosphatase; Suc, sucrose. The following prefixes indicate; 7120-, from *Anabaena* sp. PCC 7120; 7942-, from *Synechococcus elongatus* PCC 7942; 6803-, from *Synechocystis* sp. PCC 6803

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the donor substrate, nucleotide diphosphate glucose, binds to the B-domain. In 6803-SPP, the conserved residues of the I-III PHD motifs are represented by residues DLDNTW (9-14), LAYAT (37-41), and KX_{21}GDSGND (163-190), respectively.

The fact that some cyanobacterial genomes contain sequences homologous to those encoding bidomainal SPSs, together with the absence of an independent sequence coding for a putative SPP, raises the question whether bimodular SPSs could exhibit a dual activity leading to the disaccharide production.

The aim of this work was to analyse the potential of bidomainal SPSs to achieve Suc synthesis, by studying: (i) chimerical proteins generated by the fusion of functional GTD (7120-SPS) and PHD (7120-SPP); (ii) a comprehensive analysis on cyanobacterial sequences encoding putative bidomainal SPSs; and (iii) a functional characterisation of SPS from the unicellular strain *Synechococcus elongatus* PCC 7942. We present first evidence of chimerical and native SPSs with both SPS and SPP activity.

2. Materials and methods

2.1. Bacterial strains and growth

Escherichia coli DH5α and BL21(DE3):pLysS (Novagen) strains were grown in Luria Bertani medium supplemented with 50 μg/ml carbenicillin [15]. *Synechococcus elongatus* PCC 7942 was grown in BG11 medium [16] under a light/dark cycle (12:12 h) with white fluorescent light (20 μmol of photons m^{-2} s^{-1}) at 28 ± 2 °C with orbital shaking.

2.2. Cloning and expression of Suc biosynthesis genes

Genomic DNA purification, cloning, sequencing and PCR were carried out as described [1,12]. Plasmids were isolated and modified according to standard protocols [15]. Recombinant proteins were produced in *E. coli* BL21(λDE3):pLysS cells transformed with the corresponding plasmid based on the pRSET-A expression vector [17]. Each plasmid to produce a fusion protein (7120-SPS/SPP or 7120-SPP/SPS) was obtained from the reported plasmids constructed with the encoding sequence of *Anabaena* sp. PCC 7120 SPP or SPS-A (7120-SPP or 7120-SPS) [1,17], partially digested with *Eco*RI, and ligated with the other encoding sequence. In the resulting construction, two different linkers were generated, adding 3 or 4 amino acids in the recombinant 7120-SPS/SPP or 7120-SPP/SPS, respectively.

A 2127-bp DNA fragment containing an open reading frame (notated as putative HAD family hydrolase gene, accession number YP_399827) present in the *S. elongatus* PCC 7942 genome was PCR-amplified using the primer pair 5′-cggatccggcatggcagctcaaaatctctaca-3′ and 5′-cgagctcgtaactgaaaaggttaagccgcctcaa-3′. The amplification product was ligated into the expression vector to obtain the recombinant protein for further characterisation.

2.3. Purification of proteins and enzyme assays

His_6-tagged 7120-SPS, 7120-SPP, 7120-SPS/SPP, 7120-SPP/SPS and 7942-SPS fusion proteins were purified by Co^{2+}-affinity chromatography (TALON resin, Clontech). Recombinant proteins were eluted from the column with a stepwise imidazole pH 7.5 gradient (50, 100, 150 and 250 mM). Fractions containing the purified recombinant proteins were pooled and concentrated in an Amicon ultrafiltration cell (Newtown, PA). Protein concentration was determined by the Bradford's reagent or by absorbance at 280 nm. Purified enzymes were stored at −20 °C. Proteins were separated by SDS–PAGE using a MiniProtean system (BioRad, USA) and 10-well combs (0.75 mm) on 10% polyacrylamide gels

