Volume 249, number 2, 168–172 FEB 07177 June 1989

Location of phosphorylation site and DNA-binding site of a positive regulator, OmpR, involved in activation of the osmoregulatory genes of *Escherichia coli*

Masashi Kato, Hirofumi Aiba, Shin-ichi Tate*, Yoshifumi Nishimura* and Takeshi Mizuno

Laboratory of Microbiology, School of Agriculture, Nagoya University, Chikusa-ku, Nagoya 464 and **Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan*

Received 22 March 1989

The OmpR protein of *Escherichia coli* is a positive regulator involved in activation of the *ompF* and *ompC* genes which encode the major outer membrane proteins OmpF and OmpC, respectively. By employing recombinant DNA techniques, we isolated the N- and C-terminal halves of the OmpR molecule. From the results of biochemical analyses of these fragments, it was concluded that the N-terminal portion contains a site involved in phosphorylation by an OmpR-specific protein kinase EnvZ, whereas the C-terminal part possesses a DNA-binding site for the *ompC* and *ompF* promoters.

Activator protein; OmpR; Phosphorylation; DNA recognition; (*E. coli* outer membrane)

1. INTRODUCTION

Expression of the *ompF* and *ompC* genes, which encode the *Escherichia coli* outer membrane proteins OmpF and OmpC, respectively, is affected in a reciprocal manner by the osmolarity of the growth medium [1]. At least two protein factors, OmpR and EnvZ, are known to be involved in the transcriptional regulation of the expression of *ompF* and *ompC* [2]. OmpR protein is a positive regulator specific for the *ompF* and *ompC* genes [3–5]. Recently, this protein was purified to homogeneity, and its direct binding to the promoter regions of both *ompF* and *ompC* genes was demonstrated [6–8]. On the other hand, EnvZ protein is believed to sense the environmental osmotic signal and then modulate the functioning of OmpR [2]. The results obtained from biochemical analysis of a truncated form of EnvZ protein demonstrated that EnvZ protein is autophosphorylated in vitro in the presence of ATP, its phosphoryl group being subsequently transferred to OmpR protein [9]. Thus, EnvZ protein is presumably a protein kinase specific for OmpR protein.

We have proposed that OmpR protein has a two-domain structure [10]. Previously, a C-terminal fragment of OmpR protein, generated spontaneously from purified OmpR protein during storage, was characterized and demonstrated to retain the capability of binding to the *ompF* promoter DNA [11]. However, purification of this C-terminal fragment in large amounts was difficult due to its spontaneous generation being very slow (~1 month at 4°C). To elucidate further the structure and function of OmpR protein, we here constructed two recombinant *ompR* genes that encode the N- and C-terminal portions of the OmpR molecule. This enabled us to isolate not only the C-terminal part but also the N-terminal portion of the OmpR molecule. By using these fragments, we demonstrate here that the N-terminal portion contains a site involved in phosphorylation by EnvZ protein, and that the C-terminal fragment possesses a DNA-binding site for the *ompC* and *ompF* promoters.

Correspondence address: T. Mizuno, Laboratory of Microbiology, School of Agriculture, Nagoya University, Chikusa-ku, Nagoya 464, Japan

Published by Elsevier Science Publishers B.V. (Biomedical Division)

2. MATERIALS AND METHODS

2.1. *Materials*

Restriction endonucleases and Klenow fragment of DNA polymerase I were obtained from Takara Shuzo. [α-^{32}P]dCTP (3000 Ci/mmol) and [γ-^{32}P]ATP (30 Ci/mmol) were purchased from Amersham International. All other reagents were of reagent grade.

2.2. *Purification of OmpR protein and its N- and C-terminal fragments*

Intact OmpR protein and its C-terminal fragments were purified from *E. coli* JA221 carrying the plasmid pFN108 and pOMPR-C, respectively, its N-terminal fragment being obtained from *E. coli* SG480Δ76 carrying the plasmid pMR-N ([6] and see fig.1). These cells overproduce intact OmpR, and its C- and N-terminal fragments, respectively. Purification of the N-terminal fragment was carried out as in [6], that of the C-terminal portion being purified according to [11], with slight modification: CM-cellulose (CM52) column chromatography was performed in a buffer containing 20 mM sodium phosphate (pH 6.5) (linear NaCl gradient from 0 to 200 mM), followed by DEAE-cellulose (DE52) column chromatography in a buffer containing 20 mM Tris-HCl (pH 8.5) (linear NaCl gradient, 0–150 mM). Purified polypeptides were stored at 4°C in 10 mM Tris-HCl (pH 7.8) containing 50 mM KCl, 0.1 mM EDTA and 0.1 mM DTT.

