Intermolecular Cross-linking between the Periplasmic Loop$_{3-4}$ Regions of PomA, a Component of the Na$^+$-driven Flagellar Motor of Vibrio alginolyticus*

Received for publication, February 3, 2000, and in revised form, July 17, 2000 Published, JBC Papers in Press, July 18, 2000, DOI 10.1074/jbc.M000848200

Tomohiro Yorimitsu, Yukako Asai, Ken Sato, and Michio Homma‡

From the Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa-Ku, Nagoya 464-8602, Japan

PomA and PomB form a complex that conducts sodium ions and generates the torque for the Na$^+$-driven polar flagellar motor of Vibrio alginolyticus. PomA has four transmembrane segments. One periplasmic loop (loop$_{1-2}$) connects segments 1 and 2, and another (loop$_{3-4}$), in which cysteine-scanning mutagenesis had been carried out, connects segments 3 and 4. When PomA with an introduced Cys residue (Cys-PomA) in the C-terminal periplasmic loop (loop$_{3-4}$) was examined without exposure to a reducing reagent, a 43-kDa band was observed, whereas only a 25-kDa band, which corresponds to monomeric PomA, was observed under reducing conditions. The intensity of the 43-kDa band was enhanced in most mutants by the oxidizing reagent CuCl$_2$. The 43-kDa band was strongest in the P172C mutant. The motility of the P172C mutant was severely reduced, and P172C showed a dominant-negative effect, whereas substitution of Pro with Ala, Ile, or Ser at this position did not affect motility. In the presence of DTT, the ability to swim was partially restored, and the amount of 43-kDa protein was reduced. These results suggest that the disulfide cross-link disturbs the function of PomA. When the mutated Cys residue was modified with N-ethylmaleimide, only the 25-kDa PomA band was labeled, demonstrating that the 43-kDa form is a cross-linked homodimer and suggesting that the loops$_{3-4}$ of adjacent subunits of PomA are close to each other in the assembled motor. We propose that this loop region is important for dimer formation and motor function.

Many bacteria rotate flagellar filaments to swim. The flagellar filament is attached via a flexible hook to a protein complex termed the basal body, which is embedded in the cell surface. The motor is composed of the basal body and the C ring (rotor), whereas the multiple torque-generating units of the stator surround the rotor (1). The motor is driven by the flow of specific ions (H$^+$ or Na$^+$) through the stator, and mechanical force is presumably generated at the stator-rotor interface (2, 3).

Escherichia coli has H$^+$-driven flagellar motors whose torque-generating units consist of two proteins, MotA and MotB. These two proteins are believed to form a H$^+$ channel to permit the H$^+$ influx and to provide the energy for motor rotation.

‡ To whom correspondence should be addressed. Tel.: 81-52-789-2991; Fax: 81-52-789-3001; E-mail: g44416a@nucc.cc.nagoya-u.ac.jp.

* This work was supported in part by grants-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan (to M. H. and K. S.) and from the Japan Society for the Promotion of Science (to Y. A. and T. Y.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The abbreviations used are: NEM, N-ethylmaleimide; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.

The Journal of Biological Chemistry
© 2000 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

This paper is available online at http://www.jbc.org


The paper is available online at http://www.jbc.org

31387
FIG. 1. Putative secondary structure of PomA. This secondary structure is based on the hydrophobic profiles (8) and the reactivity of Cys-substituted proteins with SH-modifying reagents (16). PomA has two putative periplasmic loops, loop1–2 and loop3–4. The amino acids in loop3–4 are shown explicitly. The putative transmembrane (TM) regions are depicted as rectangles. Numbers indicate residue positions within PomA.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Growth Conditions, and Media—V. alginolyticus strains V105 (RifR, Pof−, Laf−, pomA), V10586 (RifR, Pof−, Laf−, pomA), and NMB188 (RifR, Pof−, Laf−, che−, pomA) were used (8, 11). E. coli strain DH5α (F− recA1 hsdR17 endA1 supE44 thi-1 relA1 gyrA96 DargF-lacZYA U169 δ80lacZAM15) was used for DNA manipulations. V. alginolyticus cells were cultured at 30 °C in VC medium: (0.5% (w/v) polypeptide, 0.5% (w/v) yeast extract, 0.4% (w/v) KH2PO4, 3% (w/v) NaCl, 0.2% (w/v) glucose); or VPG medium: (1% (w/v) polypeptide, 0.4% (w/v) KH2PO4, 3% (w/v) NaCl, 0.5% (w/v) glycerol). E. coli cells were cultured at 37 °C in LB medium. When necessary, kanamycin was added to V-buffer at a final concentration of 100 μg/ml for V. alginolyticus cells or 50 μg/ml for E. coli cells. Plasmid pYA301, a pSU41-based plasmid, carries the pomA gene under the control of the lac promoter (17).

