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TREHALOSE SYNTHESIS IN *EUGLENA GRACILIS* (EUGLENOPHYCEAE) OCCURS THROUGH AN ENZYME COMPLEX[1]

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The aim of this study was to isolate and characterize a trehalose-synthesizing enzyme from *Euglena gracilis* Klebs. After purification by anion exchange chromatography, gel filtration, isoelectric focusing, and native electrophoresis, trehalose-6-phosphate synthase (TPS, EC 2.4.1.15) and trehalose-6-phosphate phosphatase (TPP, EC 3.1.3.12) activities could not be separated. Consequently, a TPS/TPP enzyme complex of about 250 kDa was suggested as responsible for trehalose synthesis in *E. gracilis*. The TPS activity was shown to be highly specific for glucose-6-P, and UDP-Glc was the preferred glucose donor, but GDP-Glc and CDP-Glc could also act as TPS substrates. The TPP activity was highly specific for trehalose-6-P. *In vitro* phosphorylation assays revealed rapid decreases in TPS and TPP activities. These changes corresponded to variations in the elution profile of gel filtration chromatography after the phosphorylation treatment. Taken together, these results suggest that the proposed TPS/TPP complex might be regulated through a protein phosphorylation/dephosphorylation-mediated mechanism that could affect the association state of the complex. Such a regulatory mechanism might lead to a rapid change in trehalose synthesis in response to variations in environmental conditions.

Key index words: ***Euglena gracilis*; protein phosphorylation; trehalose biosynthesis; trehalose-phosphate phosphatase; trehalose-phosphate synthase**

Abbreviations: **M_r, relative molecular mass; PP2A, protein-phosphatase 2A; Ser/Thr, serine-threonine; TPP, trehalose-phosphate phosphatase; TPS, trehalose-phosphate synthase; Tyr, tyrosine**

Trehalose (α-D-glucopyranosil-[1,1]-α-D-glucopyranoside) is a widespread nonreducing disaccharide found in bacteria, fungi, and invertebrates (Elbein 1974). Evidence suggests that the occurrence of trehalose might also be extensive in plants (Eastmond et al. 2002, Wingler 2002). In addition to its function as a storage carbohydrate and transport sugar, trehalose plays an important role as stress protectant (Van Laere 1989, Wiemken 1990, Crowe et al. 1998, Argüelles 2000).

Three different pathways have been reported for trehalose biosynthesis: 1) a two-step process through the sequential action of trehalose-phosphate synthase (TPS, EC 2.4.1.15) and trehalose-phosphate phosphatase (TPP, EC 3.1.3.12) activities, 2) through the action of trehalose synthase (maltose glucosylmutase) (EC 5.4.99.16), and 3) through the successive activities of malto-oligosyl trehalose synthase (EC 5.4.99.15) and malto-oligosyl trehalosehydrolase (EC 3.2.1.141). The first route, involving TPS and TPP, is better characterized and was shown to be present in archaebacteria, eubacteria, yeast, fungi, invertebrates, and plants (Elbein 1974, Londesborough and Vuorio 1993, Eastmond et al. 2002). On the other hand, the trehalose synthase and the malto-oligosyl trehalose synthase/malto-oligosyl trehalosehydrolase pathways were only reported in the genera *Pimelobacter*, *Thermus*, *Pseudomonas*, and *Mycobacterium* (Ohguchi et al. 1997, Tsusaki et al. 1997a,b, De Smet et al. 2000) and in *Arthrobacter*, *Rhizobium*, *Sulfolobus*, and *Mycobacterium* (Maruta et al. 1996a,b,c, De Smet et al. 2000), respectively.

The regulation of trehalose synthesis has been studied mainly in *Saccharomyces cerevisiae* and *Escherichia coli*. Increases in the transcription of TPS and TPP encoding genes were reported as a response to different stress conditions in both organisms (Strøm and Kaasen 1993, Winderickx et al. 1996, Wolschek and Kubicek 1997, Pereira et al. 2001). In *S. cerevisiae*, where trehalose is synthesized through a trehalose synthase complex, the catalytic TPS and TPP polypeptides were shown to be combined with two different additional subunits with regulatory properties (De Virgilio et al. 1993, Vuorio et al. 1993, Reinders et al. 1997). The effect of some metabolites on TPS activity (Fruc-6-P, activator, and Pi, inhibitor) was reported as a different control level of trehalose synthesis (Londesborough and Vuorio 1993).

