

Pinpointing *Synechococcus* Rubisco Large Subunit Sections Involved in Heterologous Holoenzyme Formation in *Escherichia coli*

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Research Article

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Abstract

Background: Heterologous holoenzyme formation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) have been a challenge due to limited understanding of its biogenesis. Unlike bacterial Rubiscos, eukaryotic Rubiscos are incompatible with the *Escherichia coli* chaperone system to fold and assemble into the functional hexadecameric conformation (L_8S_8), which comprise eight large subunits (RbcL) and eight small subunits (RbcS). Our previous study reported three sections (residues 248-297, 348-397 and 398-447) within the RbcL of *Synechococcus elongatus* PCC6301 may be important for formation of L_8S_8 in *E. coli*. Present study further examined these three sections separately, by dividing them into six sections of 25 residues (i.e. residues 248-272, 273-297, 348-372, 373-397, 398-422 and 423-447).

Methods and Results: Six chimeric Rubiscos with each section within the RbcL from *Synechococcus* replaced by their respective counterpart sequence from *Chlamydomonas reinhardtii* were constructed and checked for their effect on holoenzyme formation in *E. coli*. Present study shows that Section 1 (residues 248-272; section of *Synechococcus* RbcL replaced by corresponding *Chlamydomonas* sequence), Section 2 (residues 273-297), Section 3 (residues 348-372) and Section 6 (residues 423-447) chimeras failed to fold and/or assemble despite successful expression of both RbcL and RbcS. Only Section 4 (residues 373-397) and 5 (residues 398-422) chimeras could form L_8S_8 in *E. coli*.

Conclusion: As GroEL chaperonin mediates folding of bacterial RbcL in *E. coli*, residues 248-297, 348-372 and 423-447 of *Synechococcus* RbcL may be important for interacting with the GroEL chaperonin for successful holoenzyme formation in *E. coli*.

Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, E.C 4.1.1.39) is the CO_2 fixing enzyme that catalyzes incorporation of atmospheric CO_2 onto organic carbon ribulose-1,5-bisphosphate (RuBP) during the Calvin-Benson-Bassham cycle of photosynthesis to produce two molecules of 3-phosphoglycerate (3PGA) for biomass accumulation in photoautotrophs [1]. Given its pivotal role, Rubisco is accounted as the gateway for inorganic CO_2 into the biosphere and its capability to sequester CO_2 dictates the efficiency of photosynthetic CO_2 assimilation [2]. Nevertheless, Rubiscos are slow enzymes with carboxylation turnover rates (k_{cat}^C) in a range of 1 s^{-1} to 13 s^{-1} [3]. Moreover, its inability to distinguish between CO_2 and O_2 for fixation onto RuBP greatly decreases the photosynthetic efficiency as oxygenation of RuBP lead to formation of one 3PGA and one 2-phosphoglycolate (2PG), which must be recycled into 3PGA through photorespiration at the expense of high cellular energy and loss of fixed carbon as CO_2 [4]. Slow catalytic rate and oxygenase activity of Rubiscos render CO_2 assimilation as one of the rate-limiting factors of photosynthesis [5]. As a result, Rubisco has been a prime target for genetic engineering to improve its catalytic performance in terms of CO_2/O_2 specificity factor (Ω) and carboxylation rate as a means to increase photosynthetic efficiency to raise crop productivity [6, 7].

Rubiscos found in plants, algae and photosynthetic bacteria are hexadecamers (L_8S_8 , ~560 kDa) consisting of eight large subunits (RbcL, 50-55 kDa) and eight small subunits (RbcS, 12-15 kDa) [8]. They share conserved structures and key catalytic residues, but have varying kinetic properties [9]. Extensive studies of Rubisco have been conducted in order to engineer a catalytically improved Rubisco as well as to identify the key determinants governing the catalytic properties[10–15]. Unfortunately, unsuccessful heterologous holoenzyme formation of Rubiscos in phylogenetically distant host limits the study of Rubiscos from diverse sources and selection of host for their genetic manipulation. For instance, Rubiscos from the diatom *Phaeodactylum tricornutum*, and from non-green algae *Galdieria sulphuraria* and *Griffithsia monolis* failed to form functional enzymes in tobacco chloroplast despite successful expression of both RbcL and RbcS subunits[16, 17]. Similarly, eukaryotic Rubiscos from maize, wheat (*Triticum aestivum*), tobacco (*Nicotiana tabacum*) and green alga *Chlamydomonas reinhardtii* form insoluble aggregates in *E. coli* [18–21]. This has prevented efforts in directed evolution of Rubisco using Rubisco-dependent *E. coli*, which would have been useful for evolving kinetically improved Rubisco variants and providing structure-function insights [22–26].

