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Purification and Characterization of a Hemolysin-Like Protein, Sll1951, a Nontoxic Member of the RTX Protein Family from the Cyanobacterium *Synechocystis* sp. Strain PCC 6803

Tetsushi Sakiyama, Hironori Ueno,† Hideya Homma, Osamu Numata, and Tomohiko Kuwabara*

Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba 305-8572, Japan

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The hemolysin-like protein (HLP) Sll1951, characterized by the GGXGXDXUX nonapeptide motif implicated in Ca^{2+} binding, was purified from the glucose-tolerant strain (GT) of *Synechocystis* sp. strain PCC 6803. HLP was eluted at 560 kDa after gel filtration chromatography. Atomic absorption spectroscopy indicated that the protein bound calcium. The bound Ca^{2+} was not chelated with EGTA; however, it was released after being heated at 100°C for 1 min, and it rebound to the Ca^{2+}-depleted protein at room temperature. The apparent HLP molecular mass increased to 1,000 kDa and reverted to 560 kDa during the release and rebinding of Ca^{2+}, respectively. The monomers of the respective forms appeared at 90 and 200 kDa after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. HLP showed no apparent hemolytic activity against sheep erythrocytes; however, a slight hemolytic activity was detected during the conformational change caused by the rebinding of Ca^{2+}. Immunoelectron microscopy using polyclonal antibodies against the 200-kDa monomer revealed that HLP is located in the cell surface layer. The localization and Ca^{2+}-induced reversible conformational change suggest that HLP is a member of the repeat in toxin (RTX) protein family despite its latent and low toxicity. In some other cyanobacteria, RTX proteins are reported to be necessary for cell motility. However, the GT was immotile. Moreover, the motile wild-type strain did not express any HLP, suggesting that HLP is one of the factors involved in the elimination of motility in the GT. We concluded that the involvement of RTX protein in cyanobacterial cell motility is not a general feature.

*R*epeat in *to*xin (RTX) proteins, which are characterized by the GGXGXDXUX nonapeptide motif (where X is any amino acid and U is an amino acid with a large hydrophobic side chain), are exotoxins produced by gram-negative bacteria, and hemolysin, leukotoxin, and cytotoxin are included in the RTX protein family (20, 36). These proteins are considered to form pores in cytoplasmic membranes of erythrocytes, leukocytes, and other cells, leading to the modification of cellular functions and/or lysis of host cells. One motif sequence serves as two half sites for Ca^{2+} binding, and an array of the sequences forms the parallel β-roll structure, as revealed by the crystal structure of alkaline protease of *Pseudomonas aeruginosa* (1). Biochemical and molecular biological studies have best characterized the following RTX proteins: HlyA of *Escherichia coli* (4, 6, 31) and CyaA of *Bordetella pertussis* (10, 11, 29). CyaA is a natural fusion protein of adenylate cyclase and hemolysin and exhibits toxicity that modifies the host cellular functions by increasing the intracellular concentration of cyclic AMP (20). The binding of Ca^{2+} ions is essential for these proteins to acquire the toxic conformation (4, 29). HlyA (K564 and K690) and CyaA (K983) are palmitoylated at the lysine residues by the acyltransferases HlyC (31) and CyaC (11), respectively. This palmitoylation is essential for the toxicity of the proteins. The RTX proteins are secreted with a noncleavable C-terminal signal peptide (6, 20) by the type I secretion system that consists of three members—HlyB, HlyD, and TolC for HlyA (35, 37) and CyaB, CyaD, and CyaE for CyaA (10). *hlyA* forms an operon with *hlyC*, *hlyB*, and *hlyD*, while *cyaA* forms an operon with *cyaB*, *cyaD*, and *cyaE* (10, 37).

RTX proteins of *Cyanobacteria*, including SwmA of *Synechococcus* sp. strain WH8102 (5) and oscillin of *Phormidium uncinatum* (13), have been shown to be necessary for cell motility. Thus, there may be a functional diversity of RTX proteins in pathogenic bacteria and cyanobacteria. However, the mechanisms underlying the involvement of the cyanobacterial RTX proteins in motility have not been clarified.

Synechocystis sp. strain PCC 6803 (referred to as PCC 6803) is a unicellular freshwater cyanobacterium; the wild-type strain (WT) of PCC 6803 has type IV pili that mediate motility (2). A glucose-tolerant strain (GT) which is capable of photoheterotrophic growth was generated by spontaneous mutation of the WT (38) and has been used to study the photosynthetic genes. In contrast to the WT, the GT is immotile on 1.2% agar plates due to an unknown mechanism that developed during the spontaneous mutation (33). The genomic DNA sequences of a single representative clone of the GT have been determined (14, 15). The product of sll1951 (referred to as *h*emolysin-*l*ike *p*rotein [HLP]) possesses the GGXGXDXUX nonapeptide motif sequences and is secreted in the absence of a cleavable N-terminal signal peptide (24). Thus, HLP is considered to be a cyanobacterial RTX protein; however, the other features of RTX proteins, such as Ca^{2+} binding, extracellular localization, and toxicity, have not been studied thus far. HLP is a natural fusion protein composed of the following three domains: the

* Corresponding author. Mailing address: Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba 305-8572, Japan. Phone: 81 29 853 6667. Fax: 81 29 853 6614. E-mail: kuwabara@biol.tsukuba.ac.jp.

† Present address: Gene Function Research Center, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8562, Japan.

N-terminal domain (M1-S758), which does not possess any nonapeptide motif sequences; the central domain (K759-F1101), which possesses 8 nonapeptide motif sequences, each preceded by a His residue; and the C-terminal domain (V1102-A1741), which possesses 21 nonapeptide motif sequences (24). Interestingly, all His residues in the protein are in the central region. Thus, the central domain is distinguishable from the C-terminal domain by the periodically occurring His residues. In contrast to *hlyA* or *cyaA*, sll1951 does not form an operon with the genes encoding the acyltransferase for palmitoylation or components of a type I secretion system (15). Furthermore, HLP appears to have no palmitoylation site that is represented by the lysine residues in G*K*Y/R sequences (11, 31); however, a gene encoding an acyltransferase (sll0720) is distantly present in the genome. The function of HLP remains unknown.