2.3. *Purification of a truncated form of EnvZ protein*

A truncated form of EnvZ protein (EnvZ*), known to exhibit OmpR-specific protein kinase activity, was purified as described [9].

2.4. *DNA-binding assay*

Binding of OmpR protein and the N- and C-terminal fragments to the *ompF* and *ompC* promoter DNAs was assayed essentially as in [6].

2.5. *Polyacrylamide gel electrophoresis*

An SDS-polyacrylamide gel (12.5% acrylamide) system was used [12].

3. RESULTS

3.1. *Isolation of polypeptide fragments corresponding to the N- and C-terminal portions of OmpR protein*

In order to isolate polypeptide fragments corresponding to the N- and C-terminal portions of intact OmpR protein consisting of 239 amino acid residues, two recombinant *ompR* genes on multicopy number plasmids were constructed (fig.1A). The recombinant plasmid, pMR-N, carries the entire *ompR*-coding sequence into which a termination codon (TAA) was introduced by insertion of a synthetic oligonucleotide linker (dGCTTAATTAATTAAGC). As shown in fig.1B, this plasmid

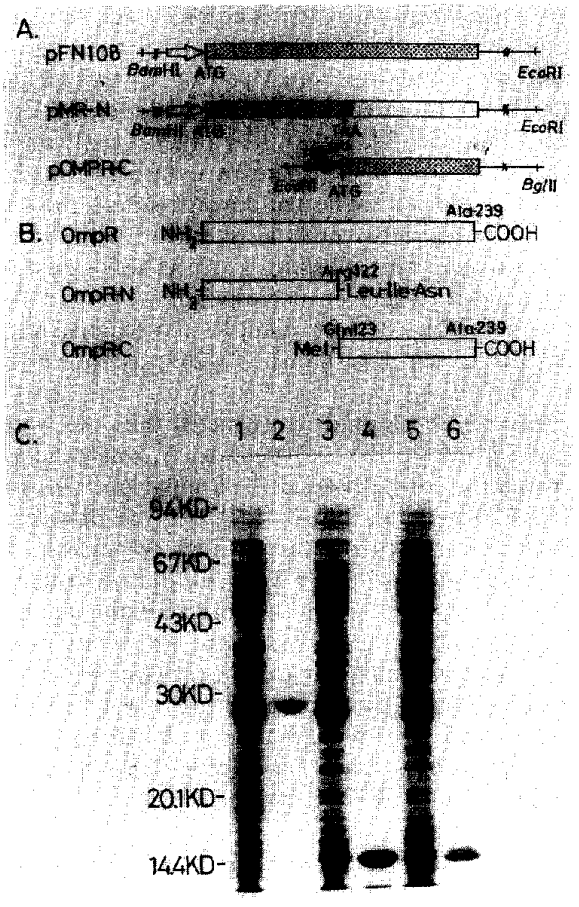

Fig.1. Schematic representation of recombinant *ompR* genes encoding the OmpR protein and its N- and C-terminal fragments. (A) Structures of recombinant *ompR* genes encoding the OmpR protein, and its N- and C-terminal fragments, the plasmids carrying these genes being designated pFN108, pMR-N and pOMPR-C, respectively. (Rectangles) *ompR* coding sequences; (open and closed arrows) *ompR* promoter and *tac* promoter-operator, respectively. (B) Scheme depicting the structures of the polypeptides expected to be synthesized by plasmids pFN108, pMR-N and pOMPR-C; polypeptides designated as OmpR, OmpR-N and OmpR-C, respectively. (C) Profiles of SDS-polyacrylamide gel electrophoresis of purified OmpR, OmpR-N and OmpR-C. *E. coli* JA221 carrying pFN108 and *E. coli* SG480 76 carrying pMR-N were cultured at 38°C to overproduce the corresponding polypeptides, OmpR and OmpR-N and total cell lysates then prepared (lanes 1,3, respectively). *E. coli* JA221 carrying pOMPR-C was grown in the presence of isopropyl β-D-thiogalactopyranoside (1 mM) to overproduce OmpR-C, followed by preparation of total lysate (lane 5). From these lysates, OmpR, OmpR-N and OmpR-C were purified as described in section 2 (lanes 2,4,6, respectively).

