

Bacterial and Eukaryotic Porins

Structure, Function, Mechanism

Edited by
Roland Benz



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Cover Illustration

Top view of a trimeric LamB maltoporin
with bound oligosaccharides
(courtesy of R. Benz)

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**Library of Congress Card No.: applied for
British Library Cataloguing-in-Publication Data**

A catalogue record for this book is available from the British Library.

**Bibliographic information published by
Die Deutsche Bibliothek**

Die Deutsche Bibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data is available in the Internet at <http://dnb.ddb.de>.

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Printed in the Federal Republic of Germany.
Printed on acid-free paper.

Typesetting Hagedorn Kommunikation GmbH, Viernheim

Printing betz-druck gmbh, Darmstadt

Bookbinding J. Schäffer GmbH & Co. KG, Grünstadt

ISBN 3-527-30775-3

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Preface

Cell wall containing eubacteria have traditionally been divided into gram-positive and gram-negative bacteria; according to their staining properties with crystal violet, also known as Gram stain. Electron microscopic analysis into the structure of the so-called gram-negative bacteria soon suggested that their cell wall contained an additional membrane, called outer membrane [1]. The lipid composition of the outer membrane composed of lipids and lipopolysaccharides is well known for a long time. Starting with the early seventies of the last century knowledge accumulated on the rather simple protein composition of the outer membrane: only a few bands were observed on SDS-PAGE of outer membrane proteins. Some of them are heat-modifiable because they change the position on SDS-PAGE when heated to 100°C. Electron microscopic analysis of the surface of *Escherichia coli* outer membrane revealed the presence of a protein with a regular structure [2]. This protein termed 'matrix protein' was considered the permeability pathway for hydrophilic solutes through the outer membrane. After identification of the pore-forming unit in the outer membrane of *Salmonella typhimurium* [3] and *E. coli* [4] the name porin for the outer membrane channels came into use.

The study of the amino acid composition of porins and the first amino acid sequence of a porin of *E. coli* deduced from the mature protein represented a big surprise [5]. The porins had an amino acid composition similar to those of water-soluble proteins, containing more than 50% hydrophilic amino acids. The primary sequences of the first and all other outer membrane porins sequenced to date do not show any indication for the presence of α -helical structures, which were considered since Kyte and Doolittle [6] to represent the typical structural elements of membrane proteins. Vogel and Jähnig [7] suggested that outer membrane proteins are arranged in amphipathic β -strands and form a β -barrel cylinder. The cylindrical structure implies that on average every second amino acid in membrane-spanning β -sheets is hydrophobic because it faces the hydrocarbon core of the membrane or it is hydrophilic and points to the channel interior. From the functional standpoint porins were divided in general diffusion pores and substrate-specific porins. Besides these more classical porins, the outer membrane contains also energy-coupled transporters and channel-tunnels (see below). General diffusion pores represent more or less structured holes in the outer membrane but they have a defined exclusion limit for the passage of hydrophilic solutes (see chapters

2 and 6). Specific porins contain binding sites for substrates such as sugars or nucleosides (see chapters 9 and 10). Luckily enough it was possible to crystallize both, general diffusion pores and specific porins of bacterial outer membranes. Chapter 2 describes structural properties of general diffusion porin and chapter 9 those of specific porins. Surprisingly, the carbohydrate-specific LamB and ScrY have two β -strands more than the general diffusion pores OmpF, OmpC and PhoE.