In the present study, we purified HLP from the GT, demonstrated that it is a member of the RTX protein family, and suggested that it is related to the absence of motility in the GT.

MATERIALS AND METHODS

Cultivation of PCC 6803. The GT and WT PCC 6803 were kindly provided by N. Murata (National Institute for Basic Biology, Okazaki, Japan) and M. Ohmori (Saitama University, Saitama, Japan), respectively. The working stock was prepared by culturing the strains in 50 ml of BG-11 medium (30) supplemented with 20 mM HEPES-KOH (pH 8.0) (referred to as BG-11) in a 300-ml Erlenmeyer flask at 30°C under continuous illumination at 60 μmol photons $m^{-2} \cdot s^{-1}$, with rotary shaking at 70 rpm to an optical density at 750 nm (OD_{750}) of 0.15 to 0.3. Twenty milliliters of the working stock was inoculated into 200 ml of BG-11 in two 100-ml test tubes, and they were precultured for 60 h to an OD_{750} of 0.7 to 1.0 with air bubbling under light and temperature conditions identical to those mentioned above, but without shaking. The 200 ml of preculture was inoculated into 1,800 ml of BG-11 supplemented with 3 μM $CuSO_4$ in two 1,000-ml turtle-shaped self-standing culture bottles. The cells were grown for 48 h to an OD_{750} of 0.3 to 0.45 under conditions similar to those used for the preculture.

Polyclonal antibodies against eHLP. A 2,000-ml culture of the GT was centrifuged at 8,000 × *g* for 10 min. The supernatant was filtered through a GF/F filter (average pore size, 0.7 μm; Whatman, Maidstone, United Kingdom) and then through a Nuclepore filter (pore size, 0.2 μm; Whatman). The cell-free supernatant was collected as the filtrate. It was concentrated 50-fold by using a dialysis membrane and polyethylene glycol 20000 (Wako, Osaka, Japan) at 4°C overnight. Subsequently, the concentrated supernatant was precipitated at 10% saturation of ammonium sulfate, incubated at 4°C for 1 h, and centrifuged at 10,000 × *g* and 4°C for 10 min. The precipitates were dissolved in 1.5 ml of 2% sodium dodecyl sulfate (SDS) and centrifuged at 10,000 × *g* for 10 min. The supernatant was subjected to the first preparative SDS-polyacrylamide gel electrophoresis (PAGE) with a 2-mm-thick gel plate. After electrophoresis, the gel plate was stained, destained, and washed twice with deionized water for 10 min each time. The band (height, 3 mm) of compact HLP (cHLP) at 90 kDa was excised and subjected to protein extraction by using Maxyield-NP (ATTO, Tokyo, Japan) as described for the manufacturer's protocol, with the exception that the reverse electrophoresis was performed at 100 V for 1 min at the end of the extraction step. The extract was concentrated 20-fold by using a Centricon YM-10 apparatus (Millipore, Bedford, Mass.), supplemented with SDS buffer, heated at 100°C for 1 min, and subjected to the second preparative SDS-PAGE. The gel plate was stained, destained, and washed as described above. The band (height, 2 mm) of extended HLP (eHLP) at 200 kDa was excised, briefly washed with deionized water, and stored at −80°C until use. Polyclonal antibodies were prepared in two rabbits by Shibayagi (Shibukawa, Japan) using a gel slurry with Freund's adjuvant as the antigen according to the manufacturer's standard protocol.

Hemolytic activity assay. A concentrate of cell-free supernatant prepared as described above was dialyzed against 10 mM KH_2PO_4-NaOH (pH 6.8) and 0.9% NaCl (buffered saline) at 4°C overnight. The concentration of HLP was about 0.02 mg · ml^{-1}. A portion of the dialyzate was heated at 100°C for 1 min to liberate Ca^{2+} ions from HLP. Fresh blood from sheep was centrifuged at 1,100 × *g* for 5 min. The erythrocytes obtained as precipitates were washed twice with the buffered saline by suspension and centrifugation and finally suspended in the buffered saline at 5% (vol/vol). Hemolytic activity was assayed by mixing the heated or nonheated HLP solution, 0.2 ml of the erythrocyte suspension, and the buffered saline to a final volume of 1 ml. In another experiment, the heated HLP was first mixed with the erythrocytes, which were then supplemented with various concentrations of $CaCl_2$. For the positive and negative controls, the erythrocyte suspension was mixed with 0.8 ml of water and the buffered saline, respectively. Following overnight incubation at 37°C, the reaction mixtures were centrifuged at 1,100 × *g* for 5 min. The hemolytic activity was measured as the A_{540} of the supernatants, and the values for the positive and negative controls were considered to be 100% and 0%, respectively.