is expected to produce a polypeptide fragment corresponding to the N-terminal half of the OmpR molecule, extending from Met-1 to Arg-122 which is followed by three extra amino acids encoded by the oligonucleotide linker. The other recombinant plasmid, pOMPR-C, carries a truncated *ompR*-coding sequence connected downstream to the *tac* promoter-operator and a synthetic ribosome-binding site. This plasmid is expected to yield a polypeptide fragment corresponding to the C-terminal half of the OmpR molecule, from Gln-123 to the C-terminal Ala-239, as shown in fig.1B.

Using both plasmids constructed thus, we were able to purify the polypeptide fragments corresponding to the N- and C-terminal portions of OmpR protein, as shown in fig.1C. The apparent molecular masses of the purified fragments on SDS-polyacrylamide gels were essentially the same as those calculated from the corresponding amino acid sequences. Furthermore, the fragments were confirmed as undergoing cross-reaction with antiserum raised against OmpR protein (not shown). The N- and C-terminal fragments of OmpR protein thus isolated are henceforth referred to as OmpR-N and OmpR-C, respectively.

3.2. *Location of DNA-binding site in OmpR protein*

We have previously proposed that the C-terminal portion of OmpR protein is responsible for the binding to *ompF* and *ompC* promoter DNAs. We have demonstrated that a C-terminal fragment, generated spontaneously from purified OmpR protein, binds to the *ompF* promoter DNA [11]. Here, by using purified OmpR-N and OmpR-C, we were able to evaluate their abilities to bind to both *ompF* and *ompC* promoter DNAs. Two *Eco*RI-*Hin*dIII fragments, one comprising 463 bp and encompassing the *ompF* promoter region (fig.2A), the other being of length 298 bp and spanning the *ompC* promoter region (fig.2B), were labeled with [α-^{32}P]dCTP at the *Hin*dIII site. After incubation of these DNA fragments with intact OmpR, OmpR-N and OmpR-C, samples were subjected to non-denaturing polyacrylamide gel electrophoresis. As shown in fig.2, OmpR-C as well as intact OmpR was capable of binding to both *ompF* and *ompC* promoter DNAs to nearly the same extent, whereas OmpR-N was unable to do so. Fur-

