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Heterologous expression and initial characterization of recombinant RbcX protein from *Thermosynechococcus elongatus* BP-1 and the role of RbcX in RuBisCO assembly

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In the cyanobacterial RuBisCO operon from *Thermosynechococcus elongatus* the *rbc*X gene is juxtaposed and cotranscribed with the *rbc*L and *rbc*S genes which encode large and small RuBisCO subunits, respectively. It has been suggested that the *rbc*X position is not random and that the RbcX protein could be a chaperone for RuBisCO. In this study, the RbcX protein from *T. elongatus* was overexpressed, purified and preliminary functional studies were conducted. The recombinant protein purified from *Escherichia coli* extracts was predominantly present in a soluble fraction in a dimeric form. Coexpression experiments have demonstrated that RbcX can mediate RbcL dimer (L_2) formation, and that it is essential for the L_8 core complex assembly. This is the first characterization of the RbcX protein from a thermophilic organism.

Keywords: RbcX, *Thermosynechococcus elongatus*, expression, purification, RuBisCO assembly

INTRODUCTION

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO, EC 4.1.1.39) is the key enzyme in photosynthesis. RuBisCO catalyzes the addition of CO_2 to ribulose-1,5-bisphosphate (RuBP), which is the slowest and rate limiting step in the photosynthetic process (for a review, see Cleland *et al.*, 1998). RuBisCO also catalyzes an additional reaction involving molecular oxygen (O_2) in the oxygenation of RuBP and, therefore, it is also a key enzyme for the photorespiration pathway (Bowes *et al.*, 1971). The importance of RuBisCO would be difficult to overestimate since this protein is the most abundant enzyme in nature and catalyzes the reaction which provides the only quantitatively significant link between the pools of inorganic carbon dioxide in the atmosphere and organic matter in the biosphere (Ellis, 1979; Whitmarsh & Govindjee, 1999).

Until now, three structural forms of RuBisCO have been reported (Tabita, 1999; Andersson & Taylor, 2003). In plants, cyanobacteria and green algae, RuBisCO exists as a holoenzyme composed of eight large (RbcL, 50–55 kDa) and eight small (RbcS; 12–18 kDa) subunits. This hexadecameric L_8S_8 complex is denoted as form I. In some photosynthetic prokaryotes (e.g., *Rhodospirillum rubrum*) the RuBisCO holoenzyme lacks the small (RbcS) subunit and exists as a dimer of the RbcL subunit (L_2). This simpler form of RuBisCO is denoted as form II. Form III, found in a thermophilic archaeon, *Thermococcus kodakaraensis*, (Kitano *et al.*, 2001) is composed of five RbcL dimers ($5L_2$).

Due to its oligomeric structure RuBisCO has gained importance in protein assembly studies. The simple form II of RuBisCO (L_2) from *R. rubrum* was expressed in *Escherichia coli* cells with bacterial GroEL/GroES system and resulted in the active en-

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Abbreviations: AP, ampicillin; BCA, bicinchoninic acid; BSA, bovine serum albumin; CBD, chitin-binding domain; CM, chloramphenicol; DTT, 1,4-dithiothreitol; IPTG, isopropyl beta-D-thiogalactopyranoside; LB, Luria-Bertani; β-ME, β-mercaptoethanol; PGA, 3-phosphoglyceric acid; RuBP, ribulose-1,5-bisphosphate.

zyme (Goloubinoff *et al.*, 1989a). In that reconstitution process, RuBisCO large subunits are folded by a chaperonin followed by spontaneous dimerization after its release into the cytoplasm (Brinker *et al.*, 2001). In plants and cyanobacteria, RuBisCO belongs to form I. Taking into consideration the structural complexity of RuBisCO form I, it is clear that before it becomes biologically active, polypeptide subunits must first be correctly folded and then assembled into an oligomeric complex. This process requires not only chaperone factors mediating polypeptide chain folding, but also specific assembly chaperones. RuBisCO form I from *Synechococcus* sp. PCC6301 is encoded by an operon containing genes coding for the large (*rbcL*) and small (*rbcS*) subunits, while the *rbcX* gene is located outside the RuBisCO operon. It has been reported that expression of RuBisCO from *Synechococcus* sp. PCC6301 in *E. coli* cells results in the production of an active holoenzyme. This process is chaperonin-dependent, and no *rbcX* gene product is required (Goloubinoff *et al.*, 1989b). However, the RuBisCO operon of some cyanobacteria contains three cotranscribed genes: between the *rbcL* and *rbcS* genes the *rbcX* gene is present (Larimer & Soper, 1993). Recently, it has been reported that the *rbcX* gene product increases the production of active RuBisCO when coexpressed with the *rbcL* and *rbcS* genes in *E. coli*. Such phenomenon was observed for recombinant RuBisCO from *Anabaena* sp. strain CA (Li & Tabita, 1997), and in *Synechococcus* sp. PCC7002, where partial inactivation of RbcX resulted in a considerable decrease of RuBisCO activity and solubility (Onizuka *et al.*, 2004). Recently, it has also been reported that the *rbcX* gene can function as a RuBisCO chaperone, but is not essential for viability of *Synechococcus* sp. PCC7942 cells (Emlyn-Jones *et al.*, 2006).

