Structure Insight into Photosystem I Octamer from Cyanobacteria

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15   **ABSTRACT**

16   Diversity of photosystem oligomers is essential to understand how photosynthetic
 17   organisms adopted to light conditions. Given by the structural and physiological significance,
 18   the assemblies of PSI supercomplex is of great interest in both chloroplast and cyanobacteria
 19   recently. In this study, two novel photosystem I supercomplexes were isolated for the first
 20   time from the low light incubated culture of filamentous cyanobacterium *Anabaena* sp. PCC
 21   7120. These complexes were defined as PSI hexamers and octamers through biochemical and
 22   biophysical characterization. Their 77K emission spectra indicated that the red forms of
 23   chlorophylls seemed not to be affected during oligomerization. By cryo-EM single particle
 24   analysis, a near-atomic (7.0 Å) resolution structure of PSI octamer was resolved, and the
 25   molecular assemblies of stable PSI octamer was revealed.

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 27   **Keywords:** cyanobacteria, photosystem I, hexamer, octamer, single particle

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30 INTRODUCTION

31   Photosystem I (PSI) is a light-driven plastocyanin: ferredoxin oxidoreductase with a
 32 quantum efficiency close to 100%, probably the most efficient photoelectron converter in
 33 nature (Amunts and Nelson, 2009). The function of this bio-macromolecule is highly
 34 conserved among cyanobacteria, algae, and plants, yet carries complicated structural
 35 diversities, especially in cyanobacteria. It has been acknowledged for years that chloroplast
 36 PSI-LHCI complex can only form monomeric state. And this classic theory was challenged
 37 by an observation of spinach thylakoid membrane with atomic force microscopy (AFM), in
 38 which PSI-LHCI complexes tense to be assembled each other to form a dimeric supercomplex
 39 in dark condition (Wood et al., 2018). And an atomic-resolution (2.97 Å) structure of novel
 40 dimeric PSI-LHCI supercomplex was resolved in low light adapted green alga
 41 *Chlamydomonas reinhardtii* very recently (Naschberger et al., 2021). Things are more
 42 complicated in cyanobacteria, in which the PSI complex is assembled as a trimer in most
 43 species (Boekema et al., 1987, Kruip et al., 1994, Jordan et al., 2001), meanwhile, the dimer
 44 and tetramer have been identified in some heterocyst-forming cyanobacteria and their close
 45 unicellular relatives (Watanabe et al., 2014; Li et al., 2014; Li et al., 2019, Zhang et al., 2019,
 46 Chen et al., 2020). Recently, high-resolution structures of PSI monomers with full
 47 physiological function were also resolved in some species of unicellular cyanobacteria
 48 (Netzer-El et al., 2018, Çoruh et al., 2021). All these studies have indicated that the dynamic
 49 oligomeric states of PSI complex are essential to help photosynthetic organism adapting the
 50 changing environments, especially under stress conditions, such as limited light energy
 51 supplying.

52   Moreover, by using blue-native PAGE, Li and co-workers observed a possible PSI
 53 hexamer in *Chroococcidiopsis* sp. PCC 6712 upon thylakoid membrane extraction (Li et al.,
 54 2019). And before that, a projection of two associated PSI tetramers was spotted with single
 55 particle analysis in filamentous cyanobacterium *Anabaena* sp. PCC 7120 (here after
 56 *Anabaena* 7120) (Watanabe et al., 2014). Due to the key role of PSI oligomer states in
 57 understanding the photosynthesis mechanism, molecular evidences and structure insights to
 58 those higher-ordered PSI oligomer complexes are of significant interests and highly desired

now. Here, two novel oligomer states of PSI complex were isolated from the low light incubated culture of *Anabaena* 7120. The pure molecules were set into biophysical analysis, and the existence of higher-ordered PSI supercomplexes in cyanobacteria was ultimately demonstrated. By employing cryo-EM single particle analysis, one of these PSI supercomplexes was subjected to high resolutions structure determination, in which a novel molecular interface for PSI complex assembling were detected.

