Rebecca Fleischer‡, Ralf Heermann‡, Kirsten Jung§, and Sabine Hunke†

From the ‡Institut für Biologie, Abteilung Physiologie der Mikroorganismen, Humboldt Universität zu Berlin, D-10115 Berlin, Germany and the §Department Biologie I, Bereich Mikrobiologie, Ludwig-Maximilians-Universität, Maria-Ward-Strasse 1a, D-80638 München, Germany

**Purification, Reconstitution, and Characterization of the CpxRAP Envelope Stress System of Escherichia coli**

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In *Escherichia coli* the Cpx sensor regulator system senses different kinds of envelope stress and responds by triggering the expression of periplasmic folding factors and proteases. It consists of the membrane-anchored sensor kinase CpxA, the response regulator CpxR, and the periplasmic protein CpxP. The Cpx pathway is induced in vivo by a variety of signals including pH variation, osmotic stress, and misfolded envelope proteins and is inhibited by overproduced CpxP. Because it is not clear how the Cpx pathway is able to recognize and respond to so many different signals we overproduced, solubilized, purified, and incorporated the complete membrane-integral CpxA protein into proteoliposomes to analyze its biochemical properties in more detail. Autokinase and phosphotransfer activities of the reconstituted CpxA-His6 protein were stimulated by KCl. NaCl also activated the stimulation but to a lesser extent. Other osmotic active solutes as glycine betaine, sucrose, and proline had no effect. The system was further characterized by testing for susceptibility to sensor kinase inhibitors. Among these, Closantel inhibited the activities of solubilized but not of the reconstituted CpxA-His6 protein. We further analyzed the effect of CpxP on CpxA activities. Purified tagless CpxP protein reduced the phosphorylation status of CpxA to 50% but had no effect on CpxA phosphotransfer or phosphatase activities. As the in vitro system excludes the involvement of other factors our finding is the first biochemical evidence for direct protein-protein interaction between the sensor kinase CpxA and the periplasmic protein CpxP resulting in a down-regulation of the autokinase activity of CpxA.

The bacterial cell wall is involved in a multitude of diverse structural, physiological, and adaptive processes including transport, elaboration of virulence factors, and cell division. These processes require specific sets of proteins whose correct folding and assembly is controlled by periplasmic folding catalysts and proteases. In *Escherichia coli* and related species, expression of some of the corresponding genes is regulated by the Cpx sensor regulator system (reviewed in Ref. 1).

The Cpx pathway consists of the sensor kinase CpxA, the response regulator CpxR, and the periplasmic CpxP protein. The cpxA gene was originally reported as a gene regulating F donor activity in bacterial conjugation (2). It encodes the 52-kDa histidine kinase CpxA, which is an integral membrane protein of the cytoplasmic membrane that contains both periplasmic and cytoplasmic domains (3). The 26-kDa response regulator CpxR is predicted to encode an OmpR-like cytolsolic transcriptional activator (4).

Signals activating the Cpx pathway include elevated pH (5, 6), altered membrane composition (7), overproduction of outer membrane lipoproteins such as NlpE (8), accumulation of misfolded variants of the maltose-binding protein (MBP) (9), accumulation of pilus subunits (10), indole (11), and increasing osmolarity (12). The molecular mechanism of signal transduction used by the Cpx sensor regulator system is not clear. CpxA functions as an autokinase, a CpxR kinase, and a CpxR-P phosphatase in vivo (13). CpxA deletion mutants are uninducible, demonstrating that CpxA is necessary for signaling (14). Indeed, cpxA gain of function mutations located in the central region of the periplasmic domain of CpxA are insensitive to normally activating signals and probably define a sensory domain (15).

Activation of the Cpx pathway results in increased production of proteins involved in protein folding and degradation in the periplasm, such as the heat shock protease DegP, the peptideyl prolyl cis/trans isomerasers PpiA and PpiD, and the disulfide oxidoreductase DsbA (1). In addition, it was shown that CpxR–P represses motility and chemotaxis genes (16).

Interestingly, overproduction of the small (19-kDa) periplasmic CpxP protein leads to decreased expression of Cpx-regulated genes and prevents Cpx activation by inducing signals (1). This effect depends on an intact CpxA sensory domain (15). Because an MBP-CpxP fusion was able to inhibit Cpx signal transduction during spheroplast formation, a strong Cpx-inducing signal, it was suggested that the interaction between CpxA and CpxP might be direct (17). On the basis of the observation that the cpxA mutants were constitutively activated (15),

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‡ To whom correspondence should be addressed: Physiologie der Mikroorganismen, Chausseestr. 117, D-10115 Berlin, Germany. Tel.: 0049-0-30-2093-8122; Fax: 0049-0-30-2093-8126; E-mail: sabine.hunke@rz.hu-berlin.de.

