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Short Communication

A study of the protein-protein interactions in the phycocyanin monomer from *Synechocystis* sp. PCC 6803 using a bacterial two-hybrid system

Simi Shao[a], Longsheng Zhao[a], Pingyi Li[a,*], Hainan Su[a,*], Xiulan Chen[a], Yuzhong Zhang[a,b,c,d]

[a] State Key Laboratory of Microbial Technology, Marine Biotechnology Research Center, Shandong University, Qingdao 266237, China
[b] College of Marine Life Sciences, Ocean University of China, Qingdao 266237, China
[c] Frontiers Science Center for Deep Ocean Multispheres and Earth System, Ocean University of China, Qingdao 266237, China
[d] Laboratory for Marine Biology and Biotechnology, Pilot National Laboratory for Marine Science and Technology, Qingdao 266237, China

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ABSTRACT

Investigations into the intramolecular interactions of the native protein in solution are important to understand its structural stability as well as its potential uses in future applications. In this study, we used a bacterial two-hybrid system to investigate the interaction between the phycocyanin α and β subunits that form the phycocyanin monomer. Key amino acid residues responsible for the interaction between the subunits were identified, providing direct experimental evidence for the intramolecular interaction.

Phycocyanin is the most widely distributed light-harvesting phycobiliprotein, and it is found in the phycobilisome complex in cyanobacteria and red algae. In addition to its important role in photosynthesis, phycocyanin has many potential applications in foods, cosmetics, and medical diagnosis, and also as fluorescent labeling probes or photosensitizers [1–4]. At present, the crystal structures of phycocyanin from several cyanobacterial and red algal species have been reported [1,5,6]. The basic building block of phycocyanin is the ($\alpha\beta$) monomer, which is composed of α and β subunits; the ($\alpha\beta$) monomer can further assemble into stable $(\alpha\beta)_3$ trimeric or $(\alpha\beta)_6$ hexameric discs [7]. The sequences of the α and β subunits of phycocyanin are highly conserved among different cyanobacterial and red algal species. Each subunit is composed of eight α helices, six of which (helices A to H) fold into a globin structure. Two additional helices (helices X and Y) serve as the association domain between the two subunits in the formation of the ($\alpha\beta$) monomer [8]. However, application of phycobiliproteins depends on a comprehensive understanding of their structural properties. In addition to the crystal structure, knowledge about the folding/unfolding properties [9,10], structural sensitivity to environmental conditions [11], intramolecular interactions [12], and thermal stability [13,14] will enable a deeper understanding of its structural properties.

From an investigation of the crystal structures and using bioinformatic calculations [15], several key amino acid residues were predicted to act as anchors in the interaction within and between the native monomers. The N-terminal X- and Y-helices of the α and β subunits were shown to contribute to the aggregation of the α and β subunits into the ($\alpha\beta$) monomers [16], and the key amino acid residues in these helices connect to residues in the other subunit through hydrogen bonds, hydrophobic interactions and electrovalent bonds. Nevertheless, there is no direct experimental biochemical evidence to support the interface hypothesis and the function of the key amino acid residues. A bacterial two-hybrid system has been used previously in cyanobacteria to detect protein-protein interactions [17–20]. In this study, we used a bacterial two-hybrid system to study the interaction between the two subunits of phycocyanin.

Structural analysis revealed that the heterodimerization of the phycocyanin monomer in *Synechocystis* sp. PCC 6803 is mainly stabilized by hydrogen bonds formed by residues Thr3, Asp13, Gly (16,89), Asn35, Arg (17,93), and Tyr97 from the α subunit and Asp (3,13), Glu17, Tyr18, Ser28, Arg (91,108), and Tyr (92,95) from the β subunit (Fig. 1). Multiple sequence alignment showed that most of the residues involved in maintaining the dimerization interface of the phycocyanin monomer are highly conserved in the proteins from *Synechocystis* sp. PCC 6803 and other cyanobacteria as well as red algae (Fig. 2).