Purification of HLP. Purification of HLP was performed at room temperature, but Superose 6 chromatography was performed at 4°C using an FPLC system (Pharmacia, Uppsala, Sweden). A cell-free supernatant was supplemented with 0.1% (wt/vol) Tween 20 and loaded on a DEAE-cellulose column (inside diameter, 1.6 by 8.5 cm) that had been equilibrated with 20 mM NaH_2PO_4-NaOH (pH 7.0) and 0.1% Tween 20 (medium A). After the column was washed with 40 ml of medium A containing 350 mM NaCl, HLP was eluted with 40 ml of the medium A containing 500 mM NaCl at a flow rate of 2 ml · min^{-1}. Fractions of 2 ml were collected. The elution of proteins was detected by measuring the A_{280} of the fractions using a spectrophotometer (model UV-1600PC; Shimadzu, Kyoto, Japan). The peak fractions were concentrated 10-fold by centrifugation at 4,000 × *g* at 4°C using a Centricon YM-10 apparatus (Millipore). The concentrate was filtered by using a cellulose acetate filter (pore size, 0.45 μm; Advantec, Tokyo, Japan) and loaded on a Superose 6 column (inside diameter, 1 by 30 cm) that had been equilibrated with 20 mM 2-morpholinoethanesulfonic acid (MES)-NaOH (pH 6.0), 50 mM NaCl, and 0.1% Tween 20 (medium B). HLP was eluted by using the same medium at a flow rate of 0.4 ml · min^{-1}. Fractions of 0.5 ml were collected.

SDS-PAGE. SDS-PAGE was performed essentially as described previously (16) using 1-mm-thick gel plates containing 6 M urea and 2× Laemmli buffer (18) at a constant power of 5 W at 7°C to minimize the effects of Joule heat during electrophoresis. Urea was necessary to have clear banding of the HLP polypeptide. The concentrations of polyacrylamide in the stacking and separation gels were 5.8% and 10%, respectively. The samples used were either not heated or heated at 100°C for 1 min in the presence or absence of SDS prior to SDS-PAGE. A sample volume of 50 μl was mixed with 30 μl of SDS buffer that contained 4.5% SDS, 13 mM dithiothreitol, 0.06% bromophenol blue, 30 mM Tris-HCl (pH 8.0), and 50% (wt/wt) glycerol. The gel plates were stained with Coomassie brilliant blue R-250 (CBB) and destained by using 20% methanol and 10% acetic acid as described previously (17).

Quantification of HLP. A concentration series of bovine serum albumin (BSA; 6 to 30 μg · ml^{-1}) was prepared with medium B as the standard. Samples and the standard were subjected to SDS-PAGE on a gel plate. The gel plate was stained, destained, washed with deionized water, and dried between two cellophane sheets. An image of the gel was taken by using a scanner that was connected to a personal computer. The densitogram of each lane was obtained using NIH Image version 1.63 (http://rsb.info.nih.gov/nih-image/download.html). The amounts of HLP in the samples were calculated by comparing densitogram peak areas from the samples with those from the standards.

Quantification of metals. Calcium and copper were quantified by atomic absorption at 423 and 325 nm, respectively, by using an atomic absorption spectrophotometer (model AA-6800G; Shimadzu). The tips of micropipettes and sample tubes were washed with 1 N HCl, rinsed with Milli Q water (Millipore), and dried in a clean oven before use. The standard curves of the respective standard solutions (Wako) diluted with 0.1 N HNO_3 were depicted after 20 μl of the solution was introduced into the instrument. Calcium was quantified according to the following scheme: it was dried by successive gradual increases in temperature from room temperature to 150°C in 20 s and then to 250°C in 10 s, charred by a gradual increase in the temperature from 250°C to 800°C in 10 s, maintained at 800°C for 13 s, and atomized at 2,200°C for 2 s, which was followed by cleaning at 2,500°C for 2 s. Copper was quantified using the identical procedure, but the atomizing temperature was 2,300°C. Each sample was measured three times, and the obtained values were averaged. The atomic absorption was linearly correlated with the concentration of the metals in the range of 0 to 60 ppb for calcium and 0 to 15 ppb for copper. For quantifying calcium, the HLP fractions obtained after Superose 6 chromatography were diluted 10-fold with the elution buffer. However, they were not diluted for quantifying copper. To eliminate the error introduced by the solvent absorbance values, the atomic absorption of the solvent was subtracted from those of the samples.

Quantification of chlorophyll. Pigments were extracted three times with 80% (vol/vol) acetone. The extracts were mixed, and the concentration of chlorophyll *a* was measured according to the method described by Mackinney (21).

Immunoelectron microscopy. The cells harvested by centrifugation were washed with 103 mM sodium cacodylate-NaOH (pH 7.2), 0.7 mM $CaCl_2$, and 30 mM sucrose (cacodylate buffer) by suspension and centrifugation. The precipitates were suspended in 0.5% paraformaldehyde in the cacodylate buffer for

fixation at 4°C for 1 h. The fixed cells were centrifuged at 8,000 × *g* for 10 min at 4°C, rinsed with the cacodylate buffer, and suspended in 6 ml of 0.1% OsO_4 in the cacodylate buffer at 4°C for 1 h. The suspension was centrifuged, and the pellet was suspended in a small volume of cacodylate buffer. The suspension was gently mixed with 1% agarose (Wako) in the cacodylate buffer. The solidified agarose was cut into small pieces, which were dehydrated with a graded ethanol series (50%, 70%, 80%, 90%, 95%, and 99.5%), with each dehydration step lasting for 10 min at 4°C. Agarose blocks were embedded in LR-White resin (London Resin, Fort Washington, United Kingdom), cut into thin sections, blocked with BSA as described previously (34), and immunolabeled for 1 h with anti-eHLP antiserum that had been diluted to 1:2,000 with 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% (wt/vol) Tween 20 (Tris buffer). After being washed six times with the Tris buffer, the sections were secondarily labeled for 1 h with colloidal gold (diameter, 10 nm)-conjugated goat anti-rabbit immunoglobulin G antiserum (British Biocell, Cardiff, United Kingdom) that had been diluted to 1:50 with the Tris buffer. After being washed six times using Tris buffer, the sections were fixed with 0.5% glutaraldehyde in 4 mM Na_2HPO_4-NaOH (pH 7.4), 1.8 mM KH_2PO_4, 140 mM NaCl, and 2.7 mM KCl for 10 min in order to stabilize the antigen-antibody interaction. Subsequently, the sections were washed six times with water, stained with 2% uranyl acetate for 30 min, again washed six times with water, and stained with 80 mM lead citrate (28) for 7 min. The specimens were washed three times with water, air dried, and examined with a transmission electron microscope (JEM1010; JEOL, Tokyo) at 70 kV.