§ The abbreviations used are: MBP, maltose-binding protein; Closantel, N-[3-chloro-4-[(R,S)-(4-chlorophenyl)cyanomethyl]-2-methylphenyl]-2-hydroxy-3,5-diodobenzamide; Ethodin, 6,9-diamino-2-oxyethyl acridine lactate; LDAO, N,N-dimethyldeoxycyclamine N-oxide; MOPS, 3-(N-morpholino)-propanesulfonic acid; NTA, nitrilotriacetic acid; TCS, 3,3′,4′-5-tetrachlorosalicylanilide; vanadate, Na-orthovanadate-decarboxylate.

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it was proposed that the presumed CpxA sensory domain normally functions to maintain the kinase in a down-regulated state mediated by direct interaction with CpxP. When CpxP is titrated out by misfolded envelope proteins, the Cpx response is activated (1, 18). The finding that the Cpx response can be further activated in a cpxP deletion strain indicates that CpxP is not required for signal transduction (19), suggesting that CpxP might be responsible for fine-tuning the response. A recent study showed that single amino acid substitutions in a predicted α-helix in the N-terminal domain of the CpxP protein affect its inhibitory function indicating that the N-terminal domain of CpxP is critical for interaction with the sensor CpxA and might be the site of inhibitory activity (20).

Most of the analysis on Cpx pathway signal transduction was done in vivo. Here we describe an efficient method to solubilize, purify, and reconstitute the complete Cpx sensor regulator system into proteoliposomes to study the biochemical properties of the signaling cascade in more detail. This in vitro system was further characterized by testing the effect of known inhibitors of sensor regulator systems of Gram-positive bacteria (21). Finally, we analyzed the inhibitory effect of the periplasmic CpxP protein on the system. Our results provide first biochemical evidence for a direct protein–protein interaction between CpxA and CpxP resulting in inhibition of the CpxA autokinase activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—[γ-32P]ATP, peroxidase-conjugated anti-mouse and anti-rabbit IgG were purchased from GE Healthcare. Ethodin and Ofloxacin were obtained from Sigma. Vanadate was from Strem Chemicals. Clostridial and TCS were gifts from Thomas Schmülling (Free University, Berlin). Ni2+-NTA resin and His₆ antibody were from Qiagen and ProtoNi–Ni from Macherey & Nagel. Detergents were obtained from Glycon Biotechnologies. Purified E. coli lipids were purchased from Avanti Polar lipids. Bio-Beads SM-2 were from Bio-Rad. Trypsin and methylamine oxide (LDAO); 1% decylmaltoside (DM); 1% dodecylmaltoside (DDM), and 1% octyl-glucoside (OG). After washing with buffer R (50 mM Tris/HCl, pH 7.5, 0.15 M NaCl, 20% glycerol (v/v), 0.1 mM phenylmethylsulfonyl fluoride). Cells were harvested and resuspended in buffer Z (50 mM Tris/HCl pH 7.5, 0.15 M NaCl, 20% glycerol (v/v), 0.1 mM phenylmethylsulfonyl fluoride). Cells were fractionated by sonication on ice and ultracentrifugation into membrane fraction (pellet) and cytosolic fraction (supernatant). The membrane fraction resuspended in buffer Z, and the cytosolic fraction were frozen in liquid nitrogen and stored at −80 °C until use.

**Purification of His₆-CpxR—His₆-CpxR fusion protein was purified by means of Ni-affinity chromatography in batch. Binding of the protein (~500 mg of cytosolic proteins/2.5 ml of Ni-NTA resin) was done in buffer R (50 mM Tris/HCl, pH 7.5, 0.15 M NaCl, 10% glycerol (v/v), 0.1 mM phenylmethylsulfonyl fluoride). Bound His₆-CpxR was eluted with an imidazole concentration of 150 mM in buffer R. His₆-CpxR-containing fractions were mixed and further purified with a Protoni Ni 2000 prepacked column kit according to the manufacturer’s instructions (Macherey & Nagel). Purified His₆-CpxR protein was passed through a PD10 column (GE Healthcare) to remove imidazole and stored at −80 °C until use.