Incapability of functional expression in heterologous host mainly stems from chaperone incompatibility and requirement of additional auxiliary factors for their complex biogenesis, which is a multi-step process requiring different kinds of chaperones for *de novo* folding and assembly [27–30]. In *E. coli*, folding of RbcL monomers are mediated by the endogenous GroEL-GroES chaperonin system prior to their assembly into oligomers [31, 32]. The importance of the GroEL-GroES chaperonin for Rubisco holoenzyme formation in *E. coli* were demonstrated by increased yield of soluble Rubiscos upon overexpression of GroEL, while mutations of GroEL or GroES abolished holoenzyme formation [33, 34]. In addition, studies have shown that Rubiscos are prone to aggregation and they are recognized as stringent substrates by the GroEL chaperonin, whereby *in vitro* reconstitution of dimeric Form II *Rhodospirillum rubrum* Rubisco and hexadecameric Form I cyanobacterial Rubisco require all GroEL, GroES, ATP and Mg²⁺ [31, 35, 36]. Therefore, unsuccessful formation of eukaryotic Rubiscos in *E. coli* suggests that their RbcLs (and more specifically, the amino acid sequences) are incompatible with bacterial GroEL/GroES chaperonin. Indeed, functional expression of *A. thaliana* Rubisco in *E. coli* require the co-expression of its chloroplast chaperonin Cpn60αβ/Cpn20, which cannot be replaced by the bacterial GroEL/GroES homolog [37].

Our previous study replaced sections of cyanobacterial RbcL from *Synechococcus elongatus* PCC6301 with their eukaryotic counterpart sequences from the green alga *Chlamydomonas reinhardtii* sequentially, and a few sections of *Synechococcus* RbcL that might be essential for successful holoenzyme formation in *E. coli* were reported [20]. Present study aimed to narrow down the range by further examining each half (25 residues) of three sections that were pinpointed (i.e. amino acids 248-297, 348-397 and 398-447). Therefore, six chimeric Rubiscos with 25-amino acid sections of *Synechococcus* RbcL (residues 248-272, 273-297, 348-372, 373-397, 398-422, and 423-447) substituted with the corresponding residues

in *Chlamydomonas* RbcL, respectively, were constructed to examine their importance for holoenzyme formation in *E. coli*.

Materials And Methods

Construction of chimeric Rubiscos

Gene fragments required for constructing chimeric *rbcL-rbcS* operons were amplified from pTrcSynLS harbouring the wild-type *Synechococcus* PCC6301 *rbcL-rbcS* operon [22] and plasmids carrying different recombinant *rbcL-rbcS* [20] using *Pfu* DNA Polymerase (Vivantis). Primers used for PCR amplification were designed with different restriction sites (*PstI*, *Ncol* and *BsmBI*) to facilitate directional cloning (Table 1; Supplementary information). Amplified products were digested with restriction enzymes and ligated into the vector backbone (pTrcHisB) of pTrcSynLS using T4 DNA ligase (Invitrogen) (Table 1; Supplementary information). XL-1 Blue *E. coli* cells were transformed with the ligation mixtures and selected on LB plates containing 100 µg ml⁻¹ ampicillin. Positive transformants were identified by colony PCR and plasmids were extracted using commercial plasmid miniprep kit for DNA sequencing.

Rubisco expression induction and protein extraction

Constructed chimeric Rubiscos were transformed into XL-1 Blue *E. coli* cells and grown on LB agar (100 µg ml⁻¹ ampicillin) overnight. Single colonies were inoculated in LB broth (100 µg ml⁻¹ ampicillin) and cultured at 37 °C, 200 rpm for 16 h. Cultures were then diluted 50X into fresh LB broth (100 µg ml⁻¹ ampicillin) and grown at 37 °C, 200 rpm. When OD₆₀₀ of 0.5 was reached, 0.5 m M IPTG was added to the cultures to induce expression of Rubisco (i.e. cultures were grown at 37 °C, 200 rpm for another 16 h). To extract the proteins (crude lysates), cells were harvested by centrifugation at 5000 g for 5 min, re-suspended in ice-cold extraction buffer (50 m M Bicine/NaOH, pH 8.0, 10 m M MgCl₂, 10 m M NaHCO₃ and 2 m M DTT) to 10 % cell suspensions, and sonicated.