RESULTS

Purification of HLP. HLP was purified from the cell-free supernatants of the GT by successive chromatography with DEAE-cellulose and Superose 6. All the media used for the purification contained 0.1% (wt/vol) Tween 20 because the protein would irreversibly bind to the columns in the absence of a detergent. In Superose 6 chromatography, HLP was eluted at 560 kDa (Fig. 1). Thus, if the molecular mass of the protomer is 178 kDa, as deduced from the DNA sequence (15), then HLP can be considered to be eluted as if it had the mass of a trimer (see the legend to Fig. 6). The HLP polypeptide was able to be stained with a GelCode glycoprotein staining kit (Pierce), indicating that HLP is a glycoprotein similar to other cyanobacterial RTX proteins (5, 13). The amount of HLP purified from 600 OD_{750} units of cells (OD_{750} by volume [ml]) was 0.1 mg.

Heat-induced reversible mobility change of HLP. Preliminary studies using cell-free supernatants indicated that the mobility of HLP reversibly changed by heat treatment in the absence of SDS prior to SDS-PAGE. To study the effects of the culture medium on the mobility change, purified HLP was supplemented with BG-11. When not heated before SDS-PAGE, HLP was electrophoresed at the position of 90 kDa; however, the apparent molecular mass increased to 200 kDa after being heated at 100°C for 1 min in the presence of SDS (Fig. 2). We designated the 90-kDa form as cHLP and the 200-kDa form as eHLP. The lowest temperature at which eHLP was produced was 60°C. When HLP was heated at 100°C for 1 min in the absence of SDS, cooled to room temperature, and subjected to SDS-PAGE, cHLP appeared (Fig. 2). Thus, in the absence of SDS, the conformation of HLP appeared to change reversibly in the presence of BG-11 due to the temperature changes. Interestingly, the similar heating and cooling treatments performed in the absence of BG-11 failed to produce cHLP (Fig. 2). This result suggests that some component in BG-11 is involved in the reversible conformational change of HLP.

Binding of calcium to HLP. The presence of the GGXGX DXUX nonapeptide motif sequences in HLP suggests that it is a Ca^{2+}-binding protein (15), whereas our previous study suggested that it might be a Cu^{2+} chelator (24). Therefore, we quantified these metals in the Superose 6 fractions by atomic absorption spectroscopy (Fig. 1A). We also quantified eHLP using SDS-PAGE by staining with CBB and using BSA as the standard to estimate metal-to-protein ratios. The estimation indicated that an HLP protomer binds about 100 atoms of calcium but does not bind copper. The calcium-to-protein ratio of 100 may be an overestimate due to the lower degree of stainability of HLP than that of BSA; this was suggested by the fact that when the gels were silver stained, the band of HLP became clear against the background. When up to two mismatches in a motif sequence are allowed, the number of the nonapeptide motif sequences in HLP is 29 (15), which supports the above consideration.

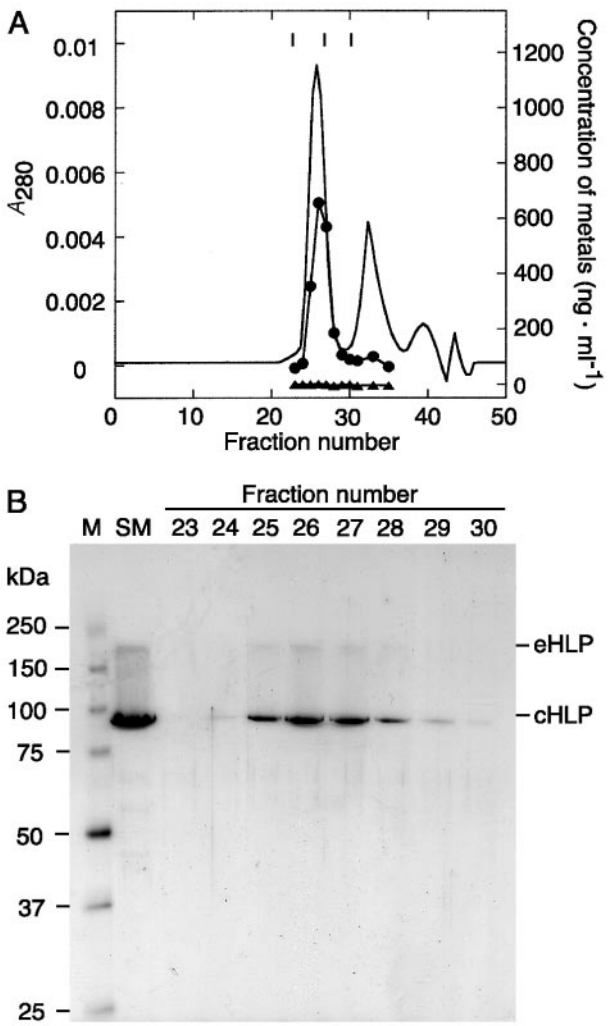

FIG. 1. Gel filtration chromatography of HLP on Superose 6. (A) Elution profiles in terms of the A_{280} (solid line) and concentrations of calcium (●) and copper (▲). The vertical lines in the upper region indicate the positions at which the molecular mass markers eluted, namely, thyroglobulin (669 kDa), ferritin (440 kDa), and catalase (232 kDa), from the left. (B) Polypeptide profile of fractions from the Superose 6 column. The fractions (50 μl each) were subjected to SDS-PAGE without heating. Note that the purified HLP preparation contained trace amounts of eHLP. M, marker; SM, starting material.