**Purification of CpxP—His₆-CpxP fusion protein was purified by means of Ni-affinity chromatography in batch. Binding of the protein (600 mg of cytosolic protein/3 ml Ni-NTA) was done in the presence of 5 mM imidazole in buffer P (50 mM Tris/HCl, pH 7.5, 0.5 M NaCl, 10% glycerol (v/v), protease inhibitor mixture (Roche Applied Science)). After washing with buffer P containing 20 mM imidazole His₆-CpxP was eluted by increasing the imidazole concentration to 150 mM. His₆-CpxP-containing fractions were passed through a PD10 column to remove imidazole and further purified by an SP-Sephadex column (Amersham Biosciences). Bound His₆-CpxP was washed extensively with buffer P2 (50 mM MOPS/K+/pH 6.6) containing increasing concentrations of NaCl (50 mM–200 mM), eluted with buffer P2 containing 0.5 M NaCl and buffered into buffer P3 (50 mM Tris/HCl, pH 7.5, 0.15 M NaCl, 10% glycerol (v/v)) by passing through a PD10 column. The His₆-fusion from the N terminus of CpxP was cleaved by thrombin using the THROMBOMBIN CleanCleaveKit essentially as described (Sigma).

**Purification of CpxA-His₆—For detergent selection, the following detergents were tested: 1% Triton X-100, 1% lauryldimethylamine oxide (LDAO); 1% decyl maltoside (DM); 1% dodecyl maltoside (DDM), and 1% octyl-glucoside (OG). After
incubation on ice for 60 min, mixtures were centrifuged, and aliquots of the supernatant containing solubilized proteins were analyzed by immunoblotting. The yield of CpxA-His₆ solubilization (%) is used as measure for the distribution of the amounts of CpxA-His₆ between the soluble and the membrane fraction after treatment of membrane vesicles with detergent.

1 ml of 10× DM was added to 9 ml of membrane vesicles (≈10 mg/ml) prepared from BL21(DE3) <pLys/pL3cpxA>. Samples were incubated on ice for 1 h and centrifuged at 200,000 × g at 4 °C for 30 min. The supernatant fraction containing solubilized CpxA-His was purified by affinity chromatography. Ni²⁺-NTA resin equilibrated with buffer A (50 mM Tris/HCl, pH 7.5, 0.15 M NaCl, 10% glycerol (v/v), 0.1% DM (w/v), 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride). Binding of CpxA-His₆ was carried out by incubating the solubilized fraction and Ni²⁺-NTA resin at 4 °C for 1 h. The protein-resin complex was washed stepwise with buffer A containing 10 and 50 mM imidazole. CpxA-His₆ was eluted by increasing the imidazole concentration to 150 mM. CpxA-His₆-containing fractions were passed through a PD10 column to remove imidazole and stored in 50 mM Tris/HCl, pH 7.5, 10% glycerol (v/v), 0.1% DM (w/v) at −80 °C.

Preparation of Proteoliposomes—Purified CpxA-His₆ was incorporated into liposomes essentially as described (23). Briefly, E. coli phospholipids (Avanti) were dried under a stream of nitrogen, and slowly redissolved in 50 mM Tris/HCl, pH 7.5, 10% glycerol (v/v), 0.47% Triton X-100 (v/v) over a period of 2 h. To this mixture purified CpxA-His₆ was added (ratio of lipid to protein, 100:1 w/w). The mixture was stirred at room temperature for 10 min. Bio-Beads were added in a bead/detergent ratio of 10:1 (w/w), and the mixture was gently stirred at 4 °C overnight. After 16 h, fresh Bio-Beads were added additionally, and the mixture was stirred for another 2 h. The proteoliposome solution was pipetted off, and proteoliposomes were collected by centrifugation for 30 min at 200,000 × g. The pellet was resuspended in 50 mM Tris/HCl, pH 7.5, 10% glycerol (v/v), and 2 mM dithiothreitol. The efficiency of CpxA incorporation into liposomes was calculated as the ratio between the amount of protein in proteoliposomes and in the supernatant after ultracentrifugation. Proteoliposomes were either used instantly or stored in liquid nitrogen. CpxP-containing proteoliposomes were prepared as described above, but before Bio-Bead treatment, purified CpxP protein was added at a 5–10-fold molar excess to the CpxA protein-lipid mixture.

Proteolysis with Trypsin—Proteoliposomes were incubated with trypsin (15 ng/μl) at a protease/protein ratio of 1:100 at room temperature. At different times, the reaction was stopped by trypsin inhibitor (2 μg/μl), and the proteins were subjected to Western blot analysis. MalK protein of Salmonella typhimurium was incorporated into proteoliposomes to control their impermeability for trypsin.