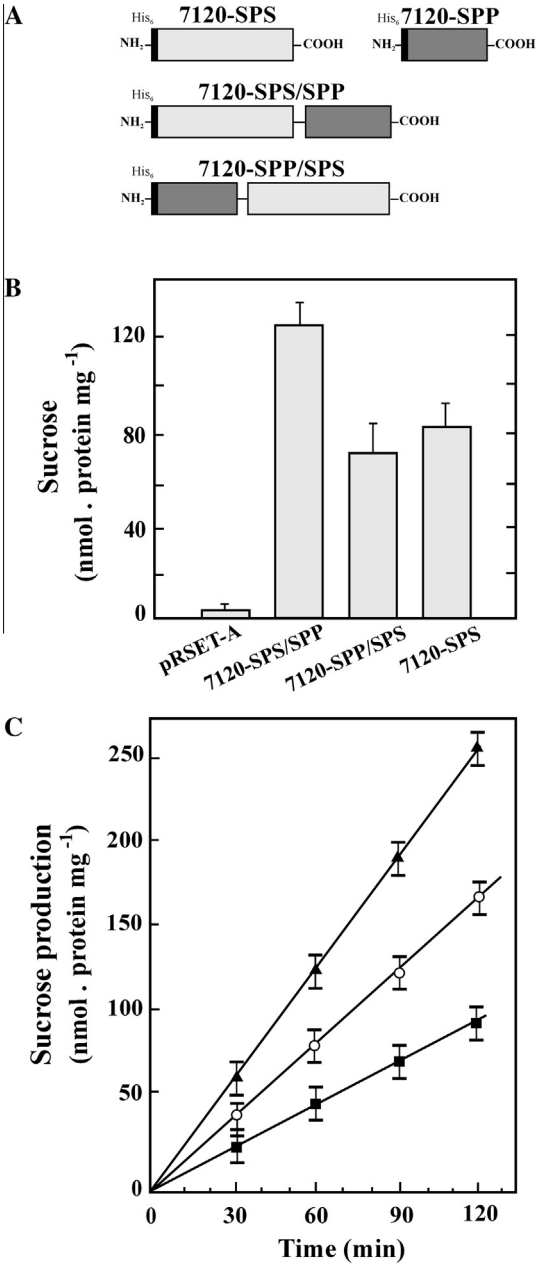

Fig. 1. Sucrose synthesis by bimodular recombinant proteins with different 7120-SPS and 7120-SPP arrangements. (A) Schematic representation of the construction of the two chimeral proteins (7120-SPS/SPP and 7120-SPP/SPS). (B) Suc accumulation in *E. coli* cells overexpressing 7120-SPS/SPP or 7120-SPP/SPS. For comparison, results from *E. coli* cells expressing 7120-SPS were included. (C) Time course for Suc production from UDP-Glc and Fru-6P by purified recombinant proteins. (▲), 7120-SPS/SPP. (○), equimolar mixture of 7120-SPP + 7120-SPS. (■), 7120-SPS/SPP. Aliquots of 10 μl were withdrawn each 30 min and Suc content was quantified.

and the recombinant polypeptides were visualized with Coomassie blue [1,17].

Protein extracts from *E. coli* cells and enzyme activity were performed as described [1,17]. Partial purification of native SPS from *S. elongatus* PCC 7942 (7942-SPS) was carried out from cultures at exponential phase grown in BG11 medium with 171 mM NaCl [8]. Protein extracts were absorbed onto a column of Deae–Sephacel and the enzymes were eluted with a linear NaCl gradient. Fractions with SPS and SPP activities were pooled and concentrated. Polypeptide sizes were determined by SDS–PAGE analyses [17].

Enzyme activities were assayed in purified recombinant proteins or in Sephadex-G50-desalted extracts. SPS was measured

Table 1
Kinetic parameters of recombinant chimeral proteins. Data are the mean values and standard deviation of three independent experiments. Values correspond to the overall synthesis of Suc from Fru-6P and UDP-Glc. For comparison, data for 7120-SPS ([a]) and 7120-SPP ([b]) are from Refs. [2] and [17], respectively.

Recombinant protein	Fru-6P			UDP-Glc			Suc-6P		
	$K_{m\ app}$ (mM)	V_{max} (nmol s^{-1} mg^{-1})	$V_{max}/K_{m\ app}$ (nmol s^{-1} mg^{-1} mM^{-1})	$K_{m\ app}$ (mM)	V_{max} (nmol s^{-1} mg^{-1})	$V_{max}/K_{m\ app}$ (nmol s^{-1} mg^{-1} mM^{-1})	K_m (mM)	V_{max} (nmol s^{-1} mg^{-1})	$V_{max}/K_{m\ app}$ (nmol s^{-1} mg^{-1} mM^{-1})
7120-SPS/SPP	0.70 ± 0.05	35 ± 5	50	1.5 ± 0.5	55 ± 10	40	0.50 ± 0.05	75 ± 8	160 ± 5
7120-SPP/SPS	5 ± 1	20 ± 5	4	2 ± 0.5	25 ± 5	12.5	0.33 ± 0.05	100 ± 10	300 ± 10
7120-SPS[a]	2 ± 0.3	75 ± 3	35	1.3 ± 0.3	78 ± 3	52	–	–	–
7120-SPP[b]	–	–	–	–	–	–	0.35 ± 0.05	135 ± 10	380 ± 10