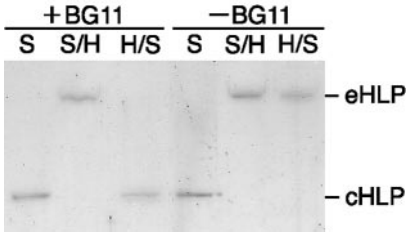

FIG. 2. Heat-induced mobility change of HLP. Purified HLP was mixed with BG-11 (15 μl to 35 μl) or not. The samples were supplemented with SDS buffer (lanes S); supplemented with SDS buffer and heated at 100°C for 1 min (lanes S/H); or heated at 100°C for 1 min, cooled to room temperature and supplemented with SDS buffer (lanes H/S) prior to SDS-PAGE.

Effects of Ca^{2+} ions on the reversible conformational change of HLP. In order to identify the component of BG-11 that is essential for the conformational change of HLP, we performed experiments similar to those described in Fig. 2 by eliminating a component(s) from BG-11. The reversible mobility change was absent only when $CaCl_2$ was removed (Fig. 3A). The result suggests that Ca^{2+} ions were released from the nonapeptide motif sequences on heating but that they rebound to the protein during subsequent cooling in the Ca^{2+}-supplemented solutions. The treatment of HLP with 4 mM EGTA without heating did not change the mobility (Fig. 3B), suggesting that the binding of Ca^{2+} ions to HLP is stronger than that to EGTA or that the Ca^{2+}-binding sites are located in the interior of the folded protein, to which EGTA has no access. Next, we studied the effects of pH on the mobility change; purified HLP was dialyzed overnight against Milli Q water (Millipore), and equal volumes of the buffers (from pH 3 to pH 10) of Teorell and Stenhagen (32) were added to the dialyzate. Upon SDS-PAGE without heating, eHLP appeared in the samples at and below pH 4 but not in those at and above pH 5, suggesting that even this apparently strong binding is sensitive to acids. Then, we studied the effects of $CaCl_2$ concentration on the reversible mobility change. HLP was heated in medium B containing

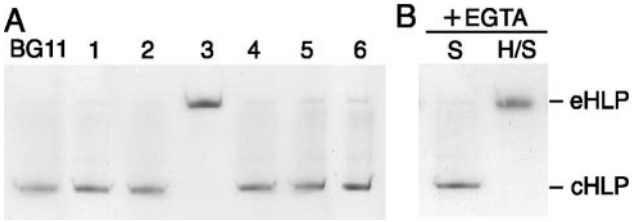

FIG. 3. Effects of BG-11 components and EGTA on the reversible mobility change of HLP. (A) Purified HLP (10 μl) was mixed with BG-11 or the related media (40 μl). The related media are BG-11 devoid of the following: lane 1, citric acid, ferric ammonium citrate, and Na_2EDTA; lane 2, $NaNO_3$, K_2HPO_4, and $MgSO_4$; lane 3, $CaCl_2$; lane 4, Na_2CO_3; lane 5, H_3BO_3, $MnCl_2$, $ZnSO_4$, $CuSO_4$, Na_2MoO_4, and $Co(NO_3)_2$; and lane 6, HEPES. The mixtures were heated, cooled, and supplemented with SDS buffer prior to SDS-PAGE. (B) The purified HLP (10 μl) was mixed with BG-11 containing 5 mM EGTA (40 μl). The mixture was supplemented with SDS buffer (lane S) or heated, cooled, and supplemented with SDS buffer (lane H/S). Concentrations of Ca^{2+} ions and total divalent cations in BG-11 were approximately 0.26 and 0.58 mM, respectively.

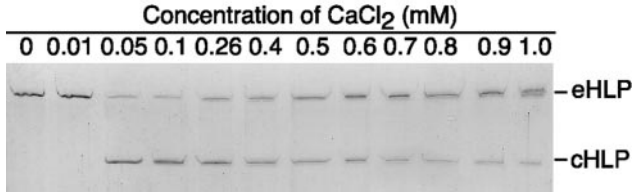

FIG. 4. Effects of the concentration of $CaCl_2$ on the reversible mobility change of HLP. Purified HLP (15 μl) was mixed with $CaCl_2$ solutions (35 μl) to yield designated concentrations of $CaCl_2$. The mixtures were heated, cooled, and supplemented with SDS buffer prior to SDS-PAGE.

various concentrations of $CaCl_2$, cooled, and subjected to SDS-PAGE. cHLP was generated when not less than 0.05 mM $CaCl_2$ was present (Fig. 4). In the $CaCl_2$ concentration range from 0.05 to 0.26 mM, the amount of cHLP produced was the maximum in the series; however, the amount of eHLP increased as the $CaCl_2$ concentration increased more than 0.26 mM. This result suggests that the nonnatural Ca^{2+}-binding sites may be present in the heat-treated HLP; the binding of Ca^{2+} ions to these sites prevents eHLP from reversing to cHLP. Electrophoresis of nonheated HLP together with up to 1 mM $CaCl_2$ never yielded eHLP, consistent with the above consideration.

Direct demonstration of the reversible conformational change of HLP by the release and rebinding of Ca^{2+} ions. To directly demonstrate the heat-induced release and subsequent rebinding of Ca^{2+} ions, we quantified the calcium bound to HLP by using Superose 6 chromatography after purifying the protein that was treated under different conditions. An HLP preparation from DEAE-cellulose was divided into three parts. The samples were unheated, heated, or heated and supplemented with 0.26 mM $CaCl_2$. They were treated with 3 mM EGTA in order to remove the nonspecifically bound Ca^{2+} ions and subjected to gel filtration chromatography. The chromatogram of the unheated HLP was essentially the same as that shown in Fig. 1A; HLP was eluted at 560 kDa, and the calcium-to-protein ratio was 100 (Fig. 5A), suggesting that there is no EGTA-removable Ca^{2+} ions bound to the protein. After the heat treatment, HLP was eluted mainly at 1,000 kDa, but some residual protein also eluted at the void volume (Fig. 5B). The calcium-to-protein ratio of the main peak was 20. After being heated and supplemented with $CaCl_2$, HLP was eluted mainly at 560 kDa; a small quantity of the remaining HLP was eluted at the void volume as well as at 1,000 kDa (Fig. 5C). The calcium-to-protein ratio of the main peak was 90. These results indicate that HLP reversibly changes its conformation by the heat-induced release and subsequent rebinding of Ca^{2+} ions. Interestingly, throughout these experiments, HLP was never eluted in the low-molecular-mass region, where its monomer is expected to be eluted, suggesting that HLP retained the oligomeric structure in the course of the conformational change. Figure 6 shows a schematic representation of the conformational change.