Because *E. coli* has unique advantages for two-hybrid screening [21], the BacterioMatch II two-hybrid system was used to detect the interaction between the phycocyanin α and β subunits. As expected, the ratio of colonies co-transformed with the positive control vectors pTRG-Gal11^{P} and pBT-LGF2 was approximately 0.9, while that of colonies co-transformed with the negative control vectors pTRG and pBT was essentially 0, which showed that it is reasonable to use the colony ratio as an indicator of the interaction strength. No colonies grew on the selective plates when the reporter *E. coli* strain was transformed with the vector pairs pTRG-*cpcA*/pBT or pBT-*cpcB*/pTRG. In contrast, the ratio was 0.2–0.3 when the vector pair pTRG-*cpcA*/pBT-*cpcB* was used for

* Corresponding authors.
E-mail addresses: lipingyipeace@sdu.edu.cn (P. Li), suhn@sdu.edu.cn (H. Su).

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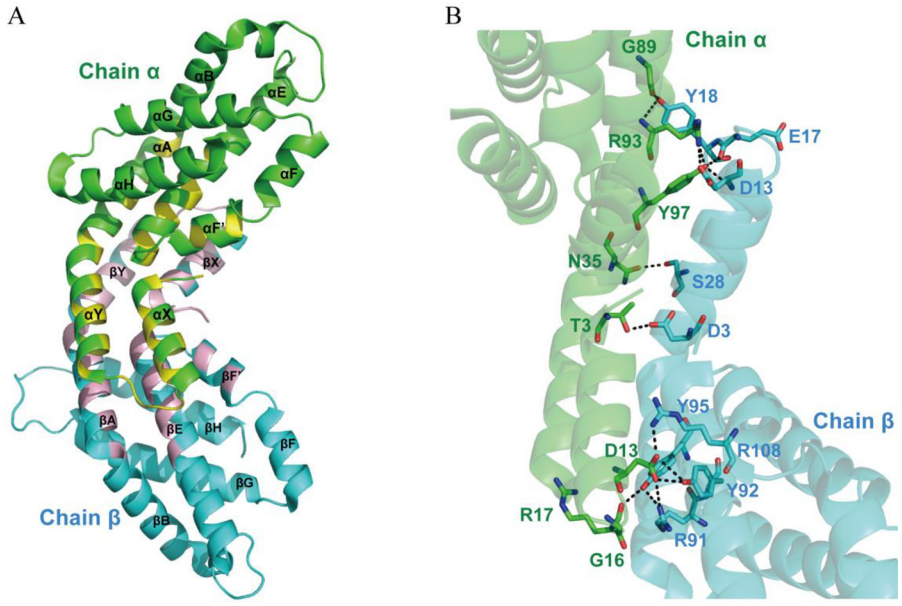

Fig. 1. Structural analysis of the phycocyanin monomer in *Synechocystis* sp. PCC 6803. (A) The heterodimerization pattern of the phycocyanin monomer in *Synechocystis* sp. PCC 6803. The *α* and *β* subunits are shown in green and cyan, respectively. On the heterodimerization interface, residues from the *α* subunit are shown in orange and residues from the *β* subunit are shown in pink. (B) The hydrogen-bond network between the interactive subunits *α* (in green) and *β* (in cyan) of the phycocyanin monomer from *Synechocystis* sp. PCC 6803.N