Table 2
Occurrence of putative bidomainal SPSs in cyanobacteria. Accession numbers of deduced amino-acid sequences and percentage amino acid identity using characterised SPP [([a]) 6803-SPP] and SPSs [([b]) 7120-SPS and ([c]) 6803-SPS] as query. The first column contains separated SPP sequences when bidomainal SPSs are present. Bidomainal SPSs with PHDs with potential to have SPP activity are in bold letters.

Cyanobacterium	SPPs Accession no.	Bidomainal SPSs %[a]	Accession no.	%[b]	%[c]
Filamentous strain					
Nodularia spumigena CCY9414	ZP_01628463	42	ZP_01629520	27	52
Unicellular strains					
Synechocystis sp. PCC 6803	NP_441739	100	NP_442711	27	97
Cyanobium sp. PCC 7001	ZP_05045012	29	ZP_05045051	30	54
Prochlorococcus marinus MIT 9303	–	–	YP_001019012	26	40
Prochlorococcus marinus MIT 9313	–	–	NP_896092	26	40
Synechochoccus sp. CC9605	–	–	YP_382969	26	40
Synechochoccus sp. CC9902	–	–	YP_378316	26	41
Prochlorococcus marinus NATL1A	–	–	YP_001016015	26	38
Prochlorococcus marinus NATL2A	–	–	YP_292514	25	38
***Synechococcus elongatus* PCC 6301**	–	–	**YP_171440**	**27**	**46**
***Synechococcus elongatus* PCC 7942**	–	–	**YP_399827**	**27**	**46**
Synechochoccus sp. WH 7803	–	–	YP_001226250	27	39
Synechochoccus sp. WH8102	–	–	NP_898609	25	39
Synechococcus sp. BL107	–	–	ZP_01469083	26	41
Synechococcus sp. CB0101	ZP_07972524	29	ZP_07974999	27	40
Synechococcus sp. CB0205	ZP_07969422	26	ZP_07969740	27	39
Synechococcus sp. PCC 7002	YP_001734147	28	YP_001734148	28	61
Synechococcus sp. RS9916	–	–	ZP_01471531	26	39
Synechococcus sp. RS9917	–	–	ZP_01079206	27	40
Synechococcus sp. WH 8109	–	–	ZP_05789248	26	41
Synechococcus sp. WH7805	–	–	ZP_01124878	27	39
***Thermosynechococcus elongatus* BP-1**	–	–	**NP_681372**	**27**	**51**

according to Porchia and Salerno [8]. The identification of Suc as a final product of the action of *Anabaena* recombinant proteins was carried out as described [18]. SPP activity was assayed by measuring the release of Pi [19]. The catalytic activity was proportional to the amount of enzyme and linear with time. Kinetic parameters (apparent K_m and V_{max} values) were determined from purified recombinant enzymes [8].

2.4. Sucrose content determination

Cells (500 mg fresh weight) were lyophilized and extracted with 80% (volume/volume) ethanol at 80 °C. Pooled extracts were evaporated, and the residue was suspended in water. Suc was determined in ethanol extracts by measuring fructose and glucose after hydrolysis with acid invertase using a coupled-enzyme method [20,21]. Enzymes and chemicals for biochemical analyses were obtained from Boehringer (Germany) and Sigma-Aldrich (USA).

2.5. Bioinformatic analyses

Nucleotide sequences with similarity to 6803-SPS and 7120-SPP (*E*-value cutoff $\leqslant 1e^{-20}$) were retrieved using BLAST and the nonredundant protein databases of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Multiple protein sequence alignments were performed using ClustalW from the MEGA5 program [22] and consensus sequence logos were made using the WebLogo 3 server [23].

2.6. Structure prediction

I-TASSER server [24] was used to predict the protein structure of 7942-SPS. Proteins with similar fold templates were retrieved from the PDB library by LOMETS [25], a meta-threading sever with nine locally installed threading programs. Consensus templates from these programs were collected and submitted for iterative structure refinement. Figures were generated using the UCSF Chimera package [26].