Expression and assembly analysis of Rubisco

Expression of RbcL and RbcS in *E. coli* was checked by denaturing SDS-PAGE. Crude lysates were mixed with sample loading buffer (30 % sucrose, 5 % SDS, 0.05 % bromophenol blue and 100 m M DTT) at 3:2 ratio and boiled for 5 min. Then, 4 µl of boiled sample mixtures was resolved on 12 % polyacrylamide gel. On the other hand, assembly or formation of Rubisco holoenzyme was examined by non-denaturing native PAGE. For native PAGE, crude lysates were mixed with sample loading buffer (50 % glycerol, 150 m M Tris-HCl, and 0.25 % bromophenol blue) at a 4:1 ratio and 25 µl of sample mixtures were resolved on 7.5 % polyacrylamide gel. For Western blot analysis, resolved proteins from SDS-PAGE and non-denaturing PAGE were transferred to nitrocellulose membranes (0.45 µm) by electro-blotting at 30 V for 2 h [38]. The membrane was then probed with rabbit anti-*Synechococcus* PCC6301 Rubisco IgG [24].

Results

In order to examine sections of *Synechococcus* RbcL essential for holoenzyme formation in *E. coli*, six chimeric Rubiscos with sections of 25 residues (i.e. residues 248-272, 273-297, 348-372, 373-397, 398-422 and 423-447) separately changed to corresponding residues in *Chlamydomonas* RbcL were constructed (Fig. 1). The number of amino acid changes in the sections ranges from one to eight amino acids (Fig. 2). SDS-PAGE and Western blot analysis of denatured total cellular proteins from *E. coli* transformed with chimeras respectively showed that both large and small subunits of all the chimeric Rubiscos were expressed in *E. coli* (Fig. 3). Therefore, any undetectable assembly of L₈S₈ was not due to non-expression. Native-PAGE and Western blot analysis showed sections 4 and 5 chimeric Rubiscos assembled into L₈S₈ whereas sections 1, 2, 3 and 6 chimeras had no detectable complexes in *E. coli* (Fig. 4). It is noteworthy that the section 4 chimera showed a reduced amount of L₈S₈ as compared to wild-type Rubisco and the section 5 chimera (Fig. 4).

Discussion

Unlike its eukaryotic isoform, RbcL from *Synechococcus* sp. PCC6301 are compatible with *E. coli* GroEL chaperonins for proper folding, followed by assembly with RbcS into holoenzyme without additional chaperones. Here we show that sections 4 and 5 are not essential for GroEL interaction as substitution with corresponding *Chlamydomonas* sequences still allowed folding and assembly into their final hexadecameric complexes (L₈S₈) in *E. coli* (Fig. 4), although it is worth mentioning that *Chlamydomonas* sequences introduced into sections 4 and 5 chimeras might have changed their kinetic properties. Notably, section 4 chimera showed reduced amount of L₈S₈ than wild-type Rubisco and section 5 chimera. Section 4 chimera has two amino acid substitutions, S395A and V396C. One possibility is that these substitutions affect the expression level of RbcL and RbcS. But looking at the SDS-PAGE and Western blot analysis of total cellular extracts, only slight differences in the expressed level of RbcL and RbcS were observed among wild-type and chimeric Rubiscos (Fig. 3). This is consistent with previous findings whereby most mutations in RbcL generally do not affect the steady-state mRNA level [22, 34]. Another possibility is that S395A and/or V396C reduced the thermal stability of the holoenzyme, as have been reported before for other substitutions in RbcL [10, 39, 40].

As sections 1, 2, 3 and 6 chimeras showed no detectable L₈S₈ (Fig. 4), sections of *Synechococcus* RbcL that might be important for interacting with GroEL can be narrowed down to these sections, which comprise residues 248-272 (Section 1), 273-297 (Section 2), 348-372 (Section 3) and 423-447 (Section 6). Considering that GroEL chaperonin form multivalent interaction with its substrate polypeptide, whereby substrate polypeptides bind to multiple subunits of heptameric GroEL ring for efficient binding [41–43], it is possible that more than one of these sections interact with GroEL. Indeed, it has been reported that Rubiscos bind to a minimum of three consecutive subunits of GroEL ring for efficient binding [41, 43]. Binding of Rubisco to GroEL mutant with less than three consecutive binding-competent apical domain

resulted in significantly reduced amounts of binary complexes of Rubisco and GroEL mutant, as compared to the wild-type GroEL [43]. In accordance with this finding, cryo-electron microscopy (cryo-EM) study of GroEL-RbcL binary complex showed the C-terminal domain of RbcL folding intermediate in contact with three consecutive apical domains while the N-terminal domain in contact with one apical domain [44]. Therefore, it may be possible that the three separate RbcL C-terminal regions (residues 248-297, 348-372 and 423-447) examined herein are involved in GroEL interaction and that loss of any favourable interactions with these three regions disrupted consecutive binding of RbcL to GroEL subunits. Consequently, binding between GroEL and RbcL was abolished or substantially reduced to an extent that only a small number of RbcLs were captured by GroEL, properly folded and assembled, resulting in the amount of L₈S₈ was undetectable.