The expression of general diffusion pores and specific porins can be regulated dependent on the requirements of the growth media. A interesting example for porin regulation represents the regulation of OmpF/OmpC porins of *E. coli* by the two-component regulatory system EnvZ/OmpR dependent on osmolarity, pH and other environmental parameters (see chapter 1). This means that bacteria are able to respond to environmental stress. Chapter 3 describes the role of bacterial porins in antibiotic susceptibility. This chapter describes the relationships between porins and antibiotic molecules and its impact on the development of resistance against certain antibiotics. Besides bacterial response against stress created by antibiotics there exist also other mechanisms of outer membrane permeability control. This type of control is described in chapter 5 of this book and deals with rapid modulation of porin function. Other gram-negative bacteria have a certain “natural” resistance against many antibiotics. A prominent example of this intrinsic antibiotic resistance is the opportunistic human pathogen *Pseudomonas aeruginosa*. Chapter 4 deals with the properties of the porins of this organism and explains the high intrinsic antibiotic resistance of this organism, which is in part the result of the control of outer membrane permeability for hydrophilic solutes. Part of this control is also OprF of *P. aeruginosa* outer membrane, which has not the “classical” trimeric form of outer membrane porins of the OmpF type as it is described in chapter 7. OprF and also OmpA of *E. coli* and other enteric bacteria exist in two different configurations. The majority of these outer membrane proteins have 8 β -strands and a very low permeability, whereas a small fraction of OprF and OmpA allow diffusion of large solutes that cannot penetrate OmpF of *E. coli* (chapter 7).

Reconstituted systems allow a meaningful study of porin function. As already mentioned, chapter 5 deals with the study of porin modulation in model membranes using the patch-clamp technique. Chapter 6 describes general diffusion porin reconstitution in lipid bilayer membranes and chapter 10 the study of specific diffusion porins containing binding-sites in the same system. Porin trimers are very stable and the lipid bilayer technique can be a useful tool in the area of development of biosensors.

Besides the classical Omp pores also several outer membrane transporters for iron chelates and one for vitamin B12 were crystallized (chapters 11 and 12). These transporters are of special interest because the outer membrane is not energized. Energy is provided through proteins of the cytoplasmic membrane and their coupling to the outer membrane transporter is of special interest. The transporters have a novel structure of outer membrane proteins. In particular, they are formed by a β -barrel cylinder of 22 β -strands. The hole in the cylinder is plugged by a cork or a hatch as it is described for FhuA (siderophore receptor, chapter 11) and BtuB

(vitamin B12 receptor, chapter 12) of *E. coli*. Another class of outer membrane permeability pathway is formed by the channel tunnel prototype TolC of *E. coli*, which has also been crystallized (chapter 8). The outer membrane channels of the TolC-type are formed by homotrimers. However, differently to the porins, the trimer contains only one channel. The channel-tunnels can be divided into different parts. The 4 nm long β -barrel cylinder of 12 β -barrels is connected to the 10 nm long α helical tunnel domain of 12 α -helices, which presumably spans the periplasmic space. The channel-tunnels are important parts of efflux pumps exports systems as discussed in chapter 8.

Because of the homology of the electron transport chains and the existence of two membranes, it has been hypothesized that mitochondria are descendants of certain strictly aerobic bacteria. In any case, the permeability properties of the mitochondrial outer membranes show some resemblance to those of bacterial outer membranes as discussed in chapter 13. A considerable part of the permeability properties of mitochondrial outer membranes is caused by the presence of a general diffusion pore, called mitochondrial porin or VDAC [8], which forms voltage-dependent channels in reconstituted systems. Mitochondrial porins have a secondary structure highly homologous to that bacterial outer membrane proteins, which means that the channel formed by a porin monomer is essentially a β -barrel cylinder (chapter 13). Several different isoforms of not well understood function exist in many organisms as discussed in chapters 14 and 15. Unfortunately, mitochondrial porin or VDAC could not be crystallized to date in order to obtain any useful 3D-structure, hence the exact 3D-structure is still not known and currently a matter of debate. Chapter 16 describes that mitochondrial porins play an important role in the physiology of these cell organelles, which means that they have a communicative function. Mitochondrial porins/VDACs regulate the movement of mitochondrial metabolites between the cytosol and the mitochondrial compartments. They are possible components of the mitochondrial permeability transition pore and may participate in very interesting mitochondrial functions such as apoptosis (see chapter 14 and 16).

I am very grateful to all authors who made valuable contributions in this book and who produced their manuscript so promptly. Many thanks go also to Christian Andersen who provided the picture of the front cover and Frank Orlik who helped me with the index.