In *Euglena gracilis*, a free-living photosynthetic protist with a remarkable ability for adaptation to environmental changes, trehalose synthesis was proposed to have a relevant role in the adaptation to salt stress (Takenaka et al. 1997, Porchia et al. 1999), and it was suggested to occur by the concomitant action of TPS and TPP. In previous studies using permeabilized cells, the incorporation of radioactivity to the disaccharide was shown to take place from UDP-[^{14}C]Glc in the

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presence of Glc-6P (Porchia et al. 1999). This study presents evidence to suggest that a TPS/TPP enzyme complex would be involved in trehalose synthesis in *E. gracilis*, which may be regulated through an association/dissociation mechanism mediated by phosphorylation.

MATERIALS AND METHODS

Biological material. Axenic cultures of *E. gracilis* Klebs strain Z were grown at 27 ± 2° C in Hutner's medium (Hutner et al. 1966) modified as described by Porchia et al. (1999). Salt treatment was performed by adding different amounts of NaCl to cell cultures at the late exponential phase. Cells were collected 24 h after salt addition.

Enzyme extraction and purification. All operations were carried out at 2–4° C unless otherwise stated. Cells were disrupted by sonication at 40 W applied three to four times for 15 s. Cell debris was removed by centrifugation at 40,000 *g*, and the supernatant was chromatographed through a DEAE-Sephacel column (Amersham-Pharmacia Biotech, Uppsala, Sweden) as previously reported (Porchia et al. 1999). Proteins were eluted with a linear NaCl gradient (0–0.5 M), and the fractions were analyzed for TPS and TPP activities. Those fractions with high enzyme activity were pooled, concentrated in an ultrafiltration membrane cone (Centriflo Amicon, Danvers, MA, USA), and further purified either by Sepharose-6B or Superose-12 (Amersham-Pharmacia Biotech) chromatographies as described elsewhere (Salerno et al. 1998, Pagnussat et al. 2002).

Fractions with high TPS and TPP activities were pooled and concentrated as described above. Sepharose-6B concentrated fractions were used for further biochemical characterization. Liquid isoelectric focusing of pooled Sepharose-6B fractions (1–2 mg protein) was performed in a Rotofor System (BioRad Laboratories, Hercules, CA, USA) containing 2% ampholytes pH 3–9 in a volume of 55 mL. Focusing was carried out at 12 W of constant power until voltage stabilization (approximately 2.5 h), and the contents of the 20 Rotofor cell compartments were collected into separated tubes. Fractions were analyzed for pH and TPS and TPP activities; simultaneously, aliquots of 0.8 mL were precipitated with 10% trichloroacetic acid. Protein pellets were taken with Laemmli's buffer (Laemmli 1970), loaded in denaturing polyacrylamide gel, and electrophoresed (SDS-PAGE). Proteins were estimated according to Bradford (1976).

Enzyme assays. Trehalose-phosphate synthase was assayed by incubating at 30° C in a total volume of 0.05 mL. The reaction mixture contained 10 mM UDP-Glc, 10 mM $MgCl_2$, 10 mM Glc-6P, 100 mM HEPES-NaOH buffer (pH 7.0), and variable amounts of enzyme preparations. The product was measured by using the anthrone method (Porchia et al. 1999). Trehalose-phosphate synthetase activity was determined according to Chifflet et al. (1988) after incubation at 30° C in a total volume of 0.05 mL containing 2 mM trehalose-6-P, 100 mM HEPES-NaOH buffer (pH 7.5), and variable amounts of enzyme preparations.