To assign possible function to the RbcX protein a search for sequence similarities of the RbcX with other sequences deposited in protein sequence databases was undertaken. The analysis revealed no significant homologues matching the complete length or only subregions of the RbcX sequence. Therefore no function could be ascribed to the RbcX protein based on the protein sequence analysis. However, it has been suggested that the position of the *rbcX* gene is not random, and the RbcX protein may serve as an assembly chaperone for RuBisCO. In a recent study of the RbcX protein from the mesophilic *Synechococcus* sp. PCC7002 it has been confirmed that cyanobacterial RbcX is indeed a RuBisCO assembly chaperone (Saschenbrecker *et al.*, 2007).

Until now, no studies have been reported on the RbcX protein from any thermophilic organism. Recently, the genome of the thermophilic cyanobacterium *Thermosynechococcus elongatus* has been completely sequenced (Nakamura *et al.*, 2002) and it was shown that the *rbcX* gene is juxtaposed and cotranscribed with the *rbcL* and *rbcS* genes as a single operon. Similarly to RuBisCO from mesophilic cyanobacteria, the thermophilic enzyme belongs to form I (Gubernator *et al.*, 2008). In this study, we investigated the influence of the RbcX protein on the production of an active RuBisCO from *T. elongatus*. Our findings reveal that the RbcX protein plays a role in RuBisCO assembly.

MATERIALS AND METHODS

Bacterial and cyanobacterial strains. *Escherichia coli* DH5α (Stratagene) and ER2566 (New England Biolabs) were maintained in Luria-Bertani (LB) medium containing 100 μg/ml or 50 μg/ml ampicillin and 25 μg/ml chloramphenicol and incubated at 14, 22, 30 or 37°C under aerobic conditions with shaking. The unicellular cyanobacterium *T. elongatus* strain BP-1 was cultured in conical flasks in liquid BG-11 medium (Rippka, 1988) at 42°C with shaking under continuous illumination provided by fluorescent lamps.

Construction of expression plasmids. The IMPACT-CN system (New England Biolabs) was used for the overexpression and purification of the RbcX protein, including the chitin-binding domain (CBD) attached to either the N- or C-terminus of the protein. For this purpose, the *rbcX* gene (381 bp) from *T. elongatus* BP-1 genomic DNA was amplified using primers (a) and (b) or (c) and (d) (all primers are listed in Table 1). The purified and digested PCR products were cloned into the pTYB1 or pTYB11 vectors (New England Biolabs) treated with *Nde*I and *Sap*I or *Sap*I and *Eco*RI restriction enzymes, respectively. The resulting plasmids pTYB1-rbcX (C-terminal fusion) and pTYB11-rbcX (N-terminal fusion) were checked by restriction analysis. The expression plasmid pUC18rbcLXS carrying the coding sequence for large (RbcL) and small (RbcS) subunits of RuBisCO and the RbcX protein from *T. elongatus* was constructed as described in Gubernator *et al.* (2008). The expression plasmid pUC18rbcL carrying the coding sequence for the large (RbcL) subunit of RuBisCO was constructed as follows: *rbcL* gene (1428 bp) from *T. elongatus* genomic DNA was amplified using primers (e) and (f) and cloned into the pUC18 vector (Fermentas) cleaved with *Eco*RI and *Bam*HI restriction enzymes. The expression plasmid pUC18rbcLS containing the genes for the RbcS and RbcL subunits of RuBisCO was also constructed. For this purpose, the *rbcS* gene (357 bp) from *T. elongatus* was amplified utilizing primers (g) and (h) and cloned into the pUC18rbcL plasmid (described above) treated with *Bam*HI and *Hin*dIII restriction enzymes. We also investigated the coexpression of

Table 1. Primers used for gene amplification

Primers	Description	Plasmid
a) [1]FW 5′-GGAATTCCATATGGATGTCAAGCACATTGCC-3′ b) [2]RV 5′-GTGGTTGCTCTTCCGCATTCAGGGGTTTCTAGGT-3′	Amplify the *rbcX* gene for expression of RbcX-CBD (C-terminal fusion)	[3]pTYB1–rbcX
c) FW 5′-GGTGGTTGCTCTTCCAACATGGATGTCAAGCACATTGC-3′ d) RV 5′-GGTGGTGAATTCCTATTCAGGGGTTTCTAGGT-3′	Amplify the *rbcX* gene for expression of CBD-RbcX (N-terminal fusion)	[3]pTYB11–rbcX
e) FW 5′-CGGAATTCTACCCATTGCAAAGGTTGC-3′ f) RV 5′-GCGGATCCCTAGATCGTGTCTTGCGCT-3′	Amplify the *rbcL* gene for expression of RbcL subunit of RuBisCO	[3]pUC18rbcL
g) FW 5′-CGGGATCCAGTTTGTTGATTGTGAACCA–3′ h) RV 5′-GCGCAAGCTTTTAATAGCGATAACCGCTGT-3′	Amplify the *rbcS* gene for expression of RbcS subunit of RuBisCO	[3]pUC18rbcLS
i) FW 5′-CGCGGTTACCCGGTCTATGGATGTCAA–3′ j) RV 5′-GCGCTCTAGAAAACAAACTATTCAGGG-3′	Amplify the *rbcX* gene for expression of RbcX subunit of RuBisCO	[4]pUC18rbcXCM