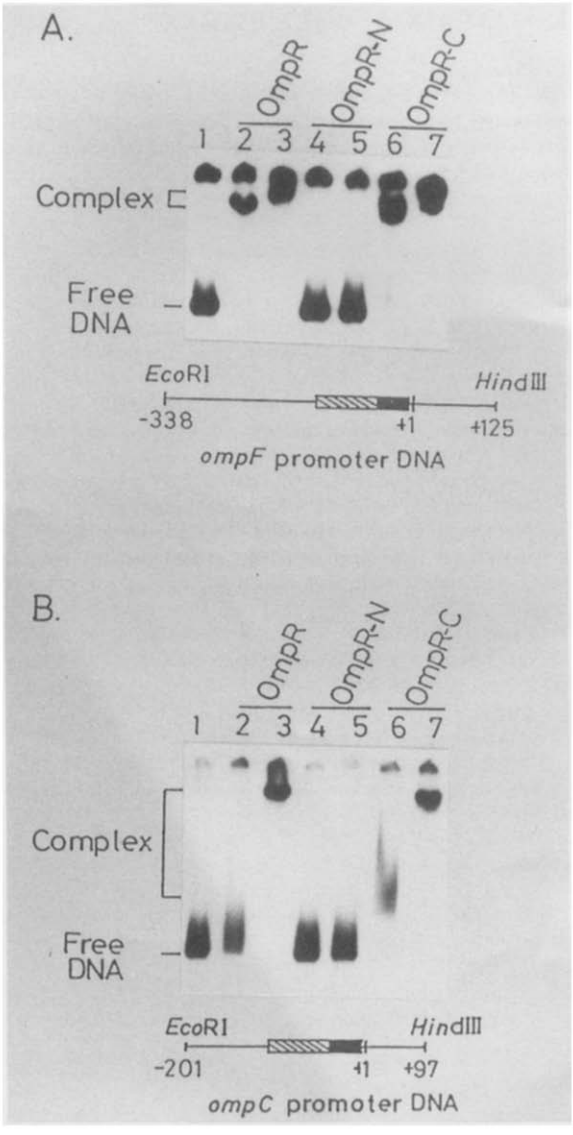

Fig.2. Binding of the N- and C-terminal fragments of OmpR protein to *ompF* and *ompC* promoter DNAs. (A) Scheme representing the 463 bp *Eco*RI-*Hin*dIII fragment encompassing the *ompF* promoter region, used for the binding assay. The position of the *ompF* transcription start site is indicated by +1. (Hatched and closed rectangles) OmpR-binding region and the −35 and −10 regions of the *ompF* promoter, respectively. The *Eco*RI-*Hin*dIII fragment was labeled with [α-^{32}P]dCTP at the *Hin*dIII site. The endo-labeled fragment (0.5 pmol), was incubated with either intact OmpR [(lane 2) 40 pmol, (lane 3) 80 pmol], OmpR-N [(lane 4) 80 pmol, (lane 5) 160 pmol] or OmpR-C [(lane 6) 80 pmol, (lane 7) 160 pmol]. After incubation, samples were subjected to non-denaturing polyacrylamide (5%) gel electrophoresis, followed by autoradiography. (B) Scheme for the 298 bp *Eco*RI-*Hin*dIII fragment encompassing the *ompC* promoter region, used for the binding assay. Other details as described for (A).

thermore, the results of DNase I footprinting analysis of both *ompF* and *ompC* promoter DNAs confirmed that OmpR-C was capable of recognizing the same specific nucleotides as in the case of intact OmpR (not shown). Thus, we conclude that OmpR-C contains a specific DNA-binding site for both *ompF* and *ompC* promoter DNAs.

3.3. *Location of phosphorylation site in OmpR protein*

We have previously demonstrated that OmpR protein is phosphorylated in vitro by EnvZ protein [9]. To localize the phosphorylation site in OmpR protein, purified OmpR-N and OmpR-C as well as intact OmpR were subjected to in vitro phosphorylation by EnvZ protein, as shown in fig.3. A truncated form of EnvZ protein (EnvZ*) is known to undergo autophosphorylation in the presence of ATP [9]. After incubation of EnvZ* with [γ-^{32}P]ATP, the radiolabelled phosphorylated EnvZ* resulting was purified and subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography (lane 1). When phosphorylated EnvZ* was incubated with intact OmpR, its phosphoryl group was rapidly transferred to OmpR (lane 2). Phosphorylated OmpR was rather stable in the absence of ATP (lane 3), whereas with ATP present, phosphorylated OmpR underwent rapidly dephosphorylation (lane 4). It was observed that OmpR-N takes part in essentially the same phosphorylation and dephosphorylation reactions as intact OmpR (lanes 5–7), whereas OmpR-C does not (not shown). Thus, we conclude that OmpR-N contains a site involved in phosphorylation by EnvZ protein.