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1

Regulation of Porin Gene Expression by the Two-component Regulatory System EnvZ/OmpR

Don Walthers, Alvin Go and Linda J. Kenney

1.1

Introduction

The major paradigm for signal transduction in bacteria is the two-component regulatory system. The first component is a sensor kinase, most often a membrane protein, which senses an environmental signal and is phosphorylated by ATP on a conserved histidine residue. The second component is the response regulator, which catalyzes the phosphotransfer of the phosphoryl group onto a conserved aspartic acid residue (see [1, 2] for recent reviews). Most response regulators are two-domain proteins and phosphorylation of the receiver domain alters the output of the effector domain, which is usually a stimulation of DNA binding. In some systems, the histidine kinase alters the level of the phosphoresponse regulator by stimulating its dephosphorylation, rather than by stimulating its phosphorylation.

The sensor kinase EnvZ and the response regulator OmpR comprise the two-component system that is responsible for the regulation of expression of the outer membrane proteins OmpF and OmpC (see Figure 1.1). Porin levels are influenced by a wide variety of environmental conditions, including osmolality, temperature, pH and growth phase. Although the total amount of OmpF and OmpC remains constant, the relative level of the two proteins fluctuates with respect to the osmolality of the growth medium. At low osmolality, the major porin present is OmpF, while at high osmolality, the expression of *ompF* is repressed and OmpC becomes the predominant porin [3]. The two porins differ from one another by the size of their pores and their flow rates, with OmpC having the smaller pore and slower flux [4]. Sensing the osmolality of its surroundings is one strategy by which *Escherichia coli* senses its environment and this ability is crucial for its survival. It is proposed that osmosensing enables *E. coli* to determine whether or not it is in a host environment (high osmolality) or a dilute environment (low osmolality).