Sequences recognized by restriction enzymes are underlined. [1]FW, forward primer; [2]RV, reverse primer; [3]plasmid carrying ampicillin resistance cassette AP^r; [4]plasmid carrying chloramphenicol resistance cassette CM^r.

the RuBisCO RbcL subunit (using pUC18rbcL plasmid) with the RbcX protein. For this purpose, the expression plasmid pUC18rbcXCM (containing chloramphenicol resistance cassette) carrying the coding region for the RbcX protein was constructed as follows: the chloramphenicol resistance cassette (CM^r) (1100 bp) from pKSCm1 vector treated with *Hin*dIII and *Xba*I was cloned into the pUC18 vector (carrying ampicillin resistance cassette; AP^r) resulting in pUC18AP^rCM^r plasmid. Subsequently, the plasmid was digested with *Eco*47I restriction enzyme causing the deletion of a 200 bp fragment from the AP^r cassette resulting in the pUC18CM vector with a non-functional AP^r region. In the next step, the *rbcX* gene was amplified using primers (i) and (j) and cloned into the pUC18CM vector cleaved with *Kpn*I and *Xba*I giving pUC18rbcXCM plasmid. To study the coexpression of the RbcL subunit with bacterial chaperonins GroES and GroEL the pGroESL plasmid (kindly provided by A. Gatenby) was used. All constructed plasmids were verified by DNA sequencing.

Overexpression of recombinant proteins. To overexpress the fusion proteins containing the CBD tag, *E. coli* ER2566 cells were transformed with pTYB1-rbcX or pTYB11-rbcX plasmid. The LB medium supplemented with ampicillin was inoculated with overnight cultures. The cells were cultured at 37°C to OD_{600} about 0.8 and expression was induced with IPTG to a final concentration of 0.3 mM. The induction was conducted at 30°C for 3 h, at 22°C for 6 h or at 14°C for 18 h. The cells were pelleted by centrifugation at 5000×*g* for 10 min at 4°C and stored at –20°C for further use. To overexpress the whole RuBisCO operon from *T. elongatus* (RbcLXS), RuBisCO large subunit (RbcL) or RuBisCO large and small subunits (RbcLS), *E. coli* DH5α cells were transformed with pUC18rbcLXS, pUC18rbcL or pUC18rbcLS plasmids, respectively. The LB medium supplemented with ampicillin was inoculated with overnight cultures. The cells were cultured at 37°C. The induction with 0.5 mM IPTG was started at OD_{600} about 0.6 and cells were grown at 37°C for 16–17 h. The cells were pelleted by centrifugation at 5000×*g* for 10 min at 4°C and stored at –20°C for further use. To study the coexpression of RuBisCO RbcL or RbcLS subunits with the RbcX protein, *E. coli* DH5α cells were cotransformed with pUC18rbcL or pUC18rbcLS and pUC18rbcXCM plasmids. The cells were grown and treated essentially as described above except that the LB medium was supplemented with ampicillin and chloramphenicol.

Purification of RbcX protein. Recombinant RbcX fusion protein with a self-cleavable intein tag may be cleaved by addition of DTT. The inducible self-cleavage results in the recombinant protein with no additional amino acids compared to the native one. To purify the fusion protein, harvested bacterial cells were disrupted by sonication in a 20 mM Tris/HCl buffer, pH 8.0, containing 500 mM NaCl and 1 mM EDTA, centrifuged at 20000×*g* for 30 min at 4°C and the supernatant was collected (soluble fraction). The resulting pellet was washed and preserved for further analysis (inclusion bodies fraction). The expressed RbcX protein containing the CBD tag was purified according to the IMPACT-CN system from the soluble fraction using 50 mM DTT for cleavage induction. The RbcX protein was dialyzed against 20 mM Tris/HCl buffer, pH 7.5, followed by concentration using an Amicon Ultra-15 device (5000 MWCO, Millipore) and stored at –80°C for further use.

Fractionation of carboxysomes and soluble proteins from *T. elongatus* BP-1 cells. Protein extracts were prepared from 200 ml cultures of cyanobacteria grown to OD_{730} about 0.7–1.1. The cells were harvested by centrifugation at 4000 × *g* for 5 min at 4°C and washed with 20 mM Hepes/NaOH buffer, pH 7.6, containing 0.6 M sucrose, then centrifuged again (5000 × *g*, 5 min at 4°C). The collected cells were resuspended in the buffer as described above and supplemented with lysozyme (2 mg/ml). After 90 min of incubation at 37°C, the suspension was centrifuged and the pelleted cells were resuspended in 50 mM Hepes/NaOH buffer, pH 8.0, containing 1 mM EDTA supplemented with protease inhibitor cocktail (Roche). The cells were broken using a Yeda press at 10 MPa nitrogen pressure. The remaining intact cells and the cell debris were removed by centrifugation (3000 × *g*, 6 min at 4°C) and an aliquot was taken for SDS/PAGE and Western blot analysis of the total cellular extract. To obtain the carboxysomal fraction, the resulting supernatant was incubated with 20 mM $MgCl_2$ for 15 min on ice, then centrifuged at 30 000 × *g* for 15 min at 4°C (Price *et al.*, 1993). The obtained supernatant (soluble proteins fraction) and the pellet containing carboxysomes were preserved for further SDS/PAGE and Western blot analysis.