RESULTS AND DISCUSSION

Discovery of higher-ordered PSI complexes

Blue-native PAGE has been recognized as a powerful tool to investigate thylakoid membrane complexes (Wittig et al., 2006, Sari et al., 2011). The bands corresponding to complexes of high molecular weight suggest the existence of higher-order oligomeric states of photosynthetic membrane complexes currently undefined, especially for PSI assembled hetero- or homo- complexes (Sari et al., 2011; Kouril et al., 2018; Zhang et al., 2010).

As shown in figure 1a₁, two previous undescribed complexes (supercomplex 1 and 2), both larger than the PSI-tetramer (1400 kDa), were extracted from *Anabaena* 7120 using 1% (w/v) β -DDM and subsequently separated on BN-PAGE gel. The naturally green colour of the bands indicates that these are chlorophyll-containing complexes, most likely the PSI-assembled complexes. To verify this hypothesis, the complexes were separated and harvested through sucrose gradient ultracentrifugation. The fraction scattering in sucrose gradients (Figure 1a₂) is highly consistent with the gradient gel results in the BN-PAGE experiment (Figure 1a₁). The fractions corresponding to supercomplex 1 and supercomplex 2 were collected and subjected to low temperature (77 K) fluorescence spectra measurement, and the PSI tetramer fraction was also harvested for comparison. As shown in Figure 1b, the emission spectra of supercomplexes 1 and 2 peaked at ~730 nm, largely overlapping with the one of PSI tetramer. Their emission spectra showed that the lowest energy level of quantum yield of chlorophylls in PSI remained ~730 nm, indicating that the red forms of chlorophylls were not energetically altered. This fluorescence spectral characteristic strongly suggests that these two complexes are both PSI homogenous supercomplexes (Watanabe et al., 2014, Lamb

87 et al., 2018, Chen et al., 2020).

88 For a closer look at the molecules, the complexes were subjected to negative staining
89 electron microscopy (EM) single particle analysis. The PSI tetramer, supercomplex 1, and
90 supercomplex 2 were stained with 0.75 % (w/v) uranyl formate and observed by transmission
91 electron microscopy (TEM). While the homogeneity of particles from all three samples is
92 acknowledged (figure 1c₁, 1c₂ and 1c₃), the remarkable diversity in particle size from different
93 samples is also revealed by negative staining EM. We subsequently using single particle
94 analysis (SPA) approach to check the oligomeric states of various samples. Using the
95 high-resolution structure of PSI tetramer as reference (Zheng et al., 2019; Kato et al., 2019;
96 Chen et al., 2020), particles in micrograph figure 1c₁ are tilted views of the PSI tetramer. Over
97 90% of the particles presented a top-view (yellow circles) orientation parallel to the thylakoid
98 membrane plane, while about 9.5% of them are side views (red circles). The contribution of
99 the top and side views is consistent with previously report (Watanabe et al., 2014). For the
100 samples of supercomplex 1 and supercomplex 2, the particle morphology in the images
101 indicated that these proteins are PSI higher-ordered oligomeric complexes (figure 1c₂ and
102 1c₃).

103 To further characterize the molecular architectures, three datasets of 16, 37, and 18
104 micrographs were collected for the PSI tetramer, supercomplex 1, and supercomplex 2,
105 respectively, from which 359, 2518, and 822 particles were manually picked. Particles were
106 then used for pre-processing and 2D classification, and the most dominant averages are
107 shown as the enlarged part in the bottom right corner of figure 1c₁, figure 1c₂, and figure 1c₃.
108 From these closer views, it is strongly suggested that supercomplex 1 is a PSI hexamer
109 consisting of a tetramer and a dimer, while supercomplex 2 is a PSI octamer formed by two
110 assembled tetramers, which agree with the previous results (Watanabe et al., 2014). This
111 conclusion is supported by a rigid fitting of PSI tetramer and dimer models over the 2D map
112 of the complexes, respectively (figure 1d). By comparing to the PSI tetramer, the PSI hexamer
113 can be identified as a complex arranged with a tetramer and dimer with substantial confidence.
114 However, it is ambiguous to identify the interface for the linkage of the PSI tetramer and
115 dimer in such low-resolution map. Furthermore, from the fitted model (figure 1d₂), the

116 PsaL-PsaA, PsaK-PsaK, and PsaM-PsaF interfaces should be addressed in further studies
117 providing high resolution details. PSI octamer revealed in figure 1d₃ is a supercomplex
118 assembled though 'side by side' arrangement of two tetramers, the fitted model reveals the
119 possible interfaces of PsaA-PsaF, PsaK-PsaK, and PsaB-PsaK. Studies providing
120 high-resolution structure would also be appreciated to investigate the detailed molecule basis
121 for the intra- and inter- connection of these supercomplexes.