Phosphorylation/dephosphorylation assays. Phosphorylation assays were carried out at 25° C in a total volume of 0.05 mL. The reaction mixtures contained 40 mM MOPS-NaOH buffer (pH 7.5), 8 mM $MgCl_2$, 2.5 mM dithiothreitol, 10 mM orthovanadate, 10 mM glycerol phosphate, 10 mM NaF, and aliquots of enzyme preparations (either crude extracts or concentrated fractions corresponding to the DEAE-Sephacel step). Where indicated, EGTA, stauroporine (a specific serine/threonine [Ser/Thr]-protein kinase inhibitor), or genistein (a specific tyrosine [Tyr]-protein kinase inhibitor) was added up to a final concentration of 5 mM, 0.5 μM, or 0.25 μM, respectively. Reactions were started by the addition of 0.1 mM ATP. At different times of incubation, aliquots were taken for measuring TPS and TPP activities. Alternatively, *in vitro* radioactive phosphorylation assays were carried out in similar reaction mixtures except 100 mM [γ^{32}P]ATP (500 cpm · pmol^{-1}) was added. Reactions were stopped by the addition of Laemmli's buffer (Laemmli 1970). Polypeptides were separated by SDS-PAGE on 12% polyacrylamide gels (Salerno et al. 1998), electroblotted onto a nitrocellulose membrane, and autoradiographed. The membrane was also immunorevealed (Western blots) with polyclonal antibodies raised in rabbits against the *E. gracilis* TPS/TPP complex (anti-*Eug*TPS/TPP) according to Salerno et al. (1998).

Dephosphorylation assays were performed at 30° C onto previously phosphorylated mixtures by adding 15 U of either protein-phosphatase 2A (PP2A, 5000 U/mg, a specific Ser/Thr-protein phosphatase, partially purified from wheat by Dr. G. Martínez Noël, CIB-FIBA, Mar del Plata, Argentina) or alkaline phosphatase type III from *E. coli* (60 U/mg, Sigma, St. Louis, MO, USA).

RESULTS

Trehalose synthesis in E. gracilis. A series of chromatography steps was performed to characterize the enzyme activities involved in trehalose biosynthesis in *E. gracilis*. Either after anion exchange chromatographies in DEAE-Sephacel at different pHs and elution conditions or after gel filtration on Superose-12 or Sepharose-6B columns, TPS and TPP activities coeluted in a single peak corresponding to a relative molecular mass (M_r) of about 250 ± 30 kDa (Fig. 1, A and B, and data not shown). Moreover, TPS and TPP activities were detected together in a single band when native gel electrophoresis and isoelectric focusing were performed (Fig. 1, C and D). Polyclonal antibodies raised against the native gel band with TPS and TPP activities (anti-*Eug*TPS/TPP) detected a single band of 80 kDa in Western blots after SDS-PAGE, in fractions corresponding to TPS and TPP activities of all the purification steps performed (Fig. 1E, fraction number 9, and data not shown). The copurification of TPS and TPP activities suggests that these enzymes may be associated in a multienzyme complex in *Euglena* cells. The low level of activity recoveries after the gel filtration steps may be due to either enzyme lability or the loss of an unknown activating/stabilizing factor of the complex. Further purification led to complete loss of enzyme activities (not shown). Table 1 summarizes the TPS/TPP purification steps performed.

Biochemical study of the trehalose biosynthesis activities. Trehalose synthesis through TPS/TPP was demonstrated to take place from Glc-6-P and, preferentially, UDP-Glc as the glucosyl donor, although GDP-Glc and CDP-Glc could also be accepted as substrates with lower affinity (Table 2). In contrast, TDP-Glc and ADP-Glc did not act as glucosyl donors, and no phosphohydrolase activity was detected in *Euglena* TPS/TPP-concentrated fractions when assayed with other sugar phosphates (Glc-6-P, Glc-1-P, Glc-1,6-P_2, Fru-6-P, Fru-1-6-P_2, or Fru-2,6-P_2, at 10 mM final concentration) than trehalose-6-P.