Production of polyclonal anti-RbcX antibodies. The purified recombinant RbcX protein in 20 mM sodium phosphate buffer, pH 7.0, was used for raising antibodies in California white rabbits. The rabbits were first immunized using 100 μg of recombinant protein in 0.25 ml of phosphate buffer and an equal volume of Freund's complete adjuvant. After 2 weeks, the rabbits were boosted three times with 100 μg recombinant protein each in complete Freund's adjuvant at 2-week intervals. The next five immunizations were conducted using 100 μg recombinant protein each in incomplete Freund's adjuvant at 2-week intervals. Sera were collected one week after the last immunization. The antibody titer was determined by immunoblotting and was found to be 1:5000.

SDS/PAGE, native-PAGE and Western blot analysis. Protein samples were analyzed by SDS/polyacrylamide gel electrophoresis (SDS/PAGE) on 12% polyacrylamide gels as described by Laemmli (1970) and stained with Coomassie Brilliant Blue R-250 (CBB R-250). Native PAGE was done under non-denaturing and non-reducing conditions essentially as described by Laemmli, except that SDS and DTT were omitted. Immunoblotting was carried out as described (Szczepaniak & Cramer, 1990). Proteins transferred onto a nitrocellulose membrane were probed with the antiserum to the recombinant protein (anti-RbcX) at a dilution of 1:2000, anti-CBD serum (New England Biolabs) at a dilution of 1:2000 and anti-RbcL antibody (Agrisera) at a dilution 1:10 000 for 2 h at room temp.

Measurements of protein and RuBP concentration. Protein concentration was measured using the BCA Protein Assay Kit (Sigma) according to manufacturer`s instructions with bovine serum albumin (BSA) as a standard. RuBP concentration was determined directly from the absorbance at 280 nm with an extinction coefficient $\varepsilon = 47.7\ M^{-1}\ cm^{-1}$ (Rice & Pon, 1978).

RuBisCO carboxylase assay. The carboxylase activity of RuBisCO was determined at 30°C as the RuBP-dependent incorporation of $^{14}CO_2$ into acid-stable 3-phosphoglyceric acid according to previously reported procedures (Whitman & Tabita, 1976). Prior to the assay, the enzyme was fully activated by incubation in 50 mM Bicine/NaOH, pH 8.0, buffer containing 20 mM $MgCl_2$, 20 mM $NaH^{14}CO_3$ (specific activity 1 Ci/mol) and 2 mM DTT at 30°C for 30 min. The final volume of the reaction mixture was 0.5 ml. The carboxylase reaction was started by the addition of RuBP to the final concentration of 0.4 mM and was terminated by adding 2 M HCl (0.1 ml of reaction mixture per 0.5 ml 2 M HCl). The samples were dried and the residues were resuspended in 0.5 ml water and mixed with 4.5 ml of scintillation fluid. The incorporation of $^{14}CO_2$ into PGA was quantified by liquid scintillation counting (LS-6500 Liquid Scintillation Counter, Beckman). Specific activity was expressed as micromoles of $^{14}CO_2$ fixed per minute per milligram of protein.

N-terminal amino acid sequencing. N-Terminal protein sequence analysis was performed at the BioCentrum Ltd. facility (Kraków, Poland). The sequentially detached phenylthiohydantoin derivatives of amino acids were identified using a Procise 491 (Applied Biosystems) automatic sequence analysis system.

Molecular mass determination. Gel filtration chromatography of the recombinant RbcX protein under non-denaturing conditions was performed using an Äkta FPLC system (GE Healthcare). For this purpose a Superdex 75 HR 10/30 gel filtration chromatography column (GE Healthcare) was used with 20 mM Tris/HCl, pH 8.5, 200 mM NaCl as a mobile phase, a flow rate of 0.5 ml/min and UV (λ = 280 nm) detection. The column calibration was carried out with proteins of known molecularmasses: bovine serum albumin (66 kDa), ovalbumin (44 kDa), trypsinogen (24 kDa) and cytochrome *c* (12 kDa). We also determined the molecular mass of the recombinant RbcX using ESI mass spectrometry at the Neurobiochemistry Unit of Jagiellonian University with an Esquire 3000 (Bruker Daltonics) instrument.

RESULTS AND DISCUSSION

Production and purification of the RbcX protein

The *rbc*X gene is commonly present in cyanobacterial genomes, but in various species it is located either within or outside the RuBisCO operon. Amino-acid sequence comparison shows an overall similarity of 57% (13% identity and 44% pseudo-identity) for cyanobacterial RbcX proteins based on 20 sequences (Fig. 1). The amino acids that are highly conserved in the primary structure of RbcX cluster in two regions of the protein, near the N-terminus (1–35 aa) and within the protein (70–108 aa). The C-terminal regions are of varying lengths and lack sequence similarities.