Site-directed Mutagenesis—To introduce the P172A, P172I, and P172S substitutions into PomA, we used the two-step polymerase chain reaction method described previously (17). We synthesized pairs of mutant primers homologous to either the sense or antisense strand of the pomA gene, with a 1–3-base mismatch at the mutation site. In addition to these primers, we used end primers and amplified the full gene. This fragment was cloned into pSU41, and the identity of the entire insert was confirmed by DNA sequencing.

Swarm Assay—An overnight culture in VC medium was spotted onto VPG plates containing 0.25% agar and 100 μg/ml kanamycin and incubated at 30 °C. Dithiothreitol (DTT) was added to a final concentration of 1 mM, as needed.

Measurement of Swimming Speed—Cells were harvested at late logarithmic phase and suspended in V-buffer (25 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 300 mM NaCl). The cell suspension was diluted 100-fold in V-buffer, and motility of the cells was observed at room temperature under a dark field microscope and recorded on video tape. Swimming speed was determined as described previously (25). If necessary, DTT was added to V-buffer at a final concentration of 1 mM.

Detection of Cys-PomA—VIO586 (pomA) cells producing wild-type or various Cys-PomA mutant proteins were cultured in VPG medium and collected by centrifugation at mid-log phase. Cells were washed with V-buffer and then resuspended in the same buffer or in V-buffer containing 10 mM DTT or 1 mM CuCl2. After 30 min of incubation at room temperature, the cells were collected, washed with V-buffer containing 2 mM N-ethylmaleimide (NEM), suspended in the same buffer, and then incubated at room temperature for 10 min. Next, an equal volume of 15% (w/v) trichloroacetic acid was added to the cell suspension, and the resulting precipitate was washed once with acetone, dried, and then dissolved in SDS sample buffer without reducing reagent and then incubated at room temperature for 10 min. After centrifugation, the cell debris was removed by centrifugation at 10,000 × g for 10 min at 4 °C, and the supernatant was then centrifuged at 100,000 × g for 60 min at 4 °C. The precipitate was resuspended in V-buffer containing 10 mM DTT, and the resulting precipitate was incubated at room temperature. After 30 min, it was centrifuged at 100,000 × g for 60 min at 4 °C, the precipitate was resuspended in 500 μl of V-buffer containing 0.5 mM [14C]NEM (20 μCi/ml; NEN Life Science Products), and the resuspended material was incubated for 60 min at room temperature. The membrane fraction was recovered by centrifugation at 100,000 × g, solubilized with TNET buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 5 mM EDTA, and 1% (w/v) Triton X-100), and incubated for 60 min at 4 °C. Immunoprecipitation with anti-PomA antibody was carried out as described previously (18). The resulting precipitates were subjected to SDS-PAGE, followed by fluorography.

RESULTS

Detection of Cys-PomA Protein—Asai et al. (20) carried out Cys-scanning mutagenesis of the two periplasmic loops (loop1–2 and loop3–4) of PomA and characterized the mutant proteins. This work showed that Cys replacements in loop3–4 affected motility more severely than those in loop1–2, and we therefore focused on the replacements in loop3–4. When we examined Cys-PomA proteins in loop3–4 by immunoblotting in the absence of reducing reagent, we observed, in addition to the 25-kDa PomA band, a 43-kDa band with Cys replacements at residues 171–174 and at residue 177 (Fig. 2B). The intensity of the 43-kDa band was greatest with the P172C protein. When cells were treated with DTT and immunoblotting was carried out, only the 25-kDa PomA band was observed (Fig. 2A). When the cells were treated with CuCl2, the intensity of the 43-kDa PomA band was correspondingly

FIG. 2. Detection of Cys mutant PomA proteins. V10586 (pomA) cells harboring pYA301 (pomA) and mutant derivatives were incubated in V-buffer (B) and buffer containing 10 mM DTT (A) or 1 mM CuCl2 (C). After the cells were treated with 2 mM NEM, proteins were precipitated by trichloroacetic acid, washed with acetone, and separated by SDS-PAGE without reducing reagent. PomA was detected by immunoblotting with anti-PomA antibody. The PomA residues substituted with Cys are indicated. Numbers with arrows on the right side are the estimated molecular masses of the indicated bands.
swimming abilities conferred by the mutant PomA proteins. Overnight cultures of VIO586 cells producing wild-type PomA (wt) and PomA P172C, P172S, or P172A were spotted onto 0.25% agar VPG plates containing 100 μg/ml kanamycin and incubated at 30 °C for 3.5 h. In all three mutants, the 43-kDa band observed with P172C PomA was the same as that seen with wild-type PomA (Fig. 3A). In all three mutants, the 43-kDa band observed with P172C PomA was absent under all conditions tested (Fig. 3B).