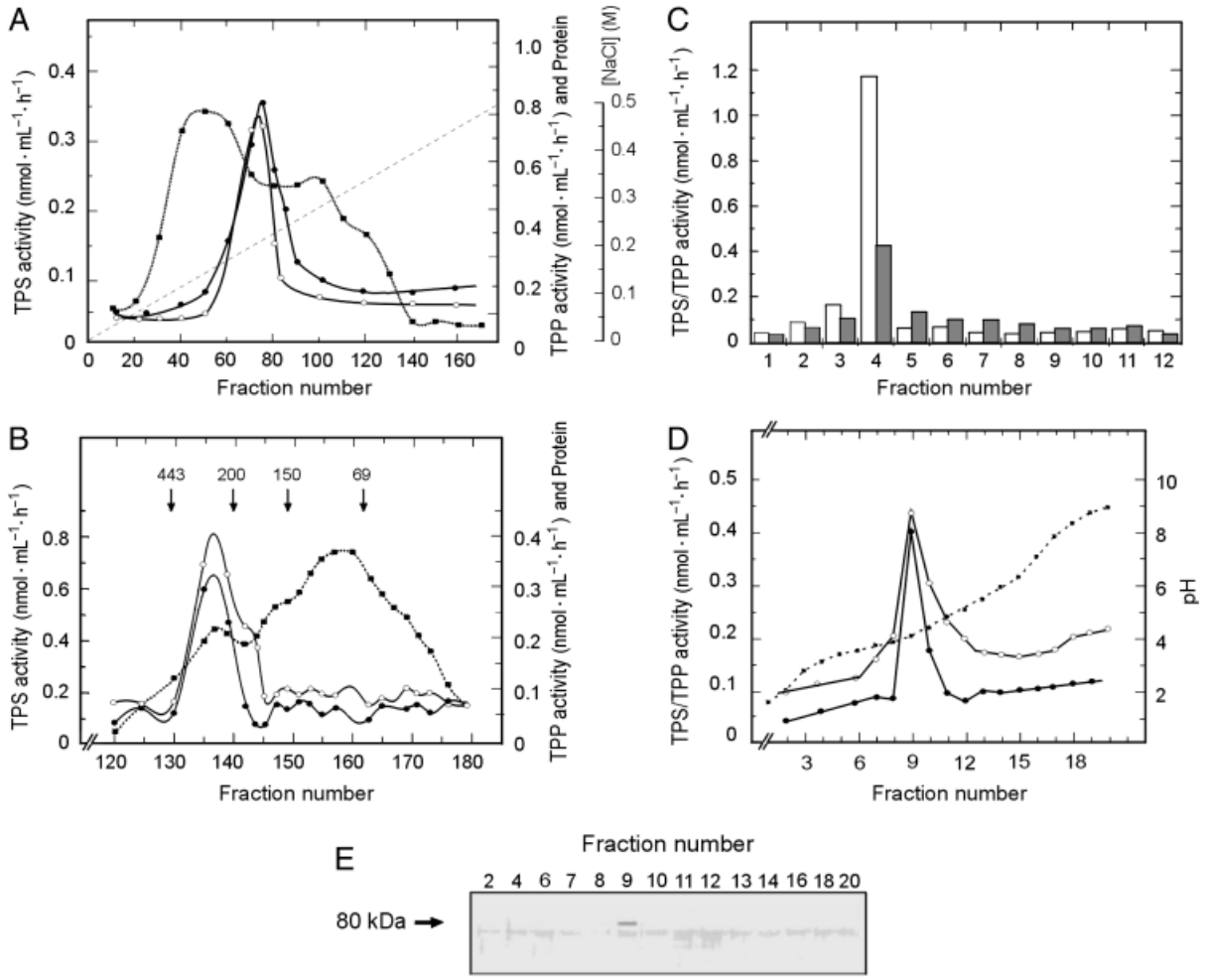

FIG. 1. Purification of TPS/TPP from *Euglena gracilis* cells. (A) DEAE-Sephacel chromatography: TPS (● - ●) and TPP (○ - ○) activities; protein (■ · · · · ■) and NaCl gradient (dashed line). (B) Sepharose-6B chromatography: TPS (● - ●) and TPP (○ - ○) activities; protein (■ · · · · ■). Arrows indicate the elution of standard proteins: BSA (~ 66 kDa), yeast alcohol dehydrogenase (~ 150 kDa), potato β-amylase (~ 200 kDa), and apoferritin (~ 443 kDa). (C) Enzyme activity in gel fractions of native gel electrophoresis: TPS (black bars) and TPP (open bars). (D) Isoelectrofocusing: TPS (● - ●) and TPP (○ - ○) activities, pH gradient (dash line). (E) Immunoblot of aliquots of fractions corresponding to TPS/TPP activity peak of isoelectric focusing revealed with polyclonal antibodies anti-*Eug*TPS/TPP. An unspecific band is revealed in each lane. Only in the lane corresponding to fraction number 9 of the isoelectric focusing was an 80-kDa polypeptide immunorecognized.

An absolute divalent-cation requirement was shown for TPP activity, estimating a $K_{a\ app}$ for Mg^{2+} of about 2.2 mM (Fig. 2A). At 10 mM, $MnCl_2$ or $CaCl_2$ also activated TPP activity but at a lower level (39% and 10%, respectively) than $MgCl_2$. Fluoride, vanadate, and molybdate (general phosphatase inhibitors) reduced TPP activity between 45% and 55% at concentrations as high as 15 mM (Fig. 2B). Trehalose synthesis activity was not modified by the addition of 20 mM Fru-6-P or Pi, reported as activator and inhibitor of yeast TPS, respectively (Londesborough and Vuorio 1993).