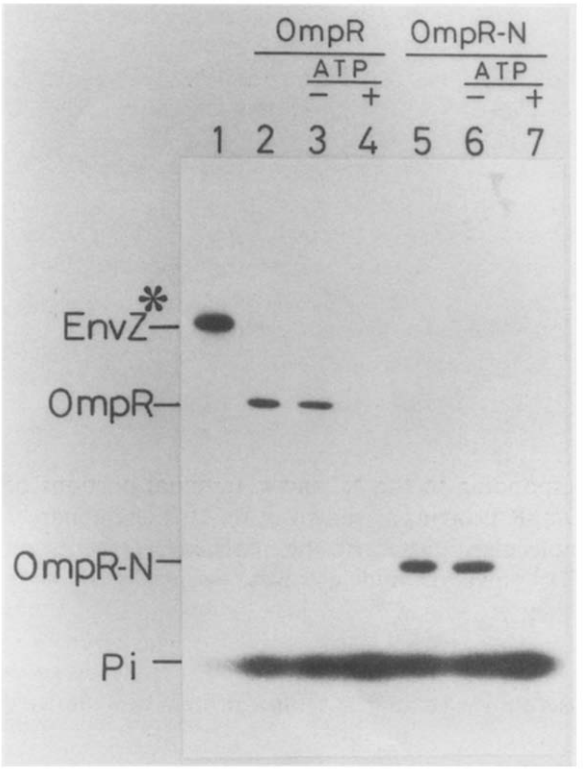

Fig.3. EnvZ-dependent phosphorylation and dephosphorylation of OmpR protein. Purified EnvZ* (1 μg) was incubated at room temperature with 0.1 mM [γ-^{32}P]ATP (10000 cpm/pmol) in the presence of 5 mM $MgCl_2$ and 50 mM KCl. Phosphorylated EnvZ* was purified by gel filtration (Sephadex G-50). The phosphorylated EnvZ* (lane 1) was incubated with either intact OmpR (lane 2, 1.5 μg) or OmpR-N (lane 5, 0.8 μg) at room temperature for 5 min. Aliquots of the same samples as lanes 2 and 5 were incubated for a further 5 min in the absence (lanes 3,6) and presence (lanes 4,7) of 0.1 mM ATP. These samples were analyzed by SDS-polyacrylamide gel electrophoresis, followed by autoradiography.

4. DISCUSSION

Here, we have succeeded in purifying the N- and C-terminal halves of an activator protein, OmpR, by employing recombinant DNA techniques. Biochemical studies on the purified fragments, OmpR-N and OmpR-C, demonstrated that the N-terminal portion of OmpR protein contains a site involved in phosphorylation by EnvZ protein, although it remains to be determined which amino acid residue(s) is (are) phosphorylated. It has also been shown that the C-terminal portion of OmpR protein contains a DNA-binding site for both *ompF* and *ompC* promoter DNAs. The present results provided us with several new clues as to the elucidation of not only the structure and function but also the interaction of OmpR protein with other components such as the *ompF* and *ompC* promoter DNAs and EnvZ protein, as summarized in fig.4.

Our results strongly support the view that OmpR protein contains at least two functional domains, which are physically separable: one is responsible for interaction with the putative protein kinase, EnvZ, whereas the other participates in interactions with the cognate promoter DNAs. This contention is consistent with the results of previous

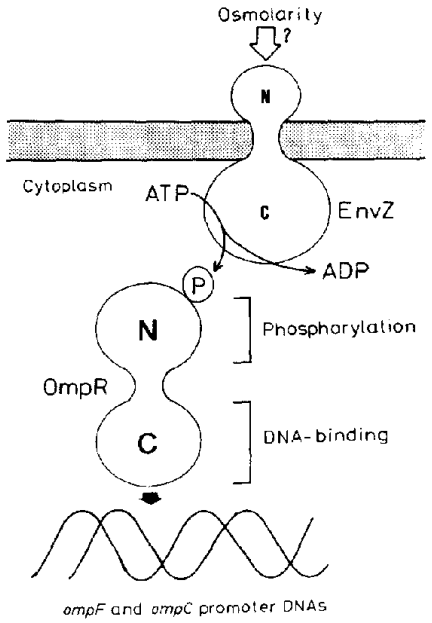

Fig.4. Scheme showing the functional interactions between OmpR and EnvZ proteins and promoter DNAs. (N,C) N- and C-terminal portions of OmpR and EnvZ proteins, respectively. (Stippled area) Cytoplasmic membrane. It should be noted that this illustration is intended to be schematic; the shapes and sizes of the OmpR and EnvZ proteins, and the diameter of the DNA helix are all arbitrary.

genetic studies on various *ompR* mutants [10,13]. Based on this view, EnvZ protein may somehow be able to sense the environmental osmotic signal and modulate its own protein kinase activity. The N-terminal portion of OmpR protein is phosphorylated by EnvZ protein, and consequently the DNA-binding properties of the C-terminal portion are altered. Alternatively, phosphorylation might affect possible interaction between OmpR protein and RNA polymerase, since a functional interaction between them has also been suggested [14,15]. In any event, since the OmpR-N and OmpR-C characterized here are readily purified in milligram quantities, biochemical and biophysical characterization of the fragments will certainly facilitate clarification of the complex mechanisms underlying the expression of *ompF* and *ompC* in response to environmental osmotic signals in prokaryotes.

Acknowledgement: This work was supported by a grant from the Ministry of Education, Science and Culture of Japan.

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