Hemolytic activity of HLP. Since Tween 20—the detergent used for the purification of HLP—caused hemolysis, we were unable to study the hemolytic activity of the purified HLP. Thus, a cell-free supernatant was concentrated and dialyzed

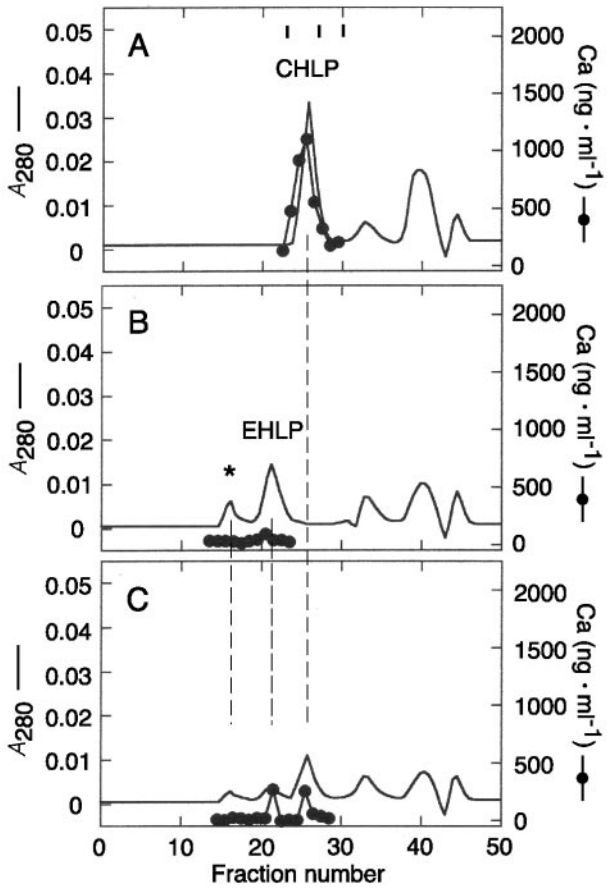

FIG. 5. Superose 6 chromatography of unheated, heated, or heated and Ca^{2+}-supplemented HLP. An HLP preparation from DEAE-cellulose was divided into three. They were unheated (A), heated (B), or heated and supplemented with 0.26 mM $CaCl_2$ (C). The samples were treated with 3 mM EGTA to remove the nonspecifically bound Ca^{2+} ions and subjected to Superose 6 chromatography. CHLP, the 560-kDa oligomer of cHLP; EHLP, the 1,000-kDa oligomer of eHLP. The asterisk indicates aggregates of HLP that eluted at the void volume.

against the buffered saline to measure the activity. The nonheated as well as heated HLP showed no hemolytic activity towards sheep erythrocytes at a protein concentration of 27 nM. Thus, HLP cannot be considered a hemolysin; *E. coli* HlyA causes complete hemolysis at a protein concentration of 10 nM (7). A portion of the Ca^{2+}-binding sites of *E. coli* HlyA as well as *B. pertussis* CyaA is EGTA sensitive, and these proteins require the binding of Ca^{2+} ions to these sites for exhibiting toxicity (4, 25, 29). We assumed that the Ca^{2+}-induced conformational change could be essential for HLP activity. Thus, we first mixed the erythrocytes and heat-treated HLP and then added various concentrations of $CaCl_2$ up to 1 mM. Hemolytic activity appeared to increase with the $CaCl_2$ concentration; however, the hemolysis that occurred at 1 mM $CaCl_2$ was only 2.5% of that occurring in the positive control, in which erythrocytes were treated with water. Thus, HLP may possess a slight, latent hemolytic activity.

$CuSO_4$ at a concentration of 3 μM never enhances the expression of HLP. Although the supplementation of $CuSO_4$ was necessary to obtain HLP in the cell-free supernatants, the effect of $CuSO_4$ on the expression of HLP remains unknown. Considering the heat-induced large mobility change of HLP, we purified eHLP from cHLP by SDS-PAGE, prepared polyclonal antibodies against eHLP, and examined the effects of 3 μM $CuSO_4$ on the expression of HLP. The cells of the GT and cell-free supernatants from cultures with or without the supplementation of 3 μM $CuSO_4$ were analyzed by Western blotting. HLP was abundant even in the cells cultured in the absence of 3 μM $CuSO_4$, and its expression was never enhanced by the supplementation of 3 μM $CuSO_4$ (Fig. 7A). Instead, 3 μM $CuSO_4$ served to slightly solubilize HLP from the cells. The cell-bound HLP did not enter the gel unless the cells were heated in the presence of SDS (Fig. 7B). We concluded that the GT constitutively expresses HLP, and the protein is slightly solubilized during culture in BG-11 supplemented with 3 μM $CuSO_4$.