In fact, by fitting the cryo-EM structure (EMD-6726) [44] and *R. rubrum* Rubisco (PDB ID 5RUB), it is found that residues 35-67, 89-116, 201-224, 236-253, 268-280, 422-457 of the *R. rubrum* RbcL might interact with GroEL. These residues correspond to *Synechococcus* RbcL residues 44-47, 104-125, 208-231, 243-260, 270-282, and 433-468. Only eleven of these residues (i.e. residues 252, 253, 259, 276, 279, 280, 435, 441, 442, 444 and 446) were changed among our unassembled section chimeras. Thus, these eleven residues could be of particular interest for mediating RbcL-GroEL binding.

Other than loss of GroEL interaction, non-assembly could be due to structural destabilizing effect of substitutions when changed to the *Chlamydomonas* counterpart. Therefore, despite of being captured by the GroEL chaperonin, the chimeric RbcL monomer might not fold into its native structure stably, and thus was unable to assemble into the final hexadecamer. Sections 1, 2, 3 and 6 chimeras have four to eight substitutions (Fig. 2). In general, amino acid substitutions could contribute to beneficial, neutral or deleterious effects [45]. In the case of our chimeras, some of these substitutions might have disrupted intra-subunit interactions or even exerted disturbances to the tertiary structure. For section 1 chimera, which has five substitutions, A269G has been reported to improve the fitness of Rubisco-dependent *E. coli* [22]. Therefore, non-assembly is more likely caused by the remaining four substitutions. As for section 6 chimera, one of the six mutations, E444S, could cause loss of intra-subunit salt-bridge with R432 in the RbcL monomer (Fig. 5a).

In addition, as there are three states of assembly: dimeric L₂ pair, (L₂)₄ core complex and final hexadecameric L₈S₈, mutations that affect the inter-subunit interactions of any of these assembled complexes could eventually lead to non-assembly. Of all mutations involved in non-assembly, only E348D in section 3 chimera is located at the interface between RbcL and RbcS. By comparing the Rubisco crystal structures of *Synechococcus* (PDB ID 1RBL) and *Chlamydomonas* (PDB ID 1GK8), this mutation could result in replacement of the carboxyethyl side-chain of Glu-348 by a shorter carboxymethyl side-chain of Asp, which could potentially disrupt the salt-bridge with Lys-96 in the small subunit [20] (Fig. 5b).

Conclusions

When six sections of *Synechococcus* RbcL are substituted by counterpart sequence from *Chlamydomonas* RbcL, respectively, only sections 4 and 5 chimeric Rubiscos form L₈S₈ in *E. coli*. Non-assembly of sections 1, 2, 3 and 6 chimeras suggest that the substituted sections of *Synechococcus* RbcL might be important for interacting with GroEL chaperonin to be properly folded. Loss of these sections might have disrupted interaction with GroEL and led to misfolding or aggregation of chimeric RbcL, which precluded subsequent assembly. Yet, non-assembly could also be due to structural destabilizing effect of mutations introduced from *Chlamydomonas* counterpart. Although the true cause of non-assembly remains to be addressed, sections of *Synechococcus* RbcL that might be essential for successful holoenzyme formation in *E. coli* are narrowed down to residues 248-297, 348-372 and 423-447 for future dissection.

Declarations

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Conflicts of interest/Competing interests

The authors declare no conflicts of interest.

Availability of data and material

The data and materials from this study are available from the corresponding author on request.

Author contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Ong Wei Chi. The first draft of the manuscript was written by Ong Wei Chi and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Consent to Participate (Ethics)

Not applicable.

Consent to Publish (Ethics)

All authors agreed to the published work.