Phosphorylation and Dephosphorylation Assays—To test autophosphorylation, solubilized CpxA, CpxA in membrane vesicles or CpxA in proteoliposomes was incubated with 40 μM [γ-³²P]ATP (1.2 Ci/mmol) in phosphorylation buffer (50 mM Tris/HCl, pH 7.5, 10% glycerol (v/v), 2 mM dithiothreitol, 50 mM KCl, 5 mM MgCl₂) at room temperature. When indicated various solutes were additionally added. At different time points, aliquots were removed and mixed with 5× SDS sample buffer. To analyze phosphotransfer, purified His₆-CpxR was added to this mixture after 10 min, additional samples were taken, and the reaction was stopped by the addition of 5× SDS sample buffer.

To test dephosphorylation, purified His₆-CpxR was phosphorylated as described above with CpxA-His₆-containing proteoliposomes. After incubation for 25 min, the phosphorylation mixture was centrifuged for 30 min (13,000 × g, 4 °C), and ATP was removed by the addition of 20 mM MgCl₂, 2 mM glucose, and 5.4 units of hexokinase. Dephosphorylation of His₆-CpxR → P was initiated by the addition of fresh CpxA-His₆ in proteoliposomes. At the indicated times, aliquots were taken, and the reaction was stopped as described above.

To test the whole signaling cascade in vitro, CpxA-His₆-containing proteoliposomes (0.5 μM) and purified CpxR (2 or 4 μM) were incubated in phosphorylation buffer at room temperature. Phosphorylation was initiated by the addition of 100 μM [γ-³²P]ATP (0.48 Ci/mmol). Samples were taken at times indicated and mixed with 5× SDS sample buffer. To analyze CpxA-His₆ phosphatase activity 1 mM ADP was added after 30 min, additional aliquots were removed at times indicated and stopped as described above.

All samples were immediately subjected to SDS-PAGE (24). Gels were dried and phosphorylated proteins were detected by a PhorShorImager system (Molecular Imager Fx, and Software Quantity One, Bio-Rad) using [γ-³²P]ATP as a standard.

Inhibitor Studies—Inhibitors (except vanadate) were dissolved in Me₃SO. Vanadate solutions were prepared in water at pH 10 as described (25). The phosphorylation assays were performed as described above in the presence of the indicated inhibitors. To reflect the presence of Me₃SO as a solvent, it was also included in control reactions in an equivalent final concentration (10% (v/v)).

Immunological Analysis—For Western blot analysis protein samples were subjected to SDS-PAGE. Proteins were electroblotted, and immunoblots were probed with antiserum to penta-His fusion (mouse) to visualize full-length CpxA-His₆ or with antiserum to MalK (rabbit). Immunodetection was performed by using the ECL-kit (GE Healthcare) with a peroxidase-conjugated anti-mouse or anti-rabbit IgG.

Analytical Methods—Protein content was determined using the BCA protein assay from Pierce according to the manufacturer’s instructions.

To determine relative protein content of proteoliposomes, proteins were separated by SDS-PAGE using 12.5% acrylamide gels and stained with Coomassie Blue. Different amounts of the corresponding purified proteins were used as a standard on the same gel. Gels were scanned with the Molecular Imager Fx (Bio-Rad), and protein bands were quantified with Quantity One (Bio-Rad).

RESULTS

Purification of Cpx Components—To allow easy purification of CpxA a hexa-His tag was attached at its C terminus. The activity of the CpxA-His₆ protein was comparable to the native protein: both autokinase and kinase activities in membrane
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fractions were only slightly lower than in authentic CpxA (data not shown). Thus, CpxA-His₆ was used for further in vitro analysis.

As a prerequisite for CpxA-His₆ purification by affinity chromatography the protein had to be efficiently solubilized. Because the yield of solubilized CpxA-His₆ was found to be 80% when decylmaltoside was used as detergent (69% n-dodecyl-maltoside, 38% Triton X-100, 25% octyl-glucoside), this detergent was used for all purification steps. Interestingly, the different detergents did not alter autokinase activity of the soluble CpxA-His₆ significantly (data not shown). The highest degree of purified CpxA-His₆ was achieved when the protein was bound to the Ni²⁺-NT-agarose in the presence of 50 mM imidazole and 0.15 M NaCl. In a typical experiment about 1 mg of CpxA-His₆ was obtained from a 1-liter culture. As demonstrated by Coomassie Blue staining (Fig. 1A), the purity of CpxA-His₆ was nearly 90%.