Intracellular localization of HLP. RTX proteins of other cyanobacteria, SwmA of *Synechococcus* sp. strain WH8102 and oscillin of *P. uncinatum*, are located in the surface layers and involved in cell motility (13, 23). With regard to PCC 6803, the

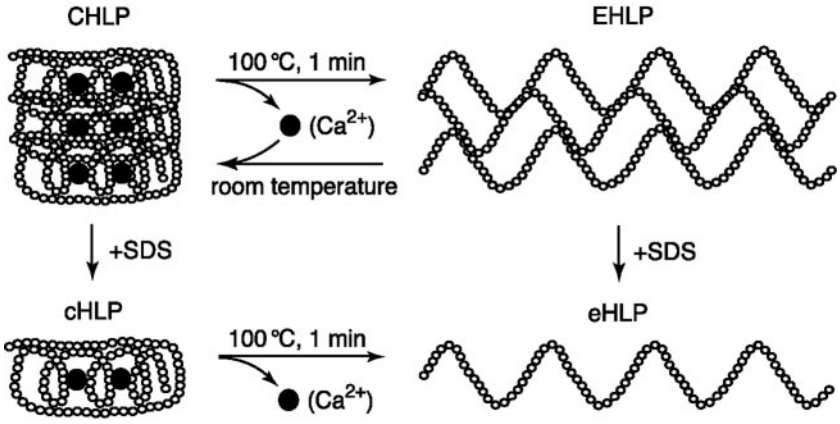

FIG. 6. Model showing the Ca^{2+}-dependent change in the tertiary structure of HLP. HLP was tentatively assumed to be a trimer in the absence of SDS based on the molecular mass of the oligomer (560 kDa) and that of the protomer deduced from the DNA sequence (178 kDa) (15). Note that it could be a hexamer if the molecular mass estimated by SDS-PAGE (90 kDa) is adopted for the protomer.

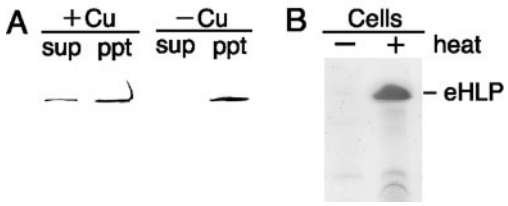

FIG. 7. Effects of copper on the expression and of heating on the solubilization of cell-bound HLP. (A) Cells of the GT were cultured in BG-11 or BG-11 supplemented with 3 μM $CuSO_4$. The cultures corresponding to 0.05 μg chlorophyll were centrifuged to separate the cells (ppt) and supernatants (sup). They were heated in the presence of SDS buffer, electrophoresed, and subjected to Western blotting with polyclonal anti-eHLP antibodies. (B) Cells of the GT cultured in BG-11, equivalent to 5 μg chlorophylls, were subjected to SDS-PAGE without or with heating in the presence of SDS buffer. The gel was stained with CBB.

HLP-possessing GT is immotile (33). In order to study the intracellular localization of HLP, immunoelectron microscopy was performed by using anti-eHLP antibodies (Fig. 8A and B). Abundant signals were observed in the surface layer. Although it is difficult to observe the double-membrane structure of the cytoplasmic membrane of cyanobacteria using transmission electron microscopy (12, 23), the surface layer is discernible as the outermost irregular layer. The following characteristics were consistent with the identification of HLP as a member of the RTX protein family, although the hemolytic activity was latent and very weak: (i) the localization of and the Ca^{2+}-induced reversible conformational change, (ii) the absence of the cleavable N-terminal signal (24), and (iii) the presence of the GGXGXDXUX motif sequences (15).

In contrast to the GT, the WT is motile (33). However, the WT showed no signals after immunoelectron microscopy (Fig. 8C and D). Furthermore, the WT did not show a GT-like surface layer (Fig. 8D); this result suggests that HLP is indispensable for the formation of the surface layer. The absence of detectable amounts of HLP in the WT was confirmed by SDS-PAGE (Fig. 8E). Considering the absence of motility in the GT, HLP appears to be related to the elimination of motility. This result is contradictory to the cases of *Synechococcus* sp. strain WH8102 (5) and *P. uncinatum* (13), in which RTX proteins are necessary for cell motility. We confirmed the absence and presence of motility in the GT and WT, respectively, by using 0.8% agar plates (39) and concluded that the involvement of cyanobacterial RTX proteins in cyanobacterial motility is not a general feature.

DISCUSSION

In the present study, we showed that HLP underwent Ca^{2+}-dependent reversible conformational change but showed no toxicity against sheep erythrocytes. The absence of toxicity may be due to the absence of EGTA-sensitive, low-affinity Ca^{2+}-binding sites. RTX toxins possess the low-affinity sites and