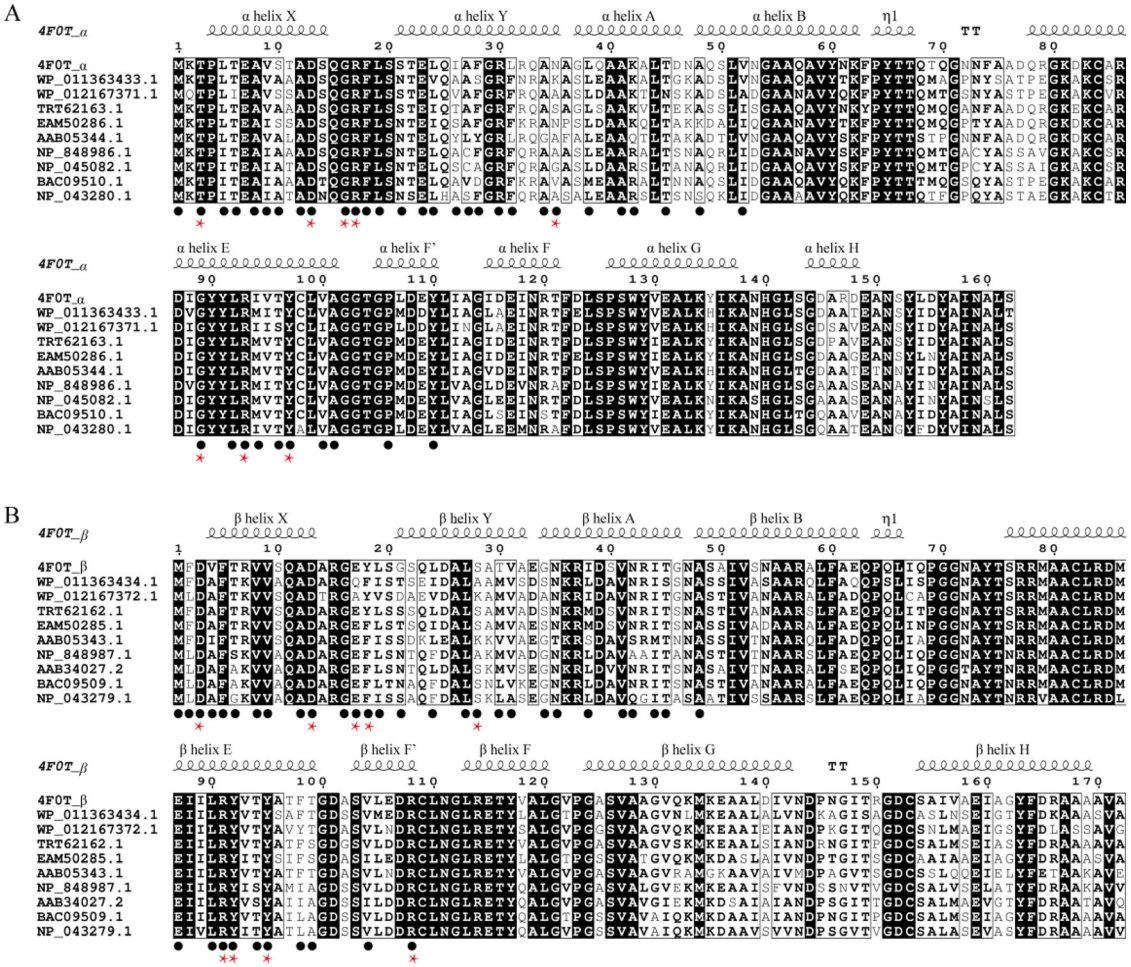

Fig. 2. Multiple-sequence alignments of the phycocyanin monomer subunits from *Synechocystis* sp. PCC 6803 and homologs from other cyanobacteria and red algae. Alignments of sequences of the phycocyanin *α* and *β* subunits from *Synechocystis* sp. PCC 6803 and other cyanobacteria as well as red algae are shown in (A) and (B), respectively. Using ESPript, the secondary structures of the phycocyanin subunits from *Synechocystis* sp. PCC 6803 are shown above the alignments. Helices are indicated by springs, strands by arrows, turns by TT letters, and 3_{10} helices by *η* letters. Identical residues are shown in white on a black background, and similar residues are shown in bold type. For the phycocyanin subunits from *Synechocystis* sp. PCC 6803, residues on the heterodimerization interface predicted by PISA are indicated by solid black circles, and the predicted key residues involved in maintaining the dimerization interface are indicated by red stars.

Table 1
Mutation sites introduced in this study.

Subunit	Mutation sites	
	Control sites[a]	Potential key residues in interface A and B
A	I26A, Y65A, *C84A*, E117A, F122A, K134A	S10L, A12L, D13A, D13N, D13R, D13Y, G16T, G16Y, G16YL19Y, R17A, L19A, L19Y, R93S, R93I, R93D, R93Y, Y97A, Y97V, Y97Q, Y110A
B	D39A, N54A, R57A, *C82A*, L83A, A128L, *C153A*	A12T, A12Y, D13A, D13N, D13R, D13Y, G16T, G16Y, E17A, R91A, Y92A, Y92V, Y92Q, Y95A, Y95V, Y95Q, R108S, R108I, R108D, R108Y

[a] The cysteines that bind to phycocyanobilins are shown in italics.