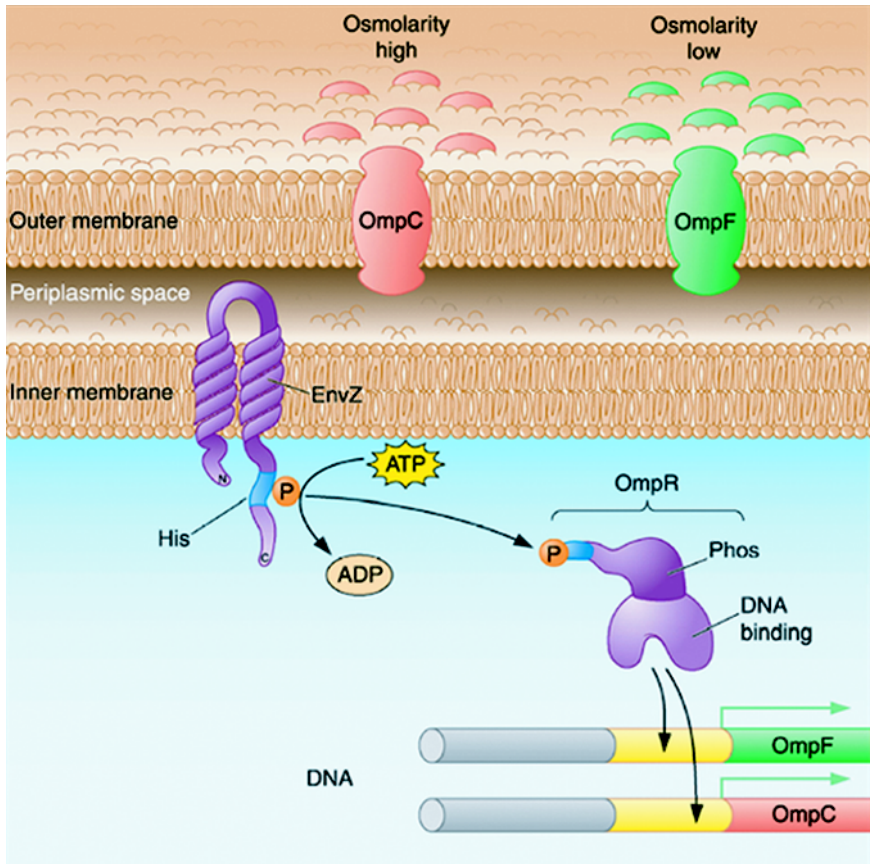


Figure 1.1 Regulation of the porin genes by EnvZ/OmpR. When the osmolality of the growth medium is low, OmpF is the predominant porin in the outer membrane. When the osmolality increases, *ompF* is repressed and OmpC becomes the major porin in the outer membrane. Regulation is mediated by the two-component regulatory system that consists of the two proteins EnvZ and OmpR. EnvZ is a sensor kinase, located in the inner membrane. ATP phosphorylates EnvZ on a conserved histidine residue and it transfers the phosphoryl group to OmpR. OmpR is a two-domain response regulator. Phosphorylation in the N-terminal receiver domain at a conserved aspartic acid residue alters the conformation of the C-terminal DNA binding domain. Phospho-OmpR (OmpR-P) binds to the regulatory regions of the porin genes *ompF* and *ompC* and alters their expression.

1.2 The Structure of EnvZ

EnvZ is a 450-amino-acid protein, located in the inner membrane (see Figure 1.2). EnvZ is comprised of two transmembrane domains flanking a 117-amino-acid periplasmic region at the N-terminus and a kinase/phosphatase catalytic domain at the C-terminus (EnvZc). EnvZc can be further separated into two functionally

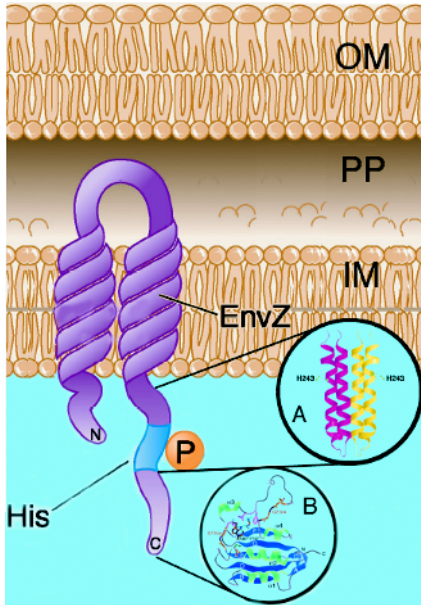


Figure 1.2 EnvZ topology. EnvZ is located in the inner membrane, with both N- and C-termini in the cytoplasm. It has two transmembrane domains and a periplasmic loop. Whether or not there is a role for the periplasmic domain in osmoregulation has not been clearly established. Structures of two domains of the cytoplasmic portion of EnvZ have been determined by nuclear magnetic resonance spectroscopy (see insets). Domain A consists of a four-helix bundle and is a dimer in solution. The histidine that is phosphorylated is located in domain A. Domain B contains the ATP binding site and is composed of a conserved α/β fold that is also found in other ATP binding proteins (see text for details). OM = outer membrane, PP = periplasm, IM = inner membrane.

distinct subdomains. Domain A (amino acids 223–289) is the phosphorylation and dimerization domain, and contains the site of autophosphorylation at His-243. Domain B (amino acids 290–450) contains the ATP-binding site as well as several regions conserved amongst all members of the histidine kinase family [5–7].

Domain A of EnvZc is located in the cytosol and is separated from the second transmembrane domain by a linker of approximately 43 amino acids. This HAMP linker (histidine kinase, adenyl cyclase, methyl-accepting chemotaxis protein and phosphatase) likely consists of two amphipathic helices, is structurally conserved among many sensor proteins and may play a role in signal transduction [8]. When expressed separately, domain A forms a stable homodimer in solution with an apparent molecular weight of 19 kDa [9]. The homodimer consists of a four-helical bundle with 2-fold symmetry. Each monomer of domain A contains two α -helices, $\alpha 1$ (residues 235–255) and $\alpha 2$ (residues 265–286), separated by a 9-amino-acid loop. The helices of the subunits pack in the dimer such that each $\alpha 1$ is surrounded by and aligns antiparallel to an $\alpha 2$ of each subunit. The core of the bundle is hydrophobic and is composed of a number of methyl-containing residues, consistent with other histidine kinases. His-243, which lies in $\alpha 1$, is oriented opposite its counterpart in $\alpha 1'$ and protrudes away from the helical bundle, where it is solvent-accessible for phosphorylation by ATP. The phosphotransfer domains of the histidine kinases CheA and ArcB also contain four-helix bundles. In each of these structures, the active histidine (His-48 in CheA and His-717 in ArcB) lies in the center of their respective helix and points outward [10, 11]. The structure