The overall goal of this study was to overexpress, purify, and conduct structural and functional preliminary investigations with the RbcX protein from *T. elongatus*. For this purpose, we established an overexpression and purification system for the RbcX protein employing the IMPACT-CN strategy, which allowed the cleavage of the fusion protein on the column. The RbcX protein was expressed in *E. coli* ER2566 cells transformed with pTYB1-rbcX or pTYB11-rbcX plasmids resulting in the recombinant RbcX-CBD protein (C-terminal fusion) or CBD-RbcX protein (N-terminal fusion), respectively. To determine the localization of the expressed fusion proteins, proteins of the soluble fraction and inclusion bodies were isolated from induced cells. The presence of the fusion protein was tested by SDS/PAGE and Western blotting using anti-CBD serum. The results showed that under all temperature conditions induction of *E. coli* cells carrying either pTYB1-rbcX or pTYB11-rbcX led to an overproduction of a fusion protein of expected size, about 70 kDa (not shown); however, expression under higher temperatures (30 and 22°C) resulted in the detection of the recom-

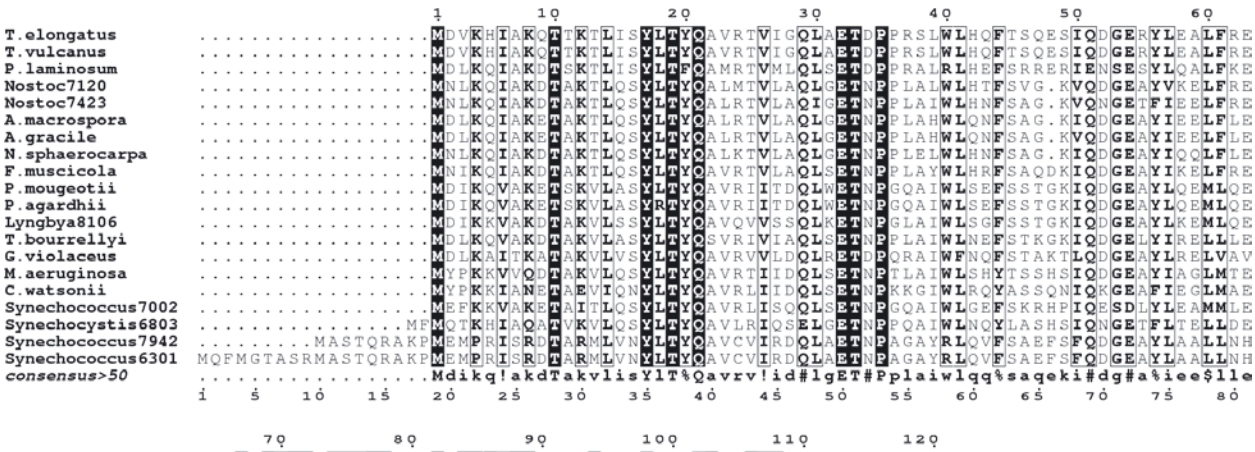

Figure 1. Amino-acid sequence alignment of RbcX proteins from cyanobacteria.
The alignment includes the RbcX protein from: *Thermosynechococcus elongatus* BP-1 (NP_682295); *Phormidium laminosum* 'OH-1-p Cl 1' (ABV04328); *Gloeobacter violaceus* PCC 7421 (NP_925103); *Synechococcus elongatus* PCC 7942 (ABB57565); *Synechococcus elongatus* PCC 6301 (YP_173231); *Synechococcus* sp. PCC 7002 (BAA03077); *Thermosynechococcus vulcanus* (BAF48768); *Synechocystis* sp. PCC 6803 (NP_442121); *Microcystis aeruginosa* PCC 7806 (CAO87108); *Tychonema bourrellyi* (CAB08210); *Nostoc* sp. PCC 7120 (NP_485565); *Planktothrix mougeotii* (CAB08195); *Planktothrix agardhii* (AAT38205); *Anabaena macrospora* (CAC51632); *Nodularia sphaerocarpa* PCC 7804 (ABB77525); *Lyngbya* sp. PCC 8106 (ZP_01622122); *Nostoc* sp. PCC 7423 (ABB77523); *Fischerella muscicola* PCC 7414 (ABB77519); *Crocosphaera watsonii* WH 8501 (ZP_00516815); *Aphanizomenon gracile* PMC9402 (CAC51627). Sequences were aligned using ClustalX program and visualized with ESPript. The residues with strict identity are shaded in black, whereas those with high similarity (score over 0.7) are shown in bold and framed. A consensus sequence was generated using criteria from MultAlin program using BLOSUM62 scoring matrix.