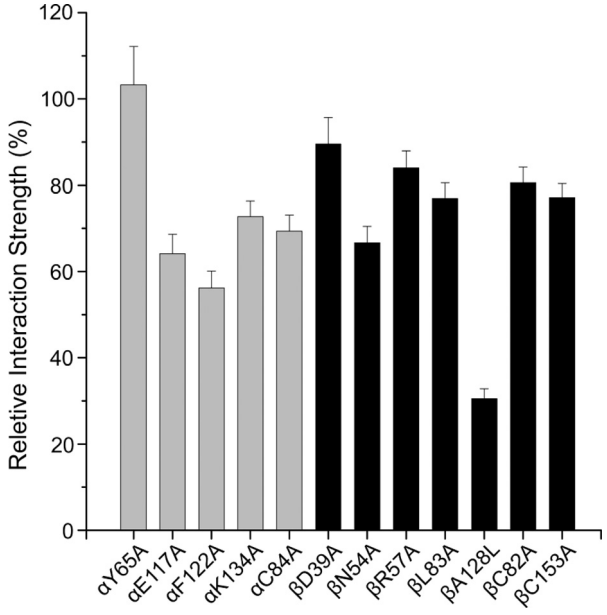

Fig. 3. Interactions between the control α and β subunits from the *Synechocystis* sp. PCC 6803 phycocyanin containing site-directed amino acid substitutions. Interactions between the control site-directed mutated α subunit and native β subunit (gray), and the native α subunit and control site-directed mutated β subunit (black) were both studied using the *E. coli* two-hybrid system. The interaction strengths were estimated by the ratios of colonies on selective plates to those on the nonselective plates. The relative interaction strength between the native α and β subunits was defined as 100%. The experiments were repeated three times.

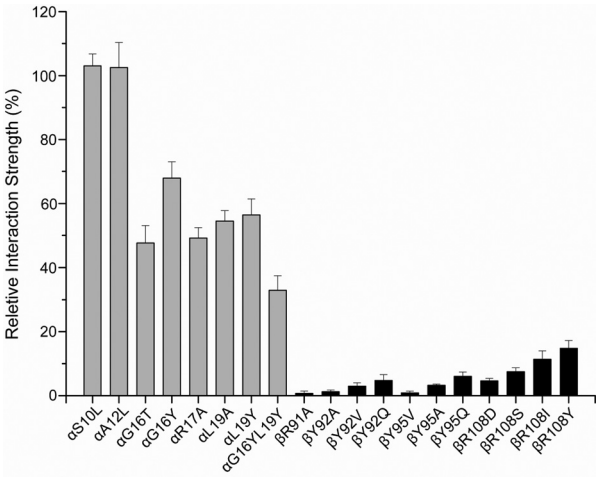

Fig. 4. Interactions between the site-directed mutated α and β subunits in interface A. Interactions between the particular amino acid residues in the X and Y helices in the mutated α subunits and native β subunits (gray), and between the native α subunits and particular amino acid residues in the E and F helices in the mutated β subunits (black) were both studied using the *E. coli* two-hybrid system.

transformation, indicating that there is strong interaction between the α and β phycocyanin subunits.

The key amino acid residues that mediate the interaction between the phycocyanin subunits were predicted by PIFPAM, and 10 candidate interaction interfaces (CIFs) were found. Based on the crystal structure of phycocyanin (PDB number: 4F0T), we found that two CIFs were located within the subunits, and the other four CIFs were located between the subunits. Several conserved residues from the two interfaces were selected and changed to other residues by targeted mutation. In addition, several amino acids selected randomly from both subunits which manifest no direct interaction with the other subunits were mutated as negative controls (Table 1).

There was no significant change in the interaction strength between the α and β subunits after the negative control sites were mutated except for the mutation A128L (Fig. 3). When the ratio of colonies co-transformed with the non-mutated vector pair pTRG-*cpcA*/pBT-*cpcB* was defined as 100%, the ratio was reduced to 30% after A128 in the β subunit was changed to leucine, suggesting that A128 plays an important role in the interaction between the α and β subunits. Even though A128, which is located inside the β "globin", has no direct interaction with the α subunit, the A128L mutation may change the structure of the β subunit and thus weaken the interaction strength between the α and β subunits. Overall, the substitutions in the randomly-selected amino acid residues showed that most of them contribute little to the interaction between the α and β subunits.