of domain A also resembles the cytoplasmic domains of the *E. coli* chemoreceptors Tar and Tsr, which are the sensory components of the chemotaxis system [12]. Like EnvZc, the functional chemoreceptor is a homodimer in which the cytoplasmic domains of each monomer form a four-helical bundle.

The structure of domain B consists of an α/β sandwich composed of a five-stranded β -sheet (strands B: residues 319–323; D: 356–362; E: 367–373; F: 420–423; and G: 431–436) on one fold and three α -helices (α 1: 301–311; α 2: 334–343; and α 3: 410–414) on the other [13]. This structure resembles the ATP-binding proteins Hsp90 and DNA gyrase B. Between these two folds is a hydrophobic core containing many structural hydrophobic residues conserved amongst other histidine kinases. Between α 3 and α 4, a long polypeptide loop extends, termed the “central loop”, which has no defined structure and may be mobile in solution. Binding of ATP occurs at α 3 and the central loop, and also involves contacts with β -strands F and G. This central loop is near the ATP-binding pocket formed by Asp-347, Asp-373, Ile-378 and Phe-387, and may interact with His-243 in domain A, possibly stabilizing phosphorylation. The triphosphate chain of the ATP molecule is exposed on the surface of the protein, to allow the transfer of the phosphate to His-243 in domain A. Several conserved glycines, forming the G1 and G2 boxes, previously shown to be essential for kinase activity, are also located in the catalytic core.

1.3

Biochemical Activities of EnvZ underlie Signaling

The EnvZ kinase has the following enzymatic activities:

- (1) $\text{EnvZ} + \text{ATP} \rightarrow \text{EnvZ-P} + \text{ADP}$ (autophosphorylation)
- (2) $\text{EnvZ-P} + \text{OmpR} \rightarrow \text{EnvZ} + \text{OmpR-P}$ (phosphotransfer)
- (3) $\text{EnvZ} + \text{OmpR-P} \rightarrow \text{EnvZ} + \text{OmpR} + \text{P}_i$ (phosphatase)

EnvZ could potentially modulate the level of OmpR-P by adjusting the activity of its autokinase (1), the phosphotransferase activity (2) or the OmpR-P phosphatase activity (3) separately, or in various combinations (see Figure 1.3).

It has been proposed that domain A contains the phosphatase activity of EnvZ [14]. The half-life of OmpR-P alone was reported to be approximately 90 min, whereas in the presence of domain A, the half-life of OmpR-P decreased to 8.7 min. This result led to the interpretation that the A domain was the source of the phosphatase activity. However, if domains A and B were intact (i.e. EnvZc), the half-life of OmpR-P further decreased to 2.5 min. It is evident that stimulation of OmpR-P turnover by EnvZc is most efficient in the presence of the intact cytoplasmic domain. Either both A and B domains contribute to OmpR-P dephosphorylation or the A domain must be in a preferred conformation that requires tethering to the B domain in order for the A domain to fully function. An important remaining question is how the A and B domains of EnvZc are organized with respect to one another in the intact protein.

1.4

What is the EnvZ Activity Regulated by the Stimulus?