TABLE 1. Purification of TPS and TPP activities from *Euglena gracilis* cells.

			TPS activity				TPP activity				
Purification step	Volume (mL)	Protein (mg)	U/mg	U	Purification (-fold)	Yield	U/mg	U	Purification (-fold)	Yield (%)	TPS/TPP ratio
Crude extract	40	300	2.05	615	1	100	2.8	840	1	100	1.4
DEAE-Sephacel	1	20	10.50	210	5.1	34	21	420	7.5	50	2
Sepharose-6B	1	1	30.01	30	17.6	4.8	48	48	17.1	5.7	1.6

One unit (U) corresponds to 1 nmol product · h^{-1}; $n = 5$.

TABLE 2. Kinetic parameters of trehalose biosynthesis enzymes in the TPS/TPP complex.

Enzyme	Substrate	$K_{m\ app.}$ (mM)	$V_{max\ app.}$ ($U \cdot \mu g^{-1}$)	$V_{max\ app}/K_{m\ app.}$ $U \cdot \mu g^{-1} \cdot mM^{-1}$
TPS	Glc-6P (+ UDP-Glc)	7.0	40.0	5.7
	UDP-Glc	1.8	27.8	15.6
	CDP-Glc	22.5	25.0	1.1
	GDP-Glc	4.1	22.0	5.4
TPP	Trehalose-6-P	0.7	28.0	40.0

One unit (U) corresponds to 1 nmol product $\cdot h^{-1}$; $n = 3$. $K_{m\ app}$, apparent Michaelis constant; $V_{max\ app}$, apparent V_{max} value.

Trehalose synthesis activity is affected by protein phosphorylation. A time-dependent decrease in trehalose synthesis was shown when either desalted crude extracts or partially purified enzyme fractions were preincubated in phosphorylating conditions (Fig. 3). A rapid decrease in both TPS and TPP activities was observed, reaching 50% inhibition after 10 min. When preincubation was carried out with the addition of staurosporine (a specific Ser/Thr-protein kinase inhibitor), it seemed that the phosphorylation did not take place, and no changes in TPS or TPP activities were detected (Fig. 3). The addition of genistein