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Figures

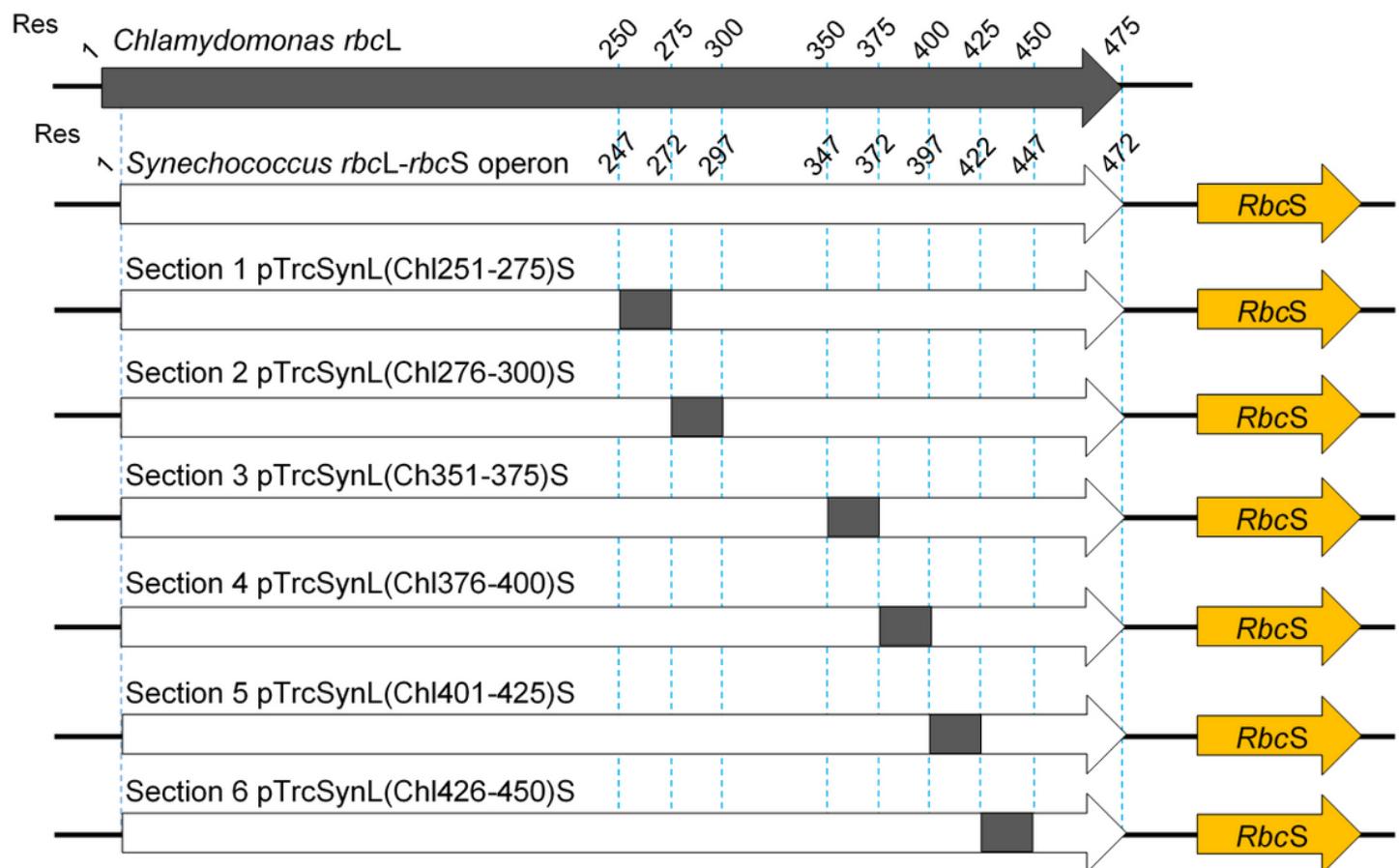


Figure 1

Schematic diagram of chimeric rbcL-rbcS operon. Six chimeric Rubiscos with respective sections of Synechococcus RbcL substituted by corresponding Chlamydomonas RbcL sequences were constructed. Res denotes residue number in RbcL genes



Figure 2

Sequence alignment of examined regions of Synechococcus and Chlamydomonas RbcL. Residue numbers (Res.) are based on Synechococcus RbcL