The relative strength of interaction between the site-directed mutated α subunit and non-mutated β subunit ranged from 30% to 105%, while the interaction strength between the non-mutated α subunit and site-directed mutated β subunit was <10% (Fig. 4). This showed that amino acid changes in the α subunit had less effect on the interaction in interface A than did changes in the β subunit. Since G16 and L19 in the α subunit showed no significant effect on the interaction after point mutation, the two residues were mutated together, which was the only double mutation used in this study. The interaction between the G16Y/L19Y-mutated α subunit and the native β subunit was weaker than either of the single G16 or L19 amino acid substitutions. Because the residues G16 and L19 are both located in the joint between the X and Y helices, they are assumed to stabilize the 3-D structure of the X and Y helices rather than interact directly with other residues.

The interactions between the Y92 and Y95 mutated β subunits and the D13, G16, or L19 mutated α subunits were also studied (Table 2). Similar to the above situation, these interactions were weak, with most of the relative interaction strength <5%, suggesting that the Y92 and Y95 residues in the β subunit E and F helices are key residues in the interaction.

Similar results were observed in the interface B interaction study (Fig. 5). The Y110A mutation in the α subunit and the E17A mutation in the β subunit showed higher interaction strengths than the others, suggesting that the Y110 and E17 residues in the α and β subunits, re-

Table 2
Interaction of parts of the site-directed mutated α and mutated β subunits on interface A.

	cpcA	α D13R	α D13A	α G16T	α G16Y	α L19A	α L19Y
cpcB	+++	++++	++	+++	++	+++	+++
β Y92A	-	+	+	+	-	+	+
β Y92V	-	+	+	+	-	+	+
β Y92Q	-	+	+	+	+	+	+
β Y95A	-	+	+	+	+	+	++
β Y95V	-	-	+	+	+	+	+
β Y95Q	+	+	+	++	+	++	+

Note: The relative strength of the interactions is indicated by the number of plus signs. The number of plus signs depends on the relative interaction strength. +, <5%; ++, 5-20%; +++, 20-150%; ++++, >150%.

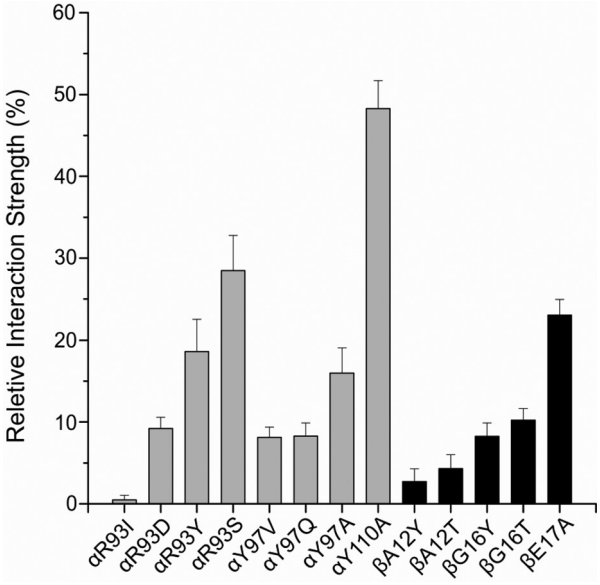

Fig. 5. Interactions between the site-directed mutated α and β subunits in interface B. Interactions between the particular amino acid residues in the E and F helices in the mutated α subunits and native β subunits (gray), and between the native α subunits and the particular amino acid residues in the X and Y helices in the mutated β subunits (black) were both studied using the *E. coli* two-hybrid system.

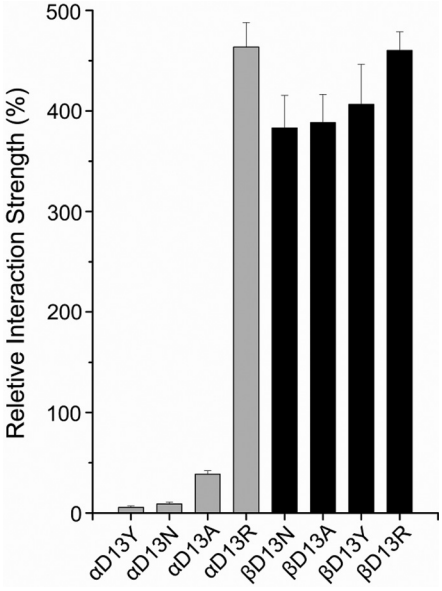

Fig. 6. Interactions between the α and β subunits containing amino acid substitutions in aspartate 13 (D13). Interactions between α subunits containing site-directed substitutions at D13 and native β subunits (gray), and between native α subunits and β subunits containing site-directed substitutions at D13 (black) were both studied using the *E. coli* two-hybrid system.