binant fusion protein predominantly in the inclusion bodies fraction (not shown). Only expression at 14°C for 18 h led to the production of the fusion protein mainly found in the soluble fraction of the cell lysate (Fig. 2, lane 3). We also found that in the case of the pTYB1-rbcX construct, most of the fusion protein present in the soluble fraction of the cell lysate underwent self-cleavage. SDS/PAGE analysis revealed the appearance of 55 kDa (related to CBD) and 14.5 kDa (related to RbcX protein) bands instead of the intact fusion of about 70 kDa (not shown). For this reason, only the pTYB11-rbcX construct was used for further studies. Various concentrations of IPTG (0.1, 0.3, 0.5 mM) were tested and 0.3 mM was chosen as optimal for overnight expression at 14°C. The expressed RbcX fusion protein represented approx. 10% of the total cellular protein as estimated from gel scan (Fig. 2, lane 2). The expressed protein was released from cells by sonication. The CBD-RbcX fusion protein was purified by a single affinity column step and the recombinant RbcX protein was analyzed by SDS/PAGE. As shown in Fig. 2, lane 4, the recombinant RbcX protein, detected as a single band of about 14.5 kDa was purified to homogeneity. Beginning with 1 liter of the bacterial culture about 3.0 mg of the purified recombinant RbcX protein could be purified. This amount was sufficient for antibody production in rabbits (for details, see Materials and Methods).

Western blot analysis of induced bacterial or cyanobacterial cells with the serum raised against the recombinant RbcX protein detected a single band of about 14.5 kDa. When the same samples were incubated with the pre-immune serum, no reaction was observed (not shown).

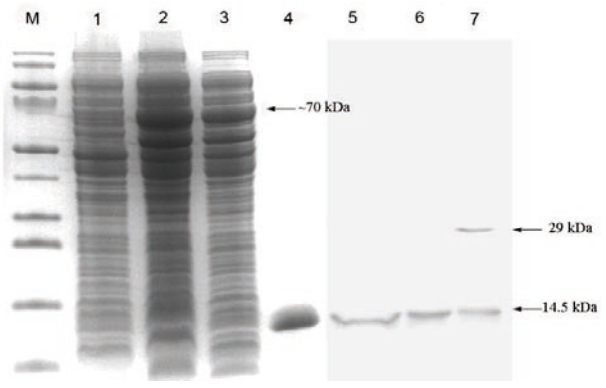

Figure 2. SDS/PAGE analysis of RbcX protein purified from *E. coli*.
Lane M, molecular mass standard (Fermentas), from the bottom: 11, 17, 26, 34, 43, 55, 72, 95, 130, 170 kDa; lane 1, extract of uninduced cells; lane 2, extract of induced cells; lane 3, fraction of soluble proteins; lane 4, purified RbcX after expression of CBD-RbcX; lane 5, RbcX reduced with β-ME; lane 6, RbcX reduced with DTT; lane 7, RbcX without reducing reagents.

The N-terminal amino-acid sequence of the expressed RbcX protein was determined to be MDVKHIAKQT. This sequence was identical with that of the native RbcX amino-acid sequence, demonstrating that CBD was correctly cleaved off. We also examined the molecular mass of the recombinant RbcX protein using ESI mass spectrometry. The obtained molecular mass of 14588 Da agrees very well with the calculated mass of 14589 Da.

All presented data show that using the IMPACT-CN system we are able to produce and purify homogeneous recombinant RbcX protein with satisfactory efficiency for antibody production and further structural studies.

RbcX may form dimers and oligomers

SDS/PAGE analysis performed in the presence or absence of reducing agents showed that the RbcX protein might exist in different forms. When DTT or β-ME were added to the RbcX sample, the monomer was the only form detected, while in the absence of the reducing agents a dimeric form was also present (Fig. 2; lanes 5–7). This data suggests that the native RbcX protein can act as a dimer. Such a suggestion is in agreement with the results presented by Saschenbrecker and coworkers (2007) where multiangle light scattering analysis revealed that RbcX from *Synechococcus* sp. PCC7002 forms a dimer in solution. We also observed that the recombinant RbcX protein at a high concentration (above 3 mg/ml) had a tendency to form higher-order oligomers. The gel filtration chromatography of RbcX in non-denaturating conditions showed up to five forms. The native-PAGE analysis also confirmed these results (not shown). The different patterns of the formed oligomers depended on protein concentration, ionic strength and the chaotropic agents used, suggesting an involvement of hydrophobic interactions in the higher-order oligomerization. However the higher-order oligomers appeared at a high concentration of the RbcX protein only and probably have no physiological meaning.

RbcX protein in *T. elongatus* BP-1 cells

To determine the subcellular localization of the RbcX protein in *T. elongatus,* the cyanobacteria cells were cultured in optimal conditions and a crude extract, soluble proteins fraction and intact carboxysomes were isolated. The RbcX protein was detected by Western blotting using the polyclonal antibody raised against the RbcX protein. The results showed that most of the RbcX protein was present in the soluble cytosolic fraction (Fig. 3; lane 2). Interestingly, quantitative studies on the distribution of RuBisCO showed that it was predominantly lo-

Figure 3. Western blot analysis of cell extracts from *Thermosynechococcus elongatus*.
Lane M, prestained protein marker (Fermentas), from the bottom: 15, 25 kDa; lane 1, crude cell extract; lane 2, soluble protein fraction; lane 3, carboxysomes fraction. Equivalent of 0.4 ml culture was applied on each lane. For immunodetection, polyclonal serum against the recombinant RbcX protein was used.

calized to carboxysomes (McKay *et al.*, 1993). In the light of the obtained results and literature data it is clear that the cellular localizations of the RbcX protein and RuBisCO are opposite. It seems that the RuBisCO subunits are folded and assembled into an oligomeric complex in the cytoplasm (RbcX could be a good candidate for the assembly chaperone) then the RuBisCO holoenzyme is accumulated in carboxysomes.