122 **Cryo-EM structure of PSI octamer complex**

123 In our experiments, the in vitro safety and stabilities of PSI octamer is better than the
124 hexamer, for this reason, a near-atomic resolution structure of stable PSI octamer was
125 resolved by cryo-EM single particle analysis in the present study. For maximal harvests and
126 minimal destroy to PSI octamer, Native-PAGE based purifying method was applied for
127 sample purification. The whole processes for concentrated pure samples preparation were
128 completed in only six to eight hours, and the fresh molecules were than frozen immediately
129 for cryo-EM single particle analysis. Protein samples with a concentration of approximately 3
130 mg/mL were loaded onto the grids for frozen and quality evaluation. Initially, very few
131 particles could be observed in the hole of the ice layer, while most of them clumped together
132 around the carbon edge (Figure 2a₁). To help particles move into the hole, an extra detergent,
133 fos-choline 8, was added to the sample immediately just before the blotting. A significantly
134 improved distribution of particles in the holes was obtained, with approximately 30 particles
135 per micrograph (Figure 2a₂). However, the presence of random orientations is a challenge
136 when considering the surface charge of the complex. By optimising the wait time between
137 sample loading and blotting, we improved the distribution of the side view by approximately
138 10% (figure 2a₃). The preferential orientation issue is biological specimen dependent, and
139 many options may proceed case by case (Han et al., 2020, Sorzano et al., 2021, Tan et al.,
140 2017). If the particles are two linked PSI tetramer, then the preferential orientation is
141 relatively inevitable as the hydrophilic area of top view is as large as around 1000 nm², while
142 it is less than 200 nm² in side view (supplementary figure 1). And top views have much higher
143 probability to be absorbed and face to the air water interface when sample being blotted and
144 frozen (Noble et al., 2018).

145 In data collection stage, 23491 movies were collected from 17 datasets using a 200 kV
146 microscope equipped with a Gatan K3 camera. As revealed in supplementary figure 2, in total
147 184882 particles were extracted during data processing, from which 124460 good particles
148 were randomly separated into 10 datasets and screened for 3D reconstruction and structure
149 determination respectively in cryoSPARC. Reconstruction of this complex proceeded without
150 applying symmetry (C1) from homogenous particle subsets. Non-uniform refinement from
151 12121 particles of subset 5 generated a map at overall resolution of 7.0 Å and highest local
152 resolution of 6.0 Å (supplementary figure 3). As shown in figure 2b, the molecular size of PSI
153 octamer are 40.6 nm, 25.7 nm and 9.8 nm on length, width, and height respectively. The
154 overall shape of the map strongly suggested that this megacomplex is a PSI oligomer
155 synclastic arranged by two linking tetramers side by side, which first proved that the complex
156 is a PSI octamer structurally. To verify this conclusion a PSI dimer model was fitted into a
157 part of the map (figure 2c₁ and 2c₂). The secondary structures in both of the transmembrane
158 and out membrane region (lumen and stroma sides) can be fitted perfectly into the densities.

159 Interestingly, a density of interface for linkage was objectively reconstructed in this map.
160 As shown in supplementary figure 4, the density (red frame) is consistent with the threshold
161 levels, and the firmness is stronger than the interfaces that forms dimers (green frame) and
162 tetramers (light blue frame). It is indicated that the newly identified PSI octamer is a
163 physiologically relevant megacomplex rather than a random aggregation during sample
164 preparation. This solid evidence was then obtained from the biochemical characterizations of
165 PSI octamer from cells growing in changing physiological conditions. As shown in
166 supplementary figure 5, by native-PAGE characterization of cells that incubated in different
167 light conditions, the PSI octamer is a low light responded supercomplex in *Anabaena* sp. PCC
168 7120. The result agrees with the conclusion from Wood's work published in 2018, in which
169 tighter packing of PSI complexes were detected in the dark (Wood et al., 2018). To find the
170 possible interfaces for the assembly of two tetramers, separated PSI tetramer model were
171 fitted rigorously into the map with ChimeraX program. As shown in figure 3, an interaction
172 between PsaK subunits from two linked PSI tetramers was found in the fitted model (figure
173 3a). As revealed in figure 3b, two residues Lys52 and Phe53 of PsaK from tetramer 1 (T1)