Table 3
Interaction of parts of the site-directed mutated α and mutated β subunits in interface B.

	cpcB	β D13A	β D13N	β D13R	β D13Y
cpcA	+++	++++	++++	++++	++++
α R93S	++	+	+	+	++
α R93I	-	+	-	+	-
α R93D	+	+	+	+	+
α R93Y	+	+	+	+	+
α Y97A	+	+	+	+	+
α Y97V	+	+	+	+	+
α Y97Q	+	+	+	+	+

Note: The relative strength of the interactions is indicated by the number of plus signs, which depends on the relative interaction strength. +, <5%; ++, 5-20%; +++, 20-150%; ++++, >150%.

spectively, play lesser roles in the interaction. When amino acid residue R93 in the α subunit was changed to isoleucine, no interaction was detected, suggesting that residue R93 is likely to interact with the neighboring residues through its polar groups. In addition, when residue R93 was changed to aspartate, the interaction was still weak, with an interaction strength of ~9%, which further suggested that the positive charge of arginine is vital to the interaction in this microenvironment. However, when residue R93 was changed to serine, the interaction strength did not change remarkably, which might be attributed to the polar groups in serine compensating for the electrovalent bond.

Amino acid residue Y97 in the α subunit has similar function to Y92 and Y95 in the β subunit. Tyrosine, which contains a large polar side chain group, helps to maintain the interaction by forming hydrogen bonds and through hydrophobic force. Therefore, the residues Y97 in the α subunit and Y92 and Y95 in the β subunit are crucial points in the interaction. Both A12 and G16 in the β subunit are small residues in interface B, and their small side chains provide enough space for the surrounding amino acid residues, which thus ensures the stability of the 3-D structure of the phycocyanin monomer. Consequently, when they were substituted by tyrosine (Fig. 5), the interaction strengths were greatly weakened.

Surprisingly, when the D13 residue in the β subunit was changed to arginine, the interaction strength between the α and β subunits increased by 4–5 folds (Fig. 6). Similar results were obtained when residue D13 in the α subunit was changed to arginine. In contrast, when all of these mutant proteins interacted with proteins containing site-directed mutations in the E and F helices of the other subunit, the interaction strengths were low, and similar results were found in the amino acid substitution mutants in the helices E and F with the active subunit (Tables 2 and 3). When residue D13 in the α subunit was changed by site-directed mutation to tyrosine, asparagine, or alanine, these amino acid substitutions led to much weaker interaction strengths compared to the control.

Based on the protein sequence alignments and structure analyses, the D13 residue is highly conserved and interacts with several other residues in the crystal structure, and thus D13 is considered to be a key amino acid in phycocyanin. Based on crystal structure analysis, D13 forms a hydrogen bond or electrovalent bond with the particular residues in the E and F helices of the other subunit. Arginine is often involved in forming hydrogen bonds and electrovalent bonds. When D13 in the α subunit was changed to arginine, the interaction strength was enhanced. We assume that arginine interacts with more amino acids in the β subunit, which resulted in the enhanced interaction strength. However, when

the D13 residue in the β subunit was substituted with alanine (a small amino acid), tyrosine (an aromatic amino acid), asparagine (a neutral amino acid), or arginine (a basic amino acid), the interactions between the two subunits were also strong.

The *E. coli* bacterial two-hybrid system is effective for studying the protein-protein interactions in phycobiliproteins, and will be a useful tool for further experiments. The results indicate that the amino acid residues in the β subunit play more important roles in the interaction, and the residues in the E and F helices are more crucial than the residues in the X and Y helices. Amino acids R93 and Y97 in the α subunit and R91, Y92, Y95, and R108 in the β subunit are crucial for the inter-subunit interactions within the ($\alpha\beta$) monomer. In contrast, although amino acids S10, A12, G16, R17, and L19 from the α subunit were predicted to be close to the residues above where hydrogen bonds or electrovalent bonds might exist, no obvious roles for these residues in the interaction were observed.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.engmic.2022.100019.

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