**Effect of DTT on Motility—**Motility was more affected by Cys substitutions near residue Pro-172 than at other positions in the loop regions (20). The P172C mutant produced swarms only after prolonged incubation, and motile cells were seldom observed in liquid culture (data not shown). We predicted that the inhibition of motility is caused by disulfide-bound formation between PomA subunits. The residual motility is probably due to incomplete cross-linking (see below).

To examine this possibility, motility was assessed on swarm plates in the presence or absence of DTT (Fig. 4A). DTT did not affect swarming of cells expressing wild-type PomA, but cells producing P172C PomA swarmed well only in the presence of DTT (Fig. 4A). This result suggests that motility is restored by reductive cleavage of the disulfide cross-link between Cys-PomA molecules. We next tested for dominant-negative effects of the cross-linked form of PomA. When P172C PomA was produced in wild-type cells (VIO5) on agar plates without DTT, motility was significantly reduced (Fig. 4B). However, motility was restored in the presence of DTT. These results suggest that the cross-linked forms can be assembled into the motor complex.

To examine the relationship between recovery of motility and reduction of the cross-linked PomA, swimming speed was measured in the presence or absence of 1 mM DTT as indicated under “Materials and Methods.” Each filled symbol indicates swimming speeds of cells (strain NMB188) producing wild-type PomA in the presence (triangles) or absence (inverted triangles) of DTT, or PomA P172C in the presence (squares) or absence (circles) of DTT. Open symbols connected by dotted lines indicate relative amounts of 43-kDa PomA P172C dimer to total PomA P172C protein in the presence (squares) or absence (circles) of DTT. The amounts were estimated from the data shown in B. B, cells of strain NMB188 producing PomA P172C were collected at late logarithmic phase and suspended in V-buffer in the presence (left) or absence (right) of DTT. At the times indicated, cells were collected, and PomA was detected as described in the legend to Fig. 2.
of about 50 kDa are the heavy chains of IgG. These results indicate that two molecules of native PomA interact with each other intimately in a torque-generating unit of the motor. Recently, PomA alone was purified as a stable homodimer even if PomB was absent (19). The purified PomA/PomB complex has been reconstituted into proteoliposomes and has been shown to catalyze Na$^+$ influx. Furthermore, a tandem PomA dimer produced as a single polypeptide is functional. Inactivation of either half of the dimer results in complete loss of PomA function.

**DISCUSSION**

The PomA protein of the Na$^+$-driven flagellar motor of *Vibrio alginolyticus* is predicted to have four transmembrane segments, whereas the PomB, MotX, and MotY proteins have only a single transmembrane segment (8–11). From the predicted topology of PomA, the N- and C-terminal regions and the large loop between transmembrane segments 2 and 3 are located in the cytoplasm. Loop1–2 and loop3–4, between transmembrane segments 1 and 2 and segments 3 and 4, respectively, are thought to be exposed to the periplasmic space (8). When the loops are reacted with biotin maleimide, the labeling pattern was different; substitutions in loop3–4 were biotinylated consistently with the membrane topology, whereas none of residues in loop1–2 was labeled (20). It has been proposed that loop1–2 is associated with other proteins, such as the motor proteins PomB, MotX, or MotY, or that it is embedded into the pore region of the channel, as is predicted for the extracellular loops of many ion channels. It has been shown that the negative charge Asp-31 of loop1–2 contributes to optimal speed and/or efficiency of the motor, although the charge is not essential (26). By random mutagenesis of the *pomA* gene, it was shown that loop3–4 and transmembrane segments 3 and 4 have residues more crucial for function than loop1–2 or transmembrane segments 1 and 2 (27).