174 interacted with the same residues from tetramer 2 (T2) with each other. This novel interface
175 should be the major contribution to stabilize PSI octamer. In summary, the map is qualified to
176 be applied to perform rigid-body fitting with an atomic model when more structural details
177 are required to be revealed. On the other hand, this map is reconstructed with datasets
178 collected from the 200 kV microscope, and higher resolution is expected by using a zero-loss
179 energy filter equipped detector, and the resolution can also be theoretically significantly
180 improved with a 300 kV microscope.

181 Higher-ordered PSI oligomers are physiologically relevant megacomplexes rather than
182 random aggregations during sample preparation. For instance, in this study, the PSI octamer was
183 strongly suggested as a low light responded supercomplex in *Anabaena* sp. PCC 7120 with the
184 results of supplementary figure 5. Similar conclusion was presented by Wood's observation
185 through atomic force microscopy, in which tighter packing of chloroplast PSI complexes are more
186 abundant in the dark (Wood et al., 2018). And the super high-resolution structure of such tight
187 packed chloroplast PSI oligomer was resolved very recently (Naschberger et al., 2021). Novel
188 type of interfaces for connection between PSI monomers were structurally detected and
189 genetically solidified. PSI assembled heterogenous or homogenous supercomplexes are often
190 detected biochemically in previous studies (Sari et al., 2011; Kouřil et al., 2018; Zhang et al.,
191 2010); here, we isolated two supercomplexes and set up biophysical characterisations of the
192 molecules. Taking the evidence from low-temperature fluorescence and negative staining EM,
193 we concluded that these two complexes are PSI hexamer and PSI octamer, respectively. To
194 our knowledge, this is the first study to isolate such higher-ordered PSI complexes in
195 photosynthetic organisms, paving the way for better understanding the molecular architecture
196 and physiological functions of each type of photosystem oligomers. And cryo-EM structure of
197 PSI octamer revealed a novel molecular interface (PsaK-PsaK) for PSI assembling in
198 photosynthetic organisms.

199 MATERIALS AND METHODS

200 Specie and growth conditions

201 Filamentous cyanobacteria *Anabaena* 7120 (FACHB-418), were purchased from Institute

202 of Hydrobiology, Chinese Academy of Sciences. Cyanobacterial cells were cultured at 20°C
203 to 30°C in BG-II (+N) media. The liquid culture in flask were incubated under illumination
204 with fluorescent lamps (5-50 $\mu\text{E m}^{-2}\text{s}^{-1}$).

205 **Membrane protein complexes solubilization**

206 For membrane complex extraction, 150 mL liquid cultures at the logarithmic phase
207 (A_{730} =1.0 to 1.3) were harvested at 4000 g centrifugation under 4°C, and cells was washed
208 once in 15 mL pre-cooled buffer A (50 mM HEPES, 10 mM MgCl_2 , 5 mM CaCl_2 and 15 mM
209 NaCl). Cells were then resuspended in 10 mL buffer A and treated with a cycle of 15 minutes
210 frozen in -80 °C refrigerator and 10 minutes melting in room temperature. Treated cells were
211 harvested at 4000 g centrifugation under 4°C and resuspended in 1.5 to 3 mL buffer A, and 10%
212 (w/v) of β -DDM were supplemented to the given final concentration for membrane
213 solubilization and complex extraction. The mixture was incubated on ice for 30 minutes, and
214 insolubilized materials were removed by 14,000 g centrifugation under 4°C for 15 minutes.
215 The supernatant in which membrane complexes resolved were harvest and store at 4°C, or
216 directly proceeded for BN-PAGE analysis.