In a recent attempt to elucidate the stimulus to which EnvZ responds, the kinase was overproduced, purified and reconstituted into proteoliposomes [15]. The EnvZ autokinase activity was stimulated by addition of potassium (activity 1), but neither phosphotransfer to OmpR (activity 2) nor the phosphatase activity of EnvZ (activity 3) were affected by the presence of potassium. However, the activities measured were extremely low, making interpretation of the experiments difficult. An osmotic upshift imposed by various sugars, glycine betaine, proline or Tris–MES was without effect. Since potassium accumulation is an early response to osmotic upshift by *E. coli*, it may be that the autokinase of EnvZ is sensitive to this step, arguing that the kinase activity (1) is the osmosensitive reaction that is regulated. A previous study also reported that potassium stimulated the level of OmpR-P, but the autokinase and phosphotransferase activities of the kinase were not separated [16]. Interestingly, the phosphorylation of an OmpR mutant (OmpR3, phenotype F^-C^+) was constitutively high at low KCl concentrations and was not stimulated by further addition of KCl [16]. This result implies that the OmpR3 mutant has altered interactions with EnvZ that lead to high levels of OmpR-P at low potassium concentrations, or that the phosphotransfer activity is the step altered by high potassium (activity 2), in contrast to the results of the proteoliposome study [15].

An earlier study by Jin and Inouye [17] proposed that at high osmolality, OmpR-P levels increase as a result of a decrease in the phosphatase of EnvZ (activity 3). This hypothesis is based on experiments with a chimeric kinase Taz, which contains the periplasmic domain of the aspartate chemoreceptor Tar fused to the cytoplasmic domain of EnvZ [18]. This construct activates *ompC* in response to aspartate. However, this construct has several serious limitations, which cast doubt on whether conclusions based on this construct are physiologically meaningful. For example, Taz requires 1–5 mM aspartate to activate *ompC* compared to Tar, which binds aspartate with a K_d of 1.2 μ M [19] and the addition of maltose, which also binds to Tar, did not enhance *ompC* expression. Furthermore, aspartate did not affect *ompF* transcription. In any case, the results from several Tar and Trg chimeras support a view that a common transmembrane signal transduction mechanism exists [20, 21].

Activation of EnvZ, by an as yet undetermined signal, leads to phosphorylation at His-243 from ATP and subsequent phosphorylation of OmpR at Asp-55. Phosphorylation of OmpR increases its affinity by at least 10-fold for the regulatory regions upstream of the *ompF* and *ompC* genes [22]. More recently, it was shown that the presence of DNA stimulates OmpR phosphorylation [23], i.e. the communication between OmpR domains is bidirectional. These experiments led to the proposal that OmpR might be activated while bound to its target DNA. This series of activation events would require that a complex exists between the membrane-embedded sensor kinase EnvZ with OmpR while complexed to the regulatory regions of *ompF* and *ompC* DNA. Genetic evidence for such a kinase/response regulator/DNA com-

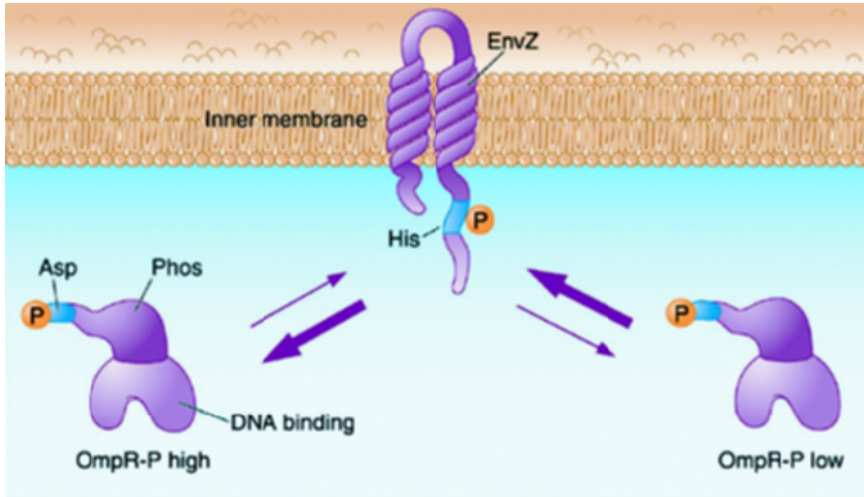


Figure 1.3 Biochemical activities of EnvZ control the concentration of OmpR-P. At low osmolality, the OmpR-P concentration is low, either because the EnvZ kinase activity is low or the phosphatase activity is high (right arrows). OmpR-P levels increase at high osmolality – this is either due to stimulation in the EnvZ kinase or a reduction in the EnvZ phosphatase activity (left arrows).