3. Results

3.1. Sucrose synthesis from bidomainal chimerical proteins

To investigate the modular arrangement of bidomainal SPSs, two hybrid proteins were produced in *E. coli* cells from an expression vector harbouring the fusion of the encoding sequences of 7120-SPS and 7120-SPP, minimal catalytic modules coincident

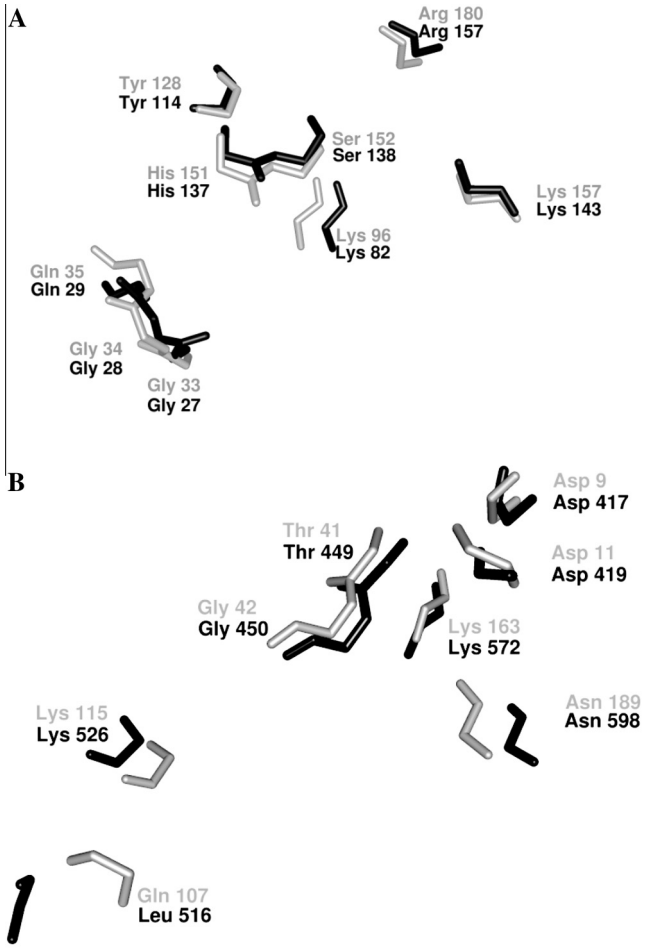

Fig. 2. Modelling of putative active sites of 7942-SPS GTD and PHD. (A) Superimposed view of the SPS of *H. orenii* in the closed conformation (gray) with the predicted structure of the GTD of 7942-SPS (black). (B) Superimposed view of the 6803-SPP active site (gray) with the predicted structure of the PHD of 7942-SPS (black).

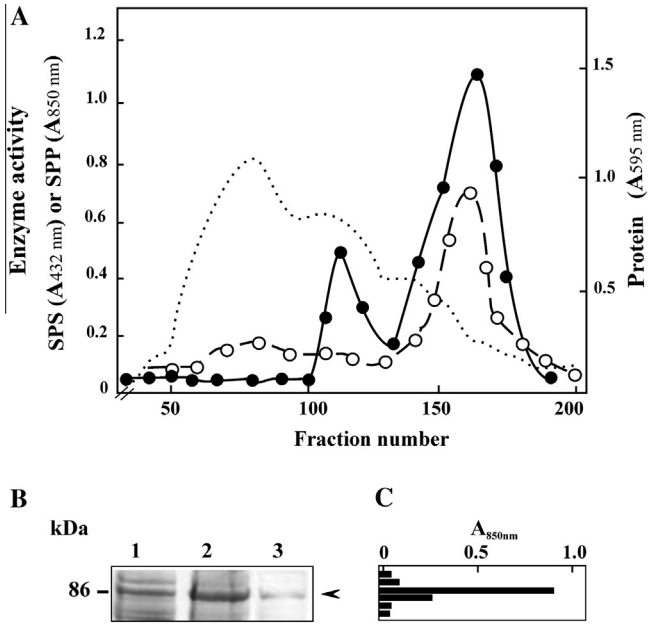

Fig. 3. SPS and SPP in *Synechococcus* sp. PCC 7942 cells. (A) Deae-Sephacel chromatography of *S. elongatus* protein extract. SPP (●) and SPS (○) activity. Dotted line, protein profile. (B) Expression of His_6::7942-SPS in *E. coli* cells. Coomassie blue staining of the recombinant protein after SDS–PAGE on a 12% gel. Lane 1, crude extract from non-induced *E. coli* cells. Lane 2, crude extract from an IPTG-induced culture. Lane 3, His_6::7942-SPS purified from a metal ion affinity chromatography. The arrowhead indicates the position of His_6::7942-SPS. (C) SPP activity determined in slices of gel of a parallel lane loaded with an aliquot of the purified recombinant protein.

with a GTD and PHD, respectively [1,17]. To generate the 7120-SPS/SPP chimera, the C-terminus of 7120-SPS encoding sequence was fused in frame with the N-terminus of that of 7120-SPP, and vice versa, for the 7120-SPP/SPS protein (Fig. 1A). Suc accumulation was shown in *E. coli* cells expressing either 7120-SPS/SPP or 7120-SPP/SPS (Fig. 1B).