217 **Isolation of membrane supercomplexes**

218 To isolate membrane supercomplexes, solubilized supernatant was collected and subjected
219 to sucrose gradient ultracentrifugation (10% to 50% (w/v) sucrose in buffer A with 0.03%
220 (w/v) digitonin) at 35000 rpm (sw41 Ti rotor, Beckman Coulter) for 16 hours at 4°C. The
221 band containing PSI tetramers and lower bands were collected with a syringe. The collected
222 fractions were buffer exchanged using a Milipore concentrator with 100 kDa molecular
223 weight cutoff into buffer A with 0.03% (w/v) digitonin to remove the sucrose and to
224 concentrate the sample.

225 **Blue-Native PAGE and reextraction**

226 BN-PAGE was performed in a cold room or a large ice bucket to maintain the low temperature
227 (4°C) during electrophoresis. A Bio-Rad Mini-PROTEAN Tetra Cell (1658006) system was used
228 for mini gel casting and electrophoresis running. The reagents for gel casting, the anode and

229 cathode native PAGE buffers were prepared according to Witting's protocol (Witting et al., 2006).
 230 A gradient generator was used to prepare the acrylamide gradient gel. In this study, 3%–10% (w/v)
 231 acrylamide gel was selected according to the large molecular weight of megacomplexes. In most
 232 cases, 4%–13% (w/v) gels are feasible to separate proteins with molecular weights ranging from
 233 10 kDa to 3,000 kDa, and 3%–13% (w/v) gels are appropriate for separating 10 kDa to 10,000
 234 kDa protein molecules. Electrophoresis power was generated using a Bio-rad PowerPac TM Basic
 235 Power Supply (1645050). In the first stage, cathode buffer B was used, and the power supply was
 236 set to 135V for electrophoresis. Move into stage two by exchanging cathode buffer B to cathode
 237 buffer B/10 when the samples were moved into a separate gel (approximately 30 min). The
 238 running was maintained at 135V for another 1.5 to 2 hours until acceptable separation efficiency
 239 was obtained.

240 For the reextraction of proteins, target bands were excised immediately after electrophoresis and
 241 stored in an ice-cooled Eppendorf tube. Gel bands were manually homogenised with a glass rod
 242 directly into the tube to minimise the loss of protein samples. To prevent heat damage to the
 243 protein complex, 10 cycles of 30 s on and 60 s off were programmed for homogenisation. The gel
 244 slurry was then mixed with reextraction buffer (buffer A with 0.03% digitonin), and the gel debris
 245 was removed by centrifugation at $14,000 \times g$ for 15 min at 4°C. The supernatant was subjected to
 246 a second run of BN-PAGE for purity and integrity check, and concentrated to qualified
 247 concentration with a 100 kDa cut off Millipore membrane filter (UFC5100BK) for single particle
 248 analysis.

249 **Cryo-EM and Image Processing**

250 Three microliters of purified PSI supercomplex was applied onto glow-discharged holey
 251 carbon grids (Cu Quantifoil R1.2/1.3) at a protein concentration of approximately 2.5 mg/ml,
 252 prior to vitrification using a Vitrobot MKIV (3.0 s blot 5, 4 °C, 100% humidity). The images
 253 were collected in 17 different sessions on 200 kV FEI Titan Krios electron microscope (50 µm
 254 C2 aperture). A Gatan K3-Summit detector was used in counting mode at a magnification of
 255 45,000 (yielding a pixel size of 0.88 Å), and a dose rate of 30 electrons per pixel per second.
 256 Exposures of 1.618 s (yielding a total dose of $63 \text{ e}^-/\text{Å}^2$). SerialEM was used to collect a total
 257 of 23491 images, which were fractionated into 27 movie frames with defocus values ranging

from 1.5 to 2.5 μm . All datasets were processed separately by using the same procedure. Collected micrographs were corrected for local-frame movement and dose-filtered using Motioncor2 (Zheng et al., 2017). The contrast transfer function parameters were estimated using Gctf (Zhang, 2016). In total, 184882 particles were autopicked using warp and subjected to reference-free two-dimensional class averaging in Relion 3.0.8 (Zivanov et al., 2018). After 2D classification, 12k good particles were generated, in which top-views were divided into 10 subsets. Each subset was combined with the side-view separately for the 3D map refinement in cryoSPARC (Punjani et al., 2017), yielding a map at an overall resolution of 7.0 $\text{\AA}$. No symmetry was applied during the final processing, as it did not improve the map. The reported resolutions are based on 3D refinement by applying 0.143 criterion on the FSC between reconstructed half-maps. All figures were generated using PyMOL (DeLano et al., 2002) and Chimaera X (Goddard et al., 2018). A local resolution map was generated using the cryoSPARC.