plex has been reported by Silhavy *et al.* in the homologous Cpx system that senses envelope stress (P. DiGiuseppi and T. J. Silhavy, personal communication). A four-state model can be described (Figure 1.4) in which OmpR exists as an equilibrium mixture between the unphosphorylated form (A), OmpR-P (B), the unphosphorylated, DNA-bound form (C) and the phosphorylated form bound to DNA (D). The reaction step that is most affected by the presence of DNA depends upon the phosphodonor employed. When phosphorylating with the small molecule phosphodonor, acetyl-phosphate, DNA binding dramatically stimulates the rate of phosphorylation with little effect on the dephosphorylation rate of OmpR-P. Estimates of initial rates indicate that phosphorylation by acetyl-phosphate is at least 25-fold faster in the presence of DNA than in its absence (i.e. C to D is much faster than A to B, Figure 1.4 [23]). Furthermore, DNA binding slows dephosphorylation about 2-fold (D to C is slightly slower than B to A, Figure 1.4 [23]). In contrast, when phosphorylating with the phosphokinase (EnvZ-P), the step most affected by DNA binding is the rate of EnvZ-stimulated OmpR-P dephosphorylation (i.e. D to C is much slower than B to A, Figure 1.4 [24]). In either case, the overall effect of DNA is to increase the net rate of OmpR-P formation on the order of 50-fold. Based on their findings, Qin *et al.* proposed that when OmpR-P binds to DNA, it is effectively made inaccessible to EnvZ and thus DNA binding inhibits EnvZ stimulation of OmpR-P breakdown [24]. If this proposal were true, it is difficult to imagine how the phosphatase activity (activity 3) could be the important physiologically regulated step [17], since OmpR-P bound to DNA would

then be inaccessible to EnvZ. In order to address this question, OmpR was labeled with a fluorescent probe and equilibrium binding was measured using fluorescence anisotropy. The K_d for EnvZ binding to OmpR was 425 nM and the presence of *ompF* or *ompC* DNA did not affect the interaction [25]. However, when OmpR was phosphorylated, the affinity of interaction with EnvZ was so low (at least 10-fold lower) that it was not measurable. These results are in conflict with the proposed role of DNA in the OmpR/OmpR-P equilibrium mediated by EnvZ, in which DNA prevents the interaction of EnvZ with OmpR [24]. However, the results are consistent with previous measurements in the chemotaxis system in which phosphorylation of the OmpR-homolog CheY reduced its affinity for the kinase CheA [26]. The lower affinity for CheA of CheY-P also favors binding to the switch proteins of the flagellar motor. In keeping with this analogy, if OmpR were phosphorylated by EnvZ-P while bound to DNA, the reduced affinity of OmpR-P for EnvZ would favor the release of EnvZ, enabling OmpR to interact with RNA polymerase and activate transcription. With a cellular concentration of OmpR of 3.5 μM and an EnvZ concentration of 180 nM [27], an apparent $K_d > 5 \mu\text{M}$ for EnvZ binding to OmpR-P indicates that these two partners would only rarely be associated. Our favored interpretation is that OmpR-P dephosphorylation is sufficiently rapid *in vivo* to promote turnover and that the more likely osmosensitive reaction of EnvZ is the autokinase activity (activity 1). This view is consistent with the observation of Jung *et al.* that potassium stimulates autophosphorylation [15].