Total reaction rate was determined from the purified chimeral proteins to analyse the effect of the physical proximity of the two functional domains. For comparison, a parallel assay was carried out with an equimolar mixture of purified 7120-SPS and 7120-SPP [1,17]. As shown in Fig. 1C, both chimeras are functional and produce Suc in a time-dependent manner. The rate of Suc synthesis by 7120-SPS/SPP was approximately 55% faster under the standard assay conditions in comparison with the equimolar mixture of the individual enzymes. In contrast, the rate of Suc synthesis by 7120-SPP/SPS was 45% lower than the mixture.

Kinetic parameters were determined for the chimerical proteins and compared with those of the individual enzymes (Table 1). Since it was not possible to separate the two sequential reactions catalysed by the chimeras, the kinetic parameters were determined for the overall synthesis of Suc from Fru-6P and UDP-Glc. The fusion 7120-SPS/SPP showed about 2.8-fold increase in affinity for Fru-6P in comparison with 7120-SPS. The V_{max}/K_m value for 7120-SPS/SPP was about 42% and 12-fold higher than that of 7120-SPS and 7120-SPP/SPS, respectively (Table 1). Additionally, the kinetic parameters for the SPP activity of 7120-SPS/SPP and 7120-SPP/SPS fusions were also determined. The K_m and V_{max}/K_m for Suc-6P dephosporylation of 7120-SPP/SPS were similar to those of SPP. In contrast, catalytic efficiency of 7120-SPS/SPP for SPP activity was lower to that of 7120-SPP, suggesting that the SPP location at the carboxyl-terminal end seems not to facilitate the substrate access to the catalytic site.

Having demonstrated that a bidomainal SPS with a GTD–PHD arrangement efficiently catalyses Suc biosynthesis, we decided to investigate the presence of bifunctional SPSs in extant cyanobacteria.

3.2. Bidomainal SPSs in cyanobacterial genomes

In addition to functionally characterised SPSs from *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 [10,12], we retrieved homologs to bidomainal SPS encoding genes from other 19 genomes of unicellular cyanobacteria and from only one genome of a filamentous strain (*Nodularia spumigena* CCY9414) (Table 2). SPSs with fused GTD-PHD were found in *Cyanobium* (also *Synechococcus*) sp. PCC 7001, *Thermosynechococcus elongatus* BP-1, in four members of *Prochlorococcus marinus* and in another 13 *Synechococcus* spp. strains.

Sequence alignment analysis of the GTD regions of bidomainal SPSs show that box I and II motifs (Supplementary Fig. 1), and especially the key residues of the SPS active site [14], are conserved. In contrast, some of the essential residues for SPP catalytic activity are substituted in the PHD regions of most bidomainal SPSs analysed (Supplementary Fig. 2). Particularly, the key amino acids for 6803-SPP activity (Asp^{185}, Ser^{186} and Asn^{188} of box III) are not conserved. However, putative SPSs from *S. elongatus* PCC 7942 (7942-SPS), *Synechococcus* PCC 6301 and *Thermosynechococcus*

elongatus BP-1 (Table 2) have all the amino acids reported as involved in SPP activity. Since to date there is no demonstration of the existence of a bifunctional SPS and there is no homologous sequence to SPP in the *S. elongatus* genome, we focused the following experiments on the putative 7942-SPS.

A structural prediction analysis of the 7942-SPS N-terminal region was performed using different methods of protein-threading to identify similar structural templates (Fig. 2A). The active site structure of the GTD region (1–411 residues) was superimposed with the crystal structure of *H. orenii* SPS (PDB2r60A) [14]. Although globally the correlation between both structures has a relatively low score (TM 0.616 and RMSD 4.92 Å), the amino-acid residues involved in the synthesis of Suc-6P is highly conserved. Also, a structure model of the C-terminal region (PHD, 412-654 residues, Fig. 2B) was obtained after superimposing with the crystal structure of 6803-SPP (PDBitj5) (TM 0.87 and RMSD 2.26 Å). The result show that the amino acids reported as involved in the binding of Mg^{2+} and Suc-6P (Asp^{417}, Asp^{419} and Asp^{598}) are conserved in 7942-SPS (Fig. 2B).