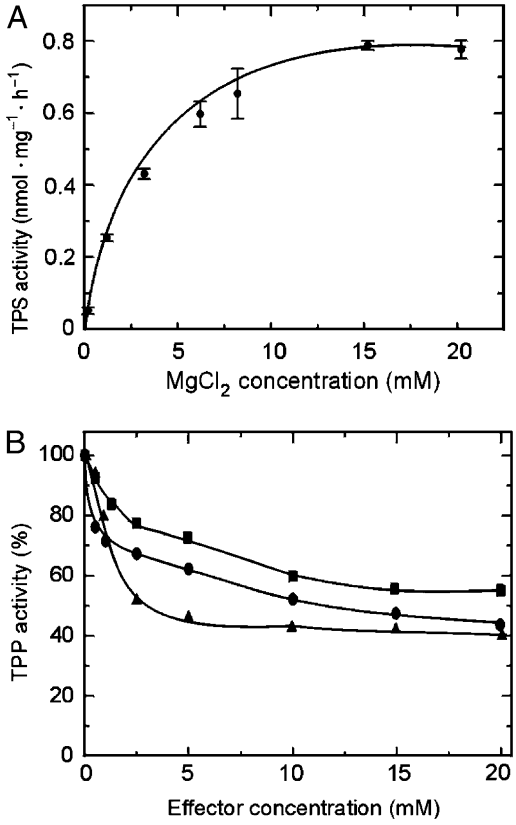

FIG. 2. Effect of $MgCl_2$ on TPS activity and of phosphatase inhibitors on TPP activity. (A) Different $MgCl_2$ concentrations up to 20 mM were added into TPS incubation mixtures. (B) Different concentrations of fluoride (▲), vanadate (●), and molybdate (■) were added into TPP assay mixtures.

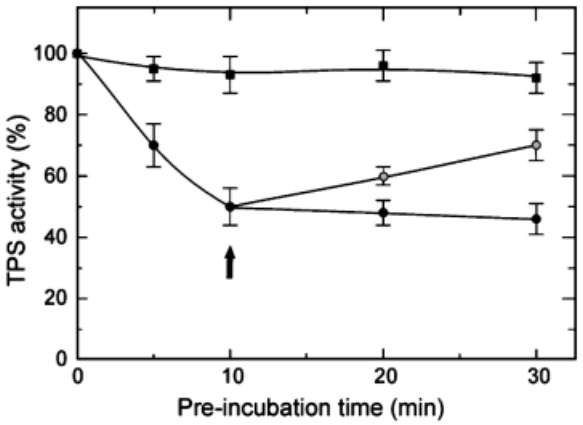

FIG. 3. Effect of phosphorylation on trehalose synthesis activity. Crude extracts were preincubated in phosphorylating conditions in absence (●) or presence (■) of staurosporine. After 10 min preincubation, PP2A was added (indicated by the arrow) and TPS activity was measured at different times (open symbols).

(a specific Tyr-protein kinase inhibitor) did not affect TPS and TPP activities (not shown), indicating that protein phosphorylation should have been taken place probably at some Ser/Thr residue. Moreover, trehalose synthesis activity was partially recovered when a Ser/Thr-protein phosphatase (PP2A) was added to the preincubation medium (Fig. 3). The presence of EGTA in the preincubation mixture did not modify enzyme activity inhibitions produced after phosphorylation, which may indicate that no divalent cations (i.e. Ca^{2+}, required for some type of protein kinase activity) are necessary for the phosphorylating activity (not shown).

Phosphorylation of TPS/TPP could be achieved in vitro. To study whether the phosphorylation-dependent decrease in TPS/TPP activity correlates with the phosphorylation of TPS and/or TPP proteins, desalted crude extracts were incubated in phosphorylating conditions in the presence of [γ^{32}P]ATP for 10 min. The resulting protein products were electrophoresed and transferred onto a nitrocellulose membrane, which was autoradiographed (Fig. 4). The position of the phosphorylation poly-

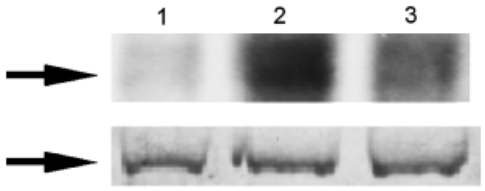

FIG. 4. Phosphorylation of the TPS/TPP complex of *Euglena gracilis*. Protein extracts were preincubated in phosphorylating conditions for 0 min (lane 1) and 10 min (lane 2) or for 10 min in the presence of 0.5 μM staurosporine (lane 3). Polypeptides were separated by SDS-PAGE in 12% polyacrylamide gels, followed by autoradiography (A) or Western blotting, revealed with anti-*Eug*TPS/TPP (B). Arrows indicate the position of an 80-kDa polypeptide.