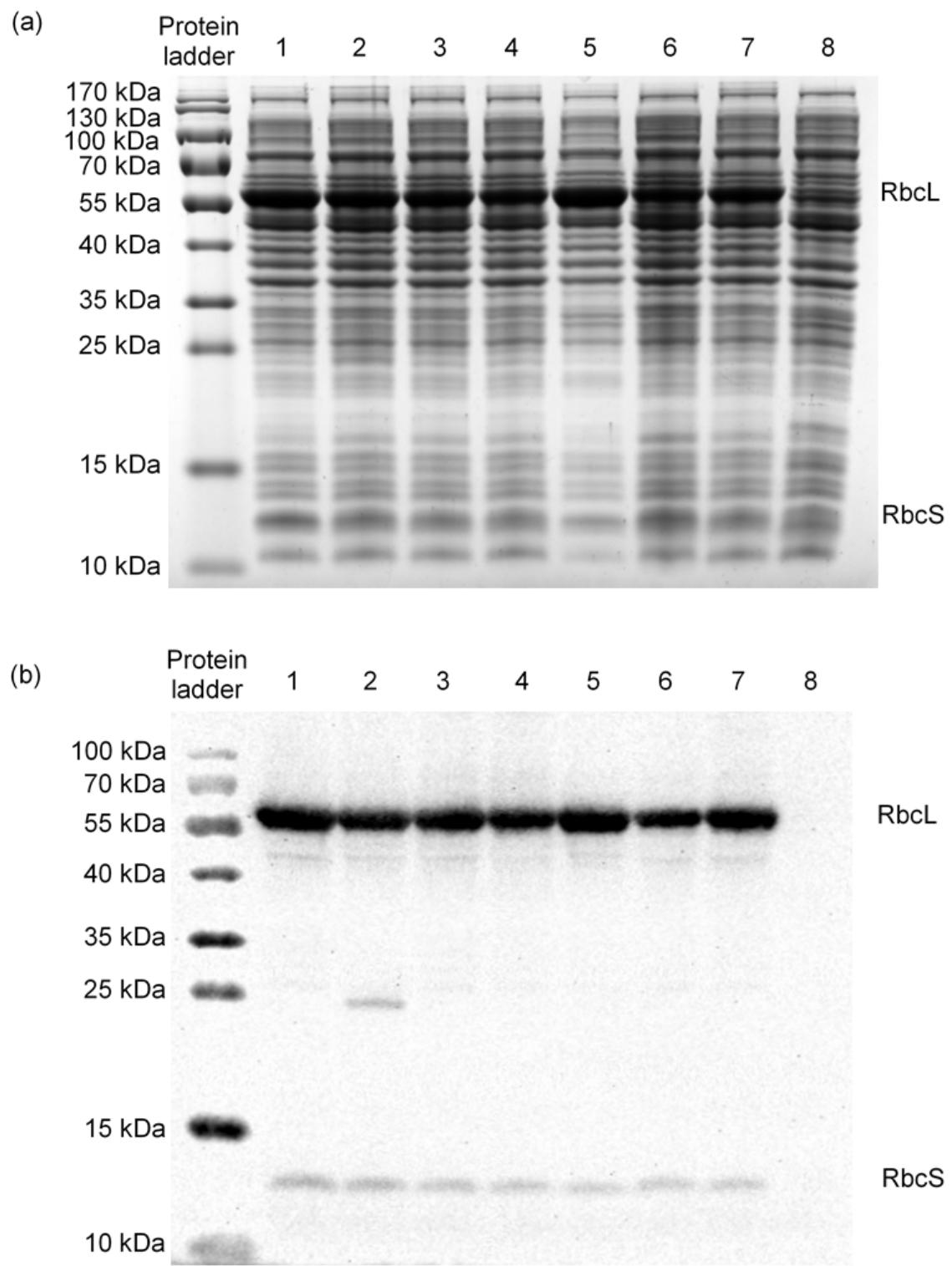


Figure 3

Expression analysis of chimeric Rubiscos in XL-1 Blue E. coli. (a) SDS-PAGE and (b) Western blot analysis total of cellular protein of E. coli transformed with (1) pTrcSynLS; (2) pTrcSynL(Chl251-275)S; (3) pTrcSynL(Chl276-300)S; (4) pTrcSynL(Chl351-375)S; (5) pTrcSynL(Chl376-400)S; (6) pTrcSynL(Chl401-425)S; (7) pTrcSynL(Chl426-450)S; (8) no plasmid