RbcX improves functional assembly of *T. elongatus* RuBisCO in *E. coli* cells

RuBisCO from *T. elongatus* belongs to form I and exists as a hexadecamer (L_8S_8). Genes for the RbcL and RbcS subunits are located in a common operon, also the RbcX protein is encoded inside the operon in the order: *rbc*L-*rbc*X-*rbc*S. It seems that the position of the *rbc*X gene is not random and may play a crucial role in RuBisCO assembly.

To determine the function of RbcX, we decided to examine the role of this protein in the production, folding and assembly of RbcL into functional RuBisCO in *E. coli.* The production of the RbcL subunit and formation of the L_8S_8 holoenzyme or L_8 complex was monitored by RuBisCO activity measurement, native-PAGE or SDS/PAGE, followed by Western blot analysis (using the antibody against RbcL).

Expression of the pUC18rbcLXS plasmid (carrying whole RuBisCO operon) in bacterial cells resulted in the production of enzymatically active RuBisCO (Gubernator *et al.*, 2008). We decided to eliminate the *rbc*X gene from the *T. elongatus* RuBisCO operon, constructing the pUC18rbcLS plasmid encoding only the large and small subunits. As a result, we observed a decrease of the RuBisCO activity in the cell extract by up to 95% compared with the complete operon (Table 2). The activity of recombinant RuBisCO in *E. coli* could be restored by coexpression of the *rbcX* gene cloned into a separate plasmid (pUC18rbcXCM). We achieved 86% of the activity provided by the intact operon expressed in *E. coli*. The results show that the expression of RbcL and RbcS subunits does not lead to a correctly assembled enzyme. Importantly, when the RbcL and RbcS subunits were coexpressed with RbcX, the RuBisCO enzymatic activity was restored, indicating a proper assembly of the subunits into an active holoenzyme. The lack or presence of correctly assembled RuBisCO (L_8S_8) was also confirmed by native-PAGE (not shown). Thus, the assembly of the RbcL and RbcS subunits from *T. elongatus* into the active holoenzyme is strongly dependent on the RbcX protein. A similar observation has been reported for the mesophile *Synechococcus* sp. PCC7002 (Saschenbrecker *et al.*, 2007).

To examine the effect of the RbcX protein on the production and assembly of RbcL in *E. coli* cells, this RuBisCO subunit was expressed in the presence or absence of the *rbc*X gene. We also tested the influence of GroEL/GroES on the production and assembly of RbcL. The expression of the *rbc*L gene alone in *E. coli* led to production of the RbcL subunit at a low level. It was detected only in the cell lysate (Fig. 4A, B; lane 1). The lack of RbcL in the soluble fraction indicates its misfolding or an inability to form the dimer. When the *rbc*L gene was coexpressed with *rbc*X, a great increase in the level of RbcL and formation of the L_8 complex were observed (Fig. 4A, B; lane 2). Western blot analysis confirmed the presence of the RbcX protein in the soluble fraction (not shown). To explain this phenomenon, it is worth noting that the simple form of RuBisCO from *R. rubrum* exists natively as the L_2 dimer (form II). It is believed that dimer formation by the RbcL subunits is a crucial step in the enzyme assembly also in the case of the more complicated RuBisCO form I (L_8S_8). The mechanism of the translation control of RuBisCO large subunit proposed by Cohen *et al.* (2006) also suggests that dimer (L_2) formation is a key step in the assembly of the active holoenzyme. According to that mechanism the N-terminus of a misfolded or unbound

Table 2. Effect of RbcX protein on RuBisCO specific activity

Expressed vectors	pUC18rbcLXS	pUC18rbcLS	pUC18rbcLS + pUC18rbcXCM
Specific activity (nmol CO_2-fixed/ mg min)[a]	289.4 ± 25.6	14.2 ± 0.8	248.6 ± 17.4
% act.[b]	100%	5%	86%

The listed plasmids were expressed/coexpressed in *E. coli* cells. The resulting cell extracts were used for RuBisCO activity measurements (for details see Materials and Methods). [a]The results are shown as the mean ±S.D. of three experiments. [b]Activities are also expressed as percentage of specific activity of pUC18rbcLXS extract.