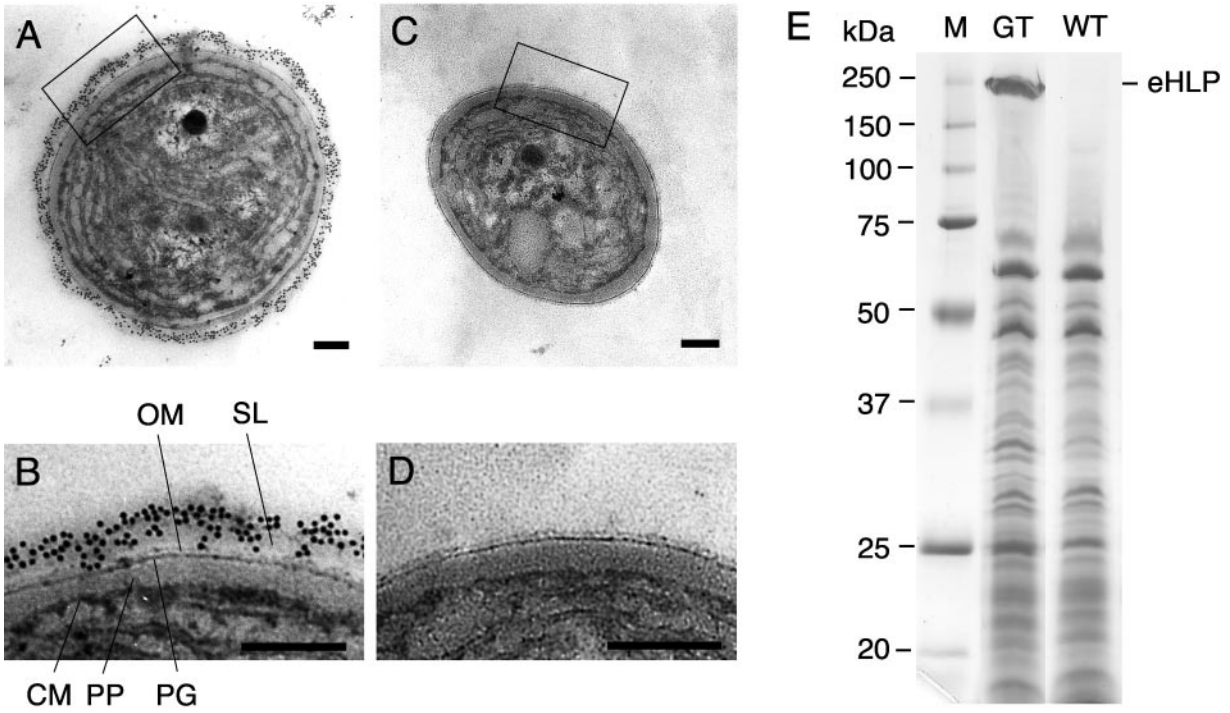

FIG. 8. Intracellular localization of HLP in the GT and the WT. The GT (A and B) and the WT (C and D) were cultured in BG-11 to the late logarithmic phases (OD_{750} of the GT, 1.1; OD_{750} of the WT, 1.2). Immunoelectron microscopy of the two strains was conducted by using a 2,000-fold-diluted polyclonal anti-eHLP antiserum and gold particle (diameter, 10 nm)-conjugated secondary antibodies. Note that in contrast to the antiserum, the normal serum generated no signals of HLP on both samples. The regions marked by squares in panels A and C are enlarged in panels B and D. CM, cytoplasmic membrane; PP, periplasm; PG, peptidoglycan; OM, outer membrane; SL, surface layer. Bars = 0.2 μm. (E) Cells of the GT and the WT, equivalent to 5 μg chlorophylls, were subjected to SDS-PAGE after being heated in the presence of SDS buffer. M, molecular mass marker.

acquire toxicity through the conformational change induced by the binding of Ca^{2+} ions to these sites (25, 29). The structure of HLP may be too rigid and devoid of flexibility to form pores. The absence of a palmitoylation site (G*K*Y/R sequence) could also contribute to the absence of toxicity (11, 15, 31). This study demonstrated that HLP is a member of the RTX protein family. This indicates that there are at least two classes of RTX proteins; one comprises the RTX toxins of pathogenic bacteria whose genes often form an operon with the genes that encode the acyltransferase and/or components of the type I secretion system (10, 27), and the other comprises the nontoxic RTX proteins of cyanobacteria, whose genes do not form such an operon (15, 26). Of 14 cyanobacterial strains whose genome DNA sequences have been sequenced (http://www.genome.ad.jp/kegg), 11 strains have genes that encode RTX proteins. Although some genes form an operon with the genes that encode the components of a type I secretion system, all the cyanobacterial RTX proteins appear to be nontoxic because the proteins lack a palmitoylation site and/or the cyanobacteria lack a gene that encodes the acyltransferase.

The genes that encode RTX toxins are supposed to be horizontally transferred (9), and thus, a similar horizontal transfer may be assumed for the genes that encode nontoxic RTX proteins. According to the selfish-operon theory (19) that explains the formation of operons through gene transfer, it is considered that a gene of nontoxic RTX protein could be transferred to pathogenic bacteria to form the operon and acquire toxicity. However, if the horizontal gene transfer of the nontoxic RTX proteins indeed occurs, then a question regarding the origin of the cyanobacterial RTX proteins would arise. It is noted that a hyperthermophilic bacterium, *Aquifex aeolicus*, has a gene that encodes a putative RTX protein (NCBI accession no. NP_213742) that lacks a palmitoylation site; this gene does not form an operon with either the gene that encodes an acyltransferase or the gene that encodes a component of a type I secretion system (8). This fact suggests a possible relationship between RTX proteins of hyperthermophilic bacteria and cyanobacteria.

Bhaya et al. (3) found that when slr1564, encoding *sigF*, was disrupted in the WT, the mutant produced HLP in the culture medium, causing a loss of motility. This finding is consistent with our result that the expression of HLP is related to the elimination of motility. The finding of Bhaya et al. (3), together with the apparent absence of HLP in the WT (Fig. 8), suggests that the expression of sll1951 is cryptic in the WT. We speculate that SigF is related to the expression of a putative transfactor that suppresses the expression of sll1951. Disruption of *sigF* might decrease the level of the transfactor, resulting in desuppression of sll1951 and expression of HLP. HLP may be one of the factors involved in the elimination of motility in the GT, functioning as a physical obstacle against type IV pilus extension and retraction for cell movement (22). Recently, by performing negative-staining electron microscopy, we found that the GT has a significantly smaller number of type IV pili (T. Sakiyama et al., unpublished). The apparent discrepancy in the functions of RTX proteins of cyanobacteria might be due to the difference in the types of motility, namely, the type IV pilus-derived twitching motility of PCC 6803, the swimming motility of *Synechococcus* sp. strain WH8102 (5), and the gliding motility of *P. uncinatum* (13); however, the function of the Ca^{2+}-binding structures of SwmA and oscillin in the activation of motility remains unclear. We are now genetically manipulating both the WT and the GT in order to elucidate the mechanism by which the expression of sll1951 affects motility as well as the physiological functions of HLP.

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