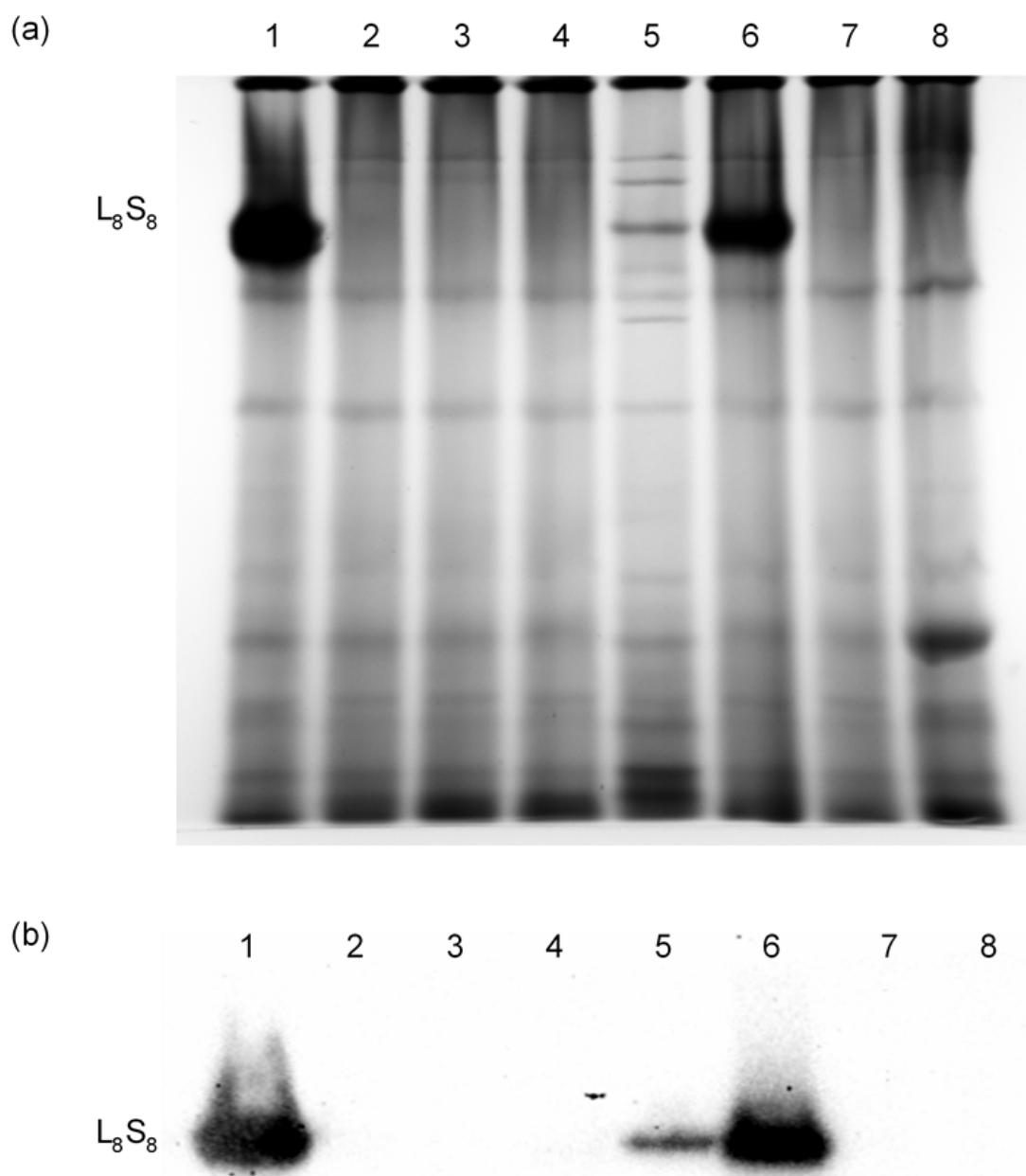


Figure 4

Assembly analysis of chimeric Rubiscos in XL-1 Blue E. coli. (a) Native PAGE and (b) Western blot analysis of total cellular protein of in E. coli transformed with (1) pTrcSynLS; ; (2) pTrcSynL(Chl251-275)S; (3) pTrcSynL(Chl276-300)S; (4) pTrcSynL(Ch351-375)S; (5) pTrcSynL(Chl376-400)S; (6) pTrcSynL(Chl401-425)S; (7) pTrcSynL(Chl426-450)S; (8) no plasmid