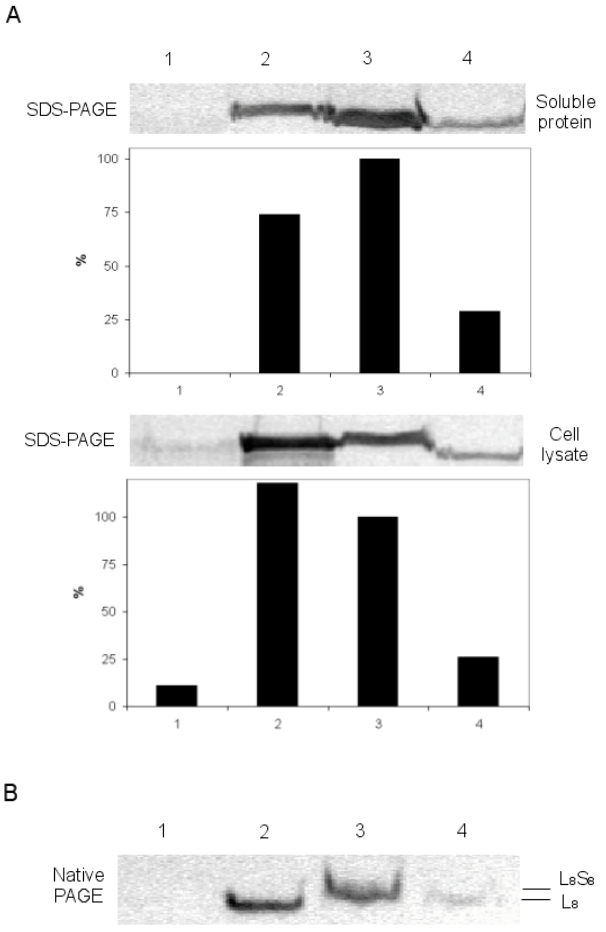

Figure 4. Effect of RbcX and GroEL/GroES on the production of RbcL and assembly of $RbcL_8$ octamer.
A. SDS/PAGE analysis of soluble fraction and total cell lysates. **B**. Native-PAGE analysis of soluble fraction. Lane 1; expression of pUC18rbcL; lane 2, coexpression of pUC18rbcL with pU18rbcXCM; lane 3, expression of pUC18rbcLXS (control); lane 4, coexpression of pUC18rbcL with pGroESL. Fifteen micrograms of total protein was applied on each lane. RbcL protein was detected by Western blotting with specific antibody (AgriSera) following native- or SDS/PAGE. The bars indicate relative densities estimated from nitrocellulose membrane scans of analyzed protein bands, where band density for pUC18rbcLXS expression was set as 100%.

within a dimer RbcL subunit can bind *rbc*L mRNA in a sequence-independent manner, causing translational arrest. When the N-terminus of RbcL is buried in the folded protein within the dimer and does not interact with the *rbc*L transcript, the translation is possible. Thus, RbcL translation is governed by its dimerization. In the light of the presented literature data, and based on our coexpression studies, the RbcX protein appears to be active in the L_2 dimer formation. In consequence, RbcX causes an increase of the RbcL level in the soluble fraction. Moreover, RbcX stimulates the formation of the L_8 core complex even in RbcS absence.

The coexpression of RbcL and RbcX in the presence of RbcS resulted in an increase of the RbcL level in the soluble fraction. (Fig. 4A, lane 3). Additionally, the native RuBisCO (L_8S_8) was stabilized (Fig. 4B; lane 3). The RbcX protein in the soluble fraction was also detected (not shown).

The coexpression of RbcL with GroEL/GroES was also studied. As a result a reduced amount (compared to RbcL and RbcX coexpression) of RbcL was found in soluble fraction (Fig. 4A, lane 4), and a small quantity of L_8 core complex was detected (Fig. 4B; lane 4). Those observations indicate that a limited amount of soluble cyanobacterial RbcL and L_8 core complex can be formed in a bacterial system *via* nonspecific folding and assembly pathways.

CONCLUDING REMARKS

We report here the overexpression, purification and initial characterization of the RbcX protein from *T. elongatus*. The expression system developed in this study provides a sufficient amount of pure recombinant RbcX protein for further studies. Our results have revealed that the RbcX protein can form a dimer in solution. RbcX proteins from various species show no homology to any other protein deposited in databases. Literature data (Saschenbrecker *et al*., 2007) and our results show that the RbcX protein may play a role of an assembly chaperone in RuBisCO biosynthesis. RbcX mediates L_2 dimer formation and is essential for L_8 core complex assembly. It has been reported for RuBisCO from *Synechococcus* sp. PCC6301 (where the *rbc*X gene is localized outside the RuBisCO operon) that RbcX was not essential for the L_8 core assembly, although the coexpression of RbcL with RcbX significantly enhanced the L_8 complex formation. In contrast, in the case of *Synechococcus* sp. PCC7002, *Anabaena* sp. CA and *T. elongatus* BP-1 (studied here), where the *rbc*X gene is cotranscribed with the *rbc*L and *rbc*S genes as one operon, the requirement of RbcX for correct RuBisCO assembly has been demonstrated. Thus, it seems that the presence of the *rbc*X gene within or outside the RuBisCO operon is closely related to its significance in RuBisCO assembly. However, understanding the RbcX function requires a detailed protein structure analysis, which is being carried out in our laboratory.

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