The issue of bifunctionality of the sensor kinase (i. e. phosphorylation and dephosphorylation functions) was addressed and a system with these features was

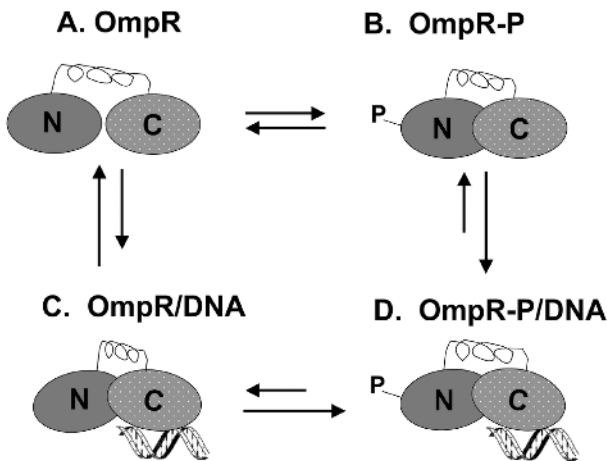


Figure 1.4 Model for OmpR phosphorylation and DNA binding. OmpR is depicted as a two-domain protein with the N-terminus joined to the C-terminus by a flexible linker region. The protein is shown alone (A), phosphorylated (B), bound to DNA (C), and phosphorylated and bound to DNA (D). The arrows depict transitions between these states. Note that the conformation of the linker changes when OmpR is phosphorylated (B), bound to DNA (C) or both (D).

compared to one in which spontaneous dephosphorylation of the response regulator was the sole means of turnover [28]. The concentrations of EnvZ and OmpR were varied independently, and the effect on *ompF* and *ompC* transcription was determined at low and high osmolality. The authors tested whether or not the system was robust with respect to the components EnvZ and OmpR. Changes in EnvZ levels had little effect on *ompF* and *ompC* transcription, whereas increasing the concentration of OmpR dramatically increased *ompC* transcription. This effect was not observed until OmpR levels had risen at least 10-fold and was especially pronounced at high osmolality. Presumably, the over-expression of OmpR enables unphosphorylated protein to occupy the low-affinity sites, and stimulate *ompC* transcription and repress *ompF*.

1.5

How is the Signal Propagated?

Reports differ as to the domains of EnvZ that are essential for signal transduction. An early study engineered large deletions (24–40 amino acids) in the periplasmic domain and examined porin phenotypes [16]. The resulting EnvZ constructs produced constitutive expression of a high osmotic phenotype, (i.e. OmpF⁻, OmpC⁻), regardless of the osmolality of the growth medium. Although the construction of the mutants resulted in the addition of a few extra amino acids and the mutants were over-expressed, the study suggested that in a low osmotic environment, there was an interaction with the periplasmic domain of EnvZ that was removed or not present at high osmolality. The authors further claimed that the mutants were defective in the EnvZ-stimulated dephosphorylation of OmpR-P (activity 3), although the autokinase activity and phosphotransferase activities were not examined in detail [16]. An interesting observation was that the EnvZ mutants produced pleiotropic phenotypes that were PhoA⁻, LamB⁻ and Mal⁻. The explanation for the effects on multiple pathways outside of the normal porin repertoire was that the accumulation of OmpR-P as a result of the altered dephosphorylation by EnvZ enabled OmpR to act on genes that it normally does not regulate. This hypothesis has not been adequately tested to determine whether or not OmpR-P levels are actually higher in these EnvZ backgrounds or whether OmpR-P directly affects these additional genes, but it remains an intriguing hypothesis.

A more recent study compared EnvZ molecules from two different organisms [29] and noted the absence of a periplasmic domain in the EnvZ from *Xenorhabdus nematophilus*. Interestingly, *envZ* from *X. nematophilus* was able to complement an *envZ*-null strain of *E. coli* and restore osmoregulation of the porin genes [29]. Replacement of the periplasmic domain of EnvZ with the non-homologous domain of PhoR (a sensor kinase not involved in porin gene expression) produced a chimera capable of osmoregulation of *ompF* and *ompC* [30]. However, a 91-amino-acid periplasmic deletion showed a similar phenotype (F⁻C⁻) reported in the Tokoshita study [16]. It would be of interest to compare a randomized amino acid sequence in the periplasmic domain, rather than the replacement with the sensing domain from