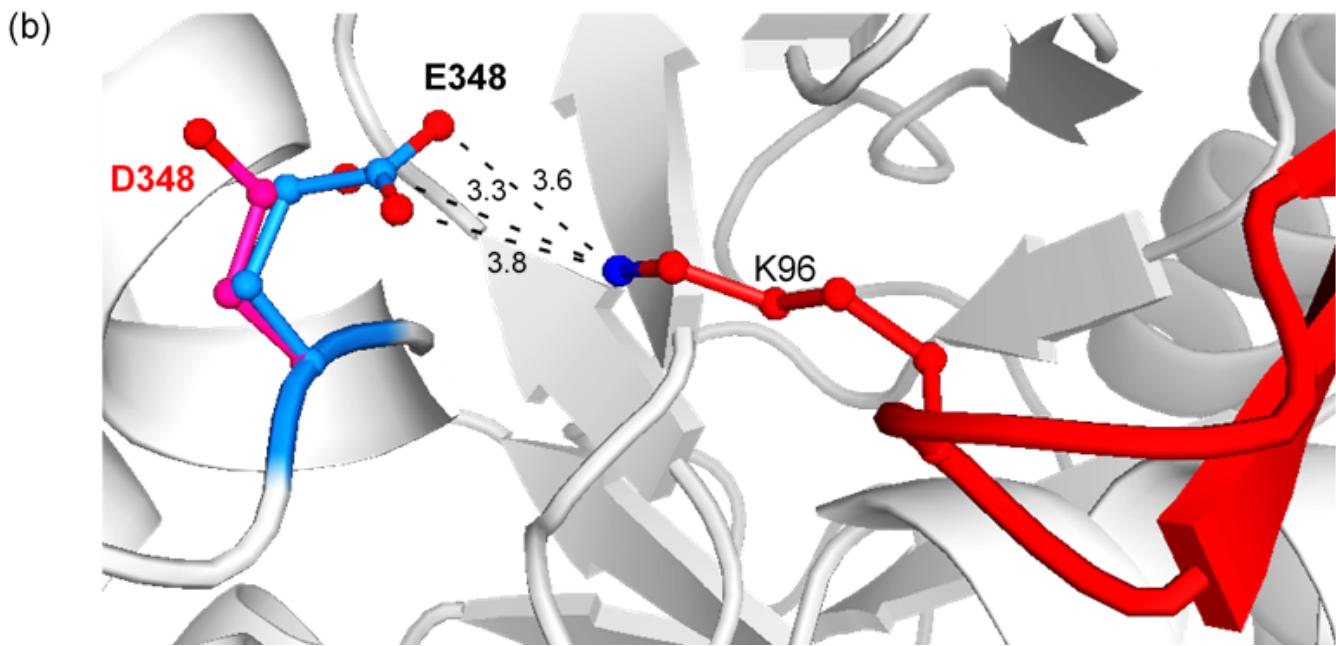
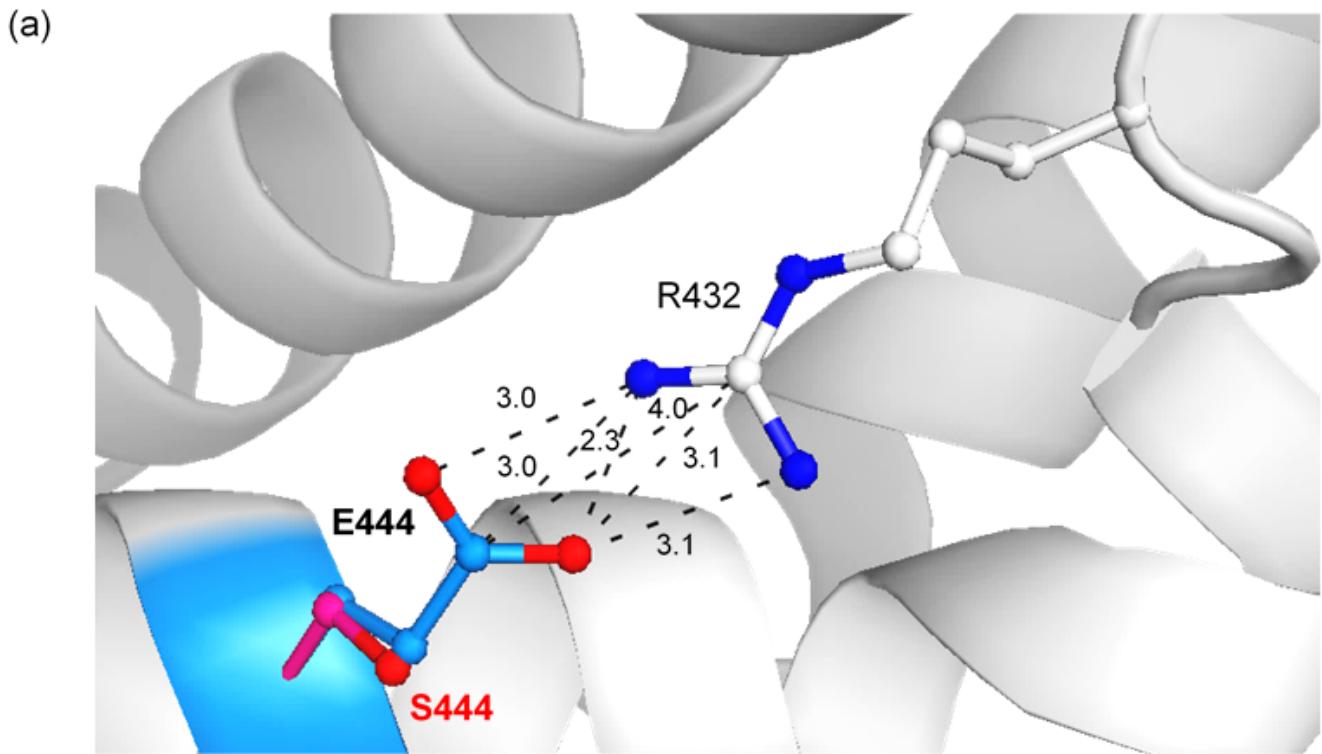


Figure 5

Potential loss of structural interaction in (a) E444S and (b) E348D. *Synechococcus* and *Chlamydomonas* Rubiscos (PDB IDs 1RBL and 1GK8) were superimposed. Mutated *Synechococcus* residue is light blue and the *Chlamydomonas* residue pink. Nitrogen atoms dark blue and oxygen atoms red. *Synechococcus* small subunit red. *Synechococcus* residue with potentially weakened/lost interaction in the chimera also shown (grey, distance measurements in Å)

Supplementary Files

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- [SupplementaryTable.docx](#)