3.3. Sucrose synthesis from the bidomainal 7942-SPS

7942-SPS was partially purified from salt-treated *S. elongatus* cells. SPS and SPP activity were assayed in the fractions eluted from a Deae-Sephacel column (Fig. 3A). SPS activity overlaps with the major peak of SPP activity. Also, both activities co-migrated in an isoelectrofocusing gel experiment (not shown), strongly suggesting that a single protein may be responsible for both activities.

Additional evidence of the bifunctionality of 7942-SPS was obtained when the predicted encoding sequence (YP_399827) was expressed in *E. coli* cells. The recombinant His_6::7942-SPS showed both SPS and SPP activities (Fig. 3B), and Suc, as final product of the sequential reactions, was also identified (Fig. 3C). Approximately, 30 nmoles of Suc per mg of total protein was accumulated after 24 h of incubation, demonstrating that bidomainal 7942-SPS dephosphorylated Suc-6P.

4. Discussion

Suc plays an important role in oxygenic-photosynthetic organisms and its biosynthesis is a typical example to follow the evolution of modular enzymes [2,27]. Different evolutionary mechanisms have been proposed for the enzymes catalysing the two-step pathway of Suc biosynthesis [2,28–29]. However, it is unlikely that SPS and SPP might evolved separately considering the instability of the intermediate (Suc-6P, the transglycosylation product of SPS), which has only one function: to be used by SPP to produce Suc. After physical and kinetic evidence, plant SPSs (bimodular proteins) and SPP were proposed to form a metabolite channel [30]. This protein–protein interaction may be attributed to sharing a PHD (pseudo enzyme SPP domain) present in bidomainal SPSs from plants, *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 [2,10,12]. In the present study, we show the first example of a native bifunctional SPS protein and, consequently, the intramolecular channelling of the intermediate Suc-6P. The fact that the chimerical 7120-SPS/SPP (GTD-PHD) arrangement was more efficient to produce Suc than the combination of separated 7120-SPS and 7120-SPP, demonstrates the catalytic improvement of the fusion of the minimal catalytic units (Fig. 1 and Table 1), and the importance of the intramolecular channelling in the bifunctional chimera.

Sequence analysis of cyanobacterial genomes uncovered that the absence of putative active SPP domain in SPS proteins correlates with the presence of a separate SPP-encoding gene (Table 2). Conversely, the occurrence of only putative bidomainal SPSs with conserved key catalytic residues in the PHD module (Supplementary Figs. 1 and 2, and Fig. 2) led to investigate the potential dual activity of 7942-SPS, which was shown to accumulate high levels of Suc (up to ~300 mM) under osmotic stress [31–32]. The C-terminal of 7942-SPS retains the critical residues of the Suc-6P binding site in accordance with the crystallized 6803-SPP [14]. Importantly, we demonstrated that 7942-SPS shows both SPS and SPP activity, demonstrating the intramolecular channelling of Suc-6P in a bidomainal protein of an extant organism. The presence of a separate SPP in *S. elongatus* PCC 7942 cannot be discarded because a minor SPP activity peak was detected in the ion exchange chromatography (Fig. 3) eluting at a salt concentration where other SPPs do [8,19]. Since there is no independent coding sequence for SPP, one possible explanation could be that an additional transcriptional starting point may be present in the 7942-SPS nucleotide sequence.

The biosynthesis of Suc has a precedent in the analogous two-step pathway involved in the synthesis of trehalose. Recently, it was shown the occurrence of a bifunctional enzyme (OtsAB) responsible of the disaccharide net synthesis in the cyanobacterium *Crocosphaera watsonii* WH8501 [33].

In summary, our results support that Suc biosynthesis enzymes may have evolved keeping its functional modularity. While in some cyanobacteria, intramolecular channelling could be taken place through bifunctional SPSs to protect the intermediate, in most strains, similarly to plants, it is likely an interaction between bidomainal SPSs, with a non-active PHD module, and SPP, may be interacting and contributing to the efficient synthesis of Suc. An outstanding question to be answered is how the transfer of the reaction intermediate (Suc-6P) is performed when unimodular enzymes, minimal catalytic units (e.g., 7120-SPS and 7120-SPP) are responsible for Suc synthesis.

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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.2013.04.012.

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