Negative staining EM and data processing.

The purified PSI tetramer and supercomplexes were adjusted to 0.15, 0.19 and 0.27 mg/ml protein respectively. In all cases, 3 μl of the protein solution was loaded on the glow-discharged copper grid. The excess protein solution was blotted by filter paper. The grid was washed twice with distilled water, and then stained with 0.75 % (w/v) uranyl formate for 30 s. The stain solution was blotted and the grid was dried in air. The grids were examined using a Tecnai G2 Spirit electron microscopy under 120 kV accelerating voltage. Micrographs were recorded with CCD camera at a normal magnification of 49,000 $\times$ with variation of defocus values from -1.5 to -2.5 μm , resulting in a pixel size of 4.3 $\text{\AA}$ at specimen level. In total 34, 16 and 18 images were manually collected for PSI dimer, tetramer and supercomplex. 359, 2518 and 822 particles were manually picked for PSI tetramer, and supercomplex in Relion 3.0.8. The selected particles were proceeded to a reference-free two-dimensional class averaging using Relion 3.0.8.

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289
290

FIGURE LEGENDS

Figure 1-Biochemical (a) and biophysical (b, c, and d) characterisation of the PSI tetramer, supercomplex 1, and supercomplex 2. a₁-membrane crude extracts were loaded on BN-PAGE at a total amount of 9 to 12 mg chlorophyll a for each lane; a₂-membrane crude extracts were loaded on sucrose gradient ultracentrifugation for the separation of PSI tetramer and supercomplexs. b-low temperature (77k) fluorescence emission detection of PSI tetramer (red line), supercomplex 1 (blue line), and supercomplex 2 (purple line). c-Negative staining electron microscopy image of PSI tetramer (c₁) supercomplex 1 (c₂) and supercomplex 2 (c₃). The yellow and red circles indicate the top and side views, respectively. The enlarged image in the right corner shows the 2D averages of the most contributed particles. d-The model fitted 2D average maps of PSI tetramer (d₁) supercomplex 1 (d₂) and supercomplex 2 (d₃).

Figure 2- The cryo-EM sample prep optimization and structure determination of PSI supercomplex 2. a-Optimization of cryo-EM sample. a₁-The protein particles were blotted immediately after sample loading; a₂-Detergent fos-coline-8 were supplemented into protein sample at final concentration of 1.0 mM around 5 seconds before blotting; a₃-Additional 3 seconds waiting time were introduced between the loading and blotting of fos-choline-8 mixed sample. b-Top (b₁) and side (b₂) views of the refined 3D density map of PSI supercomplex 2. c-Model and map fitting for structure comparison of cyanobacterial PSI dimer and the supercomplex. Top (c₁) and side (c₂) views of model fitting of PSI dimer into the density map. The model used for fitting is a published PSI dimer structure (PDB code: 6K61) from *Anabaena* sp. PCC 7120.

Figure 3-Structure details of PsaK-PsaK interaction in PSI octamer supercomplex. a-Overall view of the interface location in the complex (top view from stromal side). The original structure applied for fitting is PSI tetramer model (PDB code: 6TCL) from *Anabaena* sp. PCC 7120. b-Close-up view of structural details indicated in figure 3a. b₁-The enlarged top view of two PsaK subunits from the assembled PSI tetramers (T1 and T2), the interacted amino acids were shown as stick form. b₂-Tilted side view of interacted PsaK subunits. b₃-The focus view of intact amino acids Lys52 and Phe53 in PsaK subunits.

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416 **AUTHOR CONTRIBUTIONS**

417 M.C., X.P. and Y.Z. designed the research; Y.H, M.C. and D.L. performed the research and data
418 curation under supervision of L.T. and J.H.; M.C. wrote the manuscript with adding from Y.H., Y.P.
419 and D.L.; Y.Z., L.T and J.H. analysed the data and edit the manuscript; J.H. and Y.Z. supported the
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423

a **b** **c**

a **b₃** **b₁** **b₂**