For CpxR purification a hexa-His tag was attached at its N terminus. The activity of the protein was comparable to native CpxR as it was shown previously (11). The His₆-CpxR protein was purified by the use of two affinity chromatography steps. This procedure yielded 95% pure His₆-CpxR protein (Fig. 1B, compare lanes 2 and 3).

For the purification of CpxP we constructed an expression plasmid in which the 5′-sequence of cpxP corresponding to the signal peptide was replaced by six His codons. C-terminal fusions with the full-length protein in different vector systems could not be stably expressed in the periplasm even in a degP strain (data not shown). Therefore, the protein was overproduced and purified from the cytosol. After purification by Nickel chromatography the N-terminal histidine residues were cut off using a thrombin cleavage site resulting in a tagless CpxP protein (Fig. 1C). Purified CpxP preparations contained an additional protein with an approximate size of 70 kDa. MS spectrometry identified this protein as the chaperone DnaK (data not shown). Because DnaK was not found in proteoliposomes after reconstitution (Fig. 7A, lane 3) the preparation was not purified further.

**Incorporation of CpxA-His₆ into Proteoliposomes**—Purified CpxA-His₆ protein was incorporated into *E. coli* phospholipids using the detergent-mediated method as described (26). The efficiency of CpxA-His₆ incorporation into liposomes was ~40% (data not shown).

To analyze the orientation of CpxA-His₆ in proteoliposomes the susceptibility of its C-terminal domain to trypsin was tested. In intact cells, the C terminus is on the cytoplasmic side of the membrane. Because 82% of the CpxA-His₆ in the proteoliposomes lost a C-terminal peptide as a result of trypsin treatment (as shown by Western blotting using an anti-His antibody and calculated from the average of two independent experiments), the majority of the CpxA-His₆ protein in proteoliposomes is in the inside-out orientation (supplemental Fig. S1). As a control for the integrity of the proteoliposomes, soluble MalK ATPase was incorporated along with CpxA-His₆. No proteolytic cleavage of MalK was detected after 1.5 min, while the freely accessible MalK protein was fully degraded by trypsin after 1 min (27).

**Characterization of CpxA-His₆ Activities in Proteoliposomes**—CpxA-His₆ proteoliposomes were autophosphorylated in the presence of [γ-³²P]ATP at room temperature. The autophosphorylation increased almost linearly for 10 min (Fig. 2A). Autokinase activity of CpxA-His₆ proteoliposomes was 1.5 times higher compared with that of CpxA-His₆ in detergent (data not shown).

Next, we tested transfer of the phosphoryl group from CpxA-His₆ proteoliposomes to purified His₆-CpxR. In this experiment CpxA-His₆ in proteoliposomes was phosphorylated for
10 min, and subsequently His\textsubscript{6}-CpxR was added. The mixture was incubated, and samples were taken at the times indicated. Transfer of the phosphoryl group to His\textsubscript{6}-CpxR was observed within 0.1 min (Fig. 2B).

Next we studied the phosphatase activity of CpxA-His\textsubscript{6} in proteoliposomes. First, we tested the intrinsic stability of phospho-CpxR. Purified His\textsubscript{6}-CpxR protein was phosphorylated by CpxA-His\textsubscript{6}-containing proteoliposomes as described above. Then, His\textsubscript{6}-CpxR\textsubscript{32P} was separated from the reaction mixture and incubated in the presence and absence of ADP. Samples were taken at various time points. Phosphorylated His\textsubscript{6}-CpxR was stable for a period of 60 min independently of the presence of ADP (Fig. 2C).

To test now the dephosphorylation of His\textsubscript{6}-CpxR\textsubscript{32P} by CpxA, CpxA-His\textsubscript{6}-containing proteoliposomes were added, and dephosphorylation was monitored over a period of 60 min. The half-life of His\textsubscript{6}-CpxR\textsubscript{32P} was 55 min. In the presence of ADP the half-life was shortened to 20 min (Fig. 2C). In samples lacking ADP the level of His\textsubscript{6}-CpxR\textsubscript{32P} increased over the first 5 min. This effect was constantly observed but, as it lies in the range of the standard deviation, it was not studied further. Apparently, ADP seems to trigger the phosphatase activity of CpxA. A similar effect was reported for EnvZ and its cognate response regulator