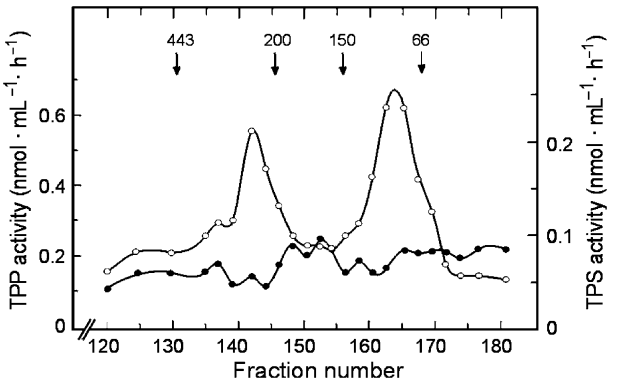

FIG. 5. Effect of phosphorylation on the TPS/TPP elution profile in a gel filtration chromatography. *Euglena gracilis* protein extracts were preincubated with a phosphorylating mixture and then chromatographed on a Superose-12 column. TPS (● - ●) and TPP (○ - ○) activity. The arrows indicate the elution position of standard proteins: BSA (~ 66 kDa), yeast alcohol dehydrogenase (≈ 150 kDa), potato β-amylase (~ 200 kDa), and apoferritin (~ 443 kDa).

peptide products was identified in the membrane by immunodetection. A single 80-kDa band was revealed with anti-*Eug*TPS/TPP antibodies. The phosphorylation was prevented when desalted extracts were preincubated in the presence of staurosporine (Fig. 4, lane 3).

Phosphorylation may promote changes in the association state of TPS/TPP. A partially purified TPS/TPP fraction from the DEAE-Sephacel step was preincubated in phosphorylating conditions and immediately submitted to gel filtration chromatography onto a Sepharose-6B column. Although TPS activity could not be detected, TPP activity eluted in two peaks: one corresponding to the position of the elution of proteins with M_r of about 250 kDa, similar to the initially described native TPS/TPP (Fig. 1B), and the other peak in the fractions corresponding to the elution of proteins with M_r of about 80 kDa (Fig. 5). A subsequent incubation of the TPS/TPP fraction in the presence of alkaline phosphatase or PP2A after the phosphorylation treatment did not revert the elution pattern shown in Figure 5 (data not shown). When the phosphorylation treatment was carried out in the absence of ATP or with the addition of staurosporine, similar elution patterns to that shown in Figure 1B were obtained (not shown). When aliquots of the column fractions were immunoanalyzed with anti-*Eug*TPS/TPP antibodies (Western blots), an 80-kDa polypeptide was detected mainly in the fractions with TPP activity but also in fractions in which the higher M_r protein complex eluted (not shown).

DISCUSSION

In this report we present the first characterization of trehalose biosynthesis activities in a protist organism, contributing to complete the trehalose pathway, initially described by Maréchal and Belocopitow (1972) and Porchia et al. (1999). It was shown that trehalose-6-P could be produced preferentially from UDP-Glc, but importantly, other sugar nucleotides may be acting as glucosyl donors (Table 2). The use of alternative sugar nucleotides may offer an adaptive advantage to *E. gracilis* cells under environmental conditions in which other UDP-Glc consuming pathways may be active and trehalose synthesis could be required.