In the present study, we focused on periplasmic loop3–4. We found that the P172C mutant protein of PomA is not functional and inhibits the motility of the wild-type cell in the absence of reducing reagents, although motility is restored by DTT. This result suggests that a cross-link formed between two molecules of Cys-PomA via loop3–4 inhibits the function of the torque-generating units. The interaction between cross-linked PomA dimers and PomB is apparently not disrupted, since PomB appears to be co-precipitated about as well with the cross-linked PomA dimer as with the wild-type PomA (data not shown). It is inferred that two molecules of native PomA interact with each other intimately in a torque-generating unit of the motor. Recently, PomA alone was purified as a stable homodimer even if PomB was absent (19). The purified PomA/PomB complex has been reconstituted into proteoliposomes and has been shown to catalyze Na$^+$ influx. Furthermore, a tandem PomA dimer produced as a single polypeptide is functional. Inactivation of either half of the dimer results in complete loss of PomA function.
tion. When a phenamil-resistant mutation was introduced into either the first or second half of the tandem PomA dimer, the resistant phenotype was identical to the phenotype of the tandem dimer in which the both halves carried the mutation. Thus, the two halves of the PomA dimer appear to function together (28).

The H\textsuperscript{+}-driven flagellar motor contains MotA and MotB, which are homologues to PomA and PomB, respectively. It is thought that the MotA/MotB complex converts H\textsuperscript{+} influx into the rotation of the flagellar motor (4, 6, 29). From the results of Trp-scanning mutagenesis, a structural model of MotA/MotB complex has been proposed in which a single transmembrane segment of one MotB protein assembles with four transmembrane segments of one MotA protein at a tilt relative to them (5). If this model holds for the H\textsuperscript{+}-driven motor, the existence of a heteromultimeric ion channel complex might be specific for the Na\textsuperscript{+}-driven flagellar motor. On the other hand, estimates of the MotA and MotB protein levels in the membrane of E. coli suggest that the ratio of MotA to MotB is about 4:1 (30). This finding is consistent with the possibility that MotA and MotB do not make 1:1 complex.

The MotA protein of the H\textsuperscript{+}-driven flagellar motor of *Rhodobacter sphaeroides* can generate torque in response to a sodium-ion flux in a *pomA* mutant of *V. alginolyticus* (31). MotA of *R. sphaeroides* may work in the Na\textsuperscript{+}-driven motor because the torque generators in the H\textsuperscript{+}-and Na\textsuperscript{+}-driven motors have similar structures, i.e., they are heteromultimeric complexes. In any case, clarification of the structure of the torque generator is clearly important for an understanding of the mechanism of coupling ion flow to flagellar rotation.

Two additional components, MotX and MotY, are required for the rotation of the Na\textsuperscript{+}-driven motor of *V. alginolyticus*, but homologous proteins are not found in the H\textsuperscript{+}-driven motor (9–12). The functions of MotX and MotY are not clear, although it is thought that they may be involved in ion recognition. MotX and MotY do not co-purify with the PomA/PomB complex (19). This observation may mean that MotX and MotY are not associated with the PomA/PomB complex, that the association is weak, or that MotX and MotY are unstable during purification. It will be interesting to determine the structural difference between the torque generators of the Na\textsuperscript{+} - and H\textsuperscript{+}-driven flagellar motors.

It has been proposed that the rotor is surrounded with multiple torque-generating units, approximately eight units in the H\textsuperscript{+}-driven flagellar motor (32) or five to nine in the Na\textsuperscript{+}-driven motor (33). We can envision two models to explain the cross-linking of PomA molecules. One is an intra-torque-generator model, in which the cross-links form between two PomA molecules in the same unit. In this argument, loop_3–4 must face into the unit. The other possibility is an inter-torque-generator model, in which the cross-link occurs between two PomA molecules in different units, so that the loop_3–4 faces outward. The former model is supported by following criteria; the distance between two generators may be too great for disulfide cross-link to form, PomA forms a stable dimer in the cell, and genetically connected tandem PomA is functional. We need more experiments to elucidate which model is correct.

**Acknowledgment**—We thank R. M. Macnab for critically reading the manuscript.

**REFERENCES**

Intermolecular Cross-linking between the Periplasmic Loop$_{3-4}$ Regions of PomA, a Component of the Na$^+$-driven Flagellar Motor of *Vibrio alginolyticus*

Tomohiro Yorimitsu, Yukako Asai, Ken Sato and Michio Homma

doi: 10.1074/jbc.M000848200 originally published online July 18, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M000848200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 33 references, 20 of which can be accessed free at http://www.jbc.org/content/275/40/31387.full.html#ref-list-1