The possibility that TPS and TPP associate to form a multienzyme complex is supported by the copurification of both activities submitted to several experimental approaches based on distinct separation principles (Fig. 1). These results led us to arrive at a similar conclusion to that reported in yeast, where it was demonstrated that TPS/TPP form a complex with an M_r of about 600–800 kDa (De Virgilio et al. 1993, Vuorio et al. 1993, Reinders et al. 1997). However, the biochemical characteristics of *Euglena* TPS/TPP (M_r of about 250 kDa) differ from those of the yeast TPS/TPP complex, with respect to M_r, substrate specificity (sugar nucleotides as glucose donor, Table 2) and effect of Pi or Fru-6-P on enzyme activity. The presence of extra regulatory subunits in the *Euglena* TPS/TPP complex, as reported in yeast, is a possibility. Recently, a TPS/TPP complex composed of three polypeptides with M_r of about 50, 67, and 115 kDa was purified and characterized from *Selaginella lepidophylla* (Valenzuela-Soto et al. 2004). This complex showed three different aggregation forms with M_r of 660, 440, and 230 kDa, but only the 440-kDa form was enzymatically active.

Although it has been reported that protein phosphorylation may be involved in trehalose biosynthesis in yeast (Panek et al. 1987), a later work could not confirm those results (Vandercammen et al. 1989). Also, the occurrence of phosphorylation in trehalose biosynthesis-related enzymes was suggested in plants, based on an interaction between a TPS/TPP-like and 14-3-3 proteins (Moorhead et al. 1999).

Our results demonstrate that *Euglena* TPS/TPP activity may be reversibly regulated by a protein phosphorylation/dephosphorylation mechanism (Fig. 3). When crude extracts were incubated in phosphorylating conditions, a decrease in TPS/TPP activity was observed. The phosphorylation-dependent decrease correlated with the phosphorylation of a polypeptide of about 80 kDa that was immunorevealed with anti-*Eug*TPS/TPP antibodies. Both the decrease in TPS/TPP activity and the phosphorylation of the 80-kDa polypeptide were prevented when desalted extracts were preincubated in the presence of a Ser/Thr-protein kinase inhibitor.

The association state of the TPS/TPP protein complex was affected after phosphorylation. A phosphorylation pretreatment resulted in substantial changes in the elution profile of TPS/TPP activities in Sepharose-6B columns. Whereas TPS activity could not be detected in the chromatography fractions, TPP activity eluted in two peaks. The maximum of the first peak was at a position where eluted proteins of about 250 kDa (similar to the M_r of the TPS/TPP complex),

and the maximum of the other peak corresponded to the elution of proteins of about 80 kDa. These results could indicate that phosphorylation may be producing a TPS/TPP dissociation and that TPS activity should be highly unstable when the complex is not assembled. Furthermore, TPS activity might be regulated by a mechanism involving the association state of the complex. On the other hand, TPP activity could be ascribed to an 80-kDa protein not assembled in the complex. The *in vitro* phosphorylation experiments showed the incorporation of [^{32}P] in an 80-kDa polypeptide that was also immunorevealed with anti-*Eug*TPS/TPP antibodies (Fig. 4). Although the identity of the 80-kDa polypeptide could not be assessed, our data suggest that TPP could be a phosphorylation target. Therefore, this mechanism may be regulating the association state of the TPS/TPP complex and, consequently, trehalose synthesis activity. However, it was also shown that dephosphorylation was not enough to reverse the effect and to restore TPS/TPP activities in a complex under the assayed conditions. The mere change of the phosphorylation state of the TPS/TPP proteins did not seem sufficient to reverse the dissociation of the complex.

The ability of *E. gracilis* cells to adapt to habitat alterations suggests that this organism may be prepared to respond rapidly to those changes through mechanisms that lead to fast changes in metabolic pathways in response to new cell demands and conditions. Trehalose synthesis in *Euglena* was believed to have a relevant role in the adaptation to salt stress (Takenaka et al. 1997, Porchia et al. 1999). Assuming the occurrence of a TPS/TPP complex in *E. gracilis*, the resulting metabolic channeling of the intermediate metabolite (trehalose-6-P) may offer catalytic advantages for the disaccharide net production (Ovadi 1991), and additionally, the regulatory mechanism of association/dissociation mediated by phosphorylation proposed herein may contribute to rapid changes in trehalose biosynthesis in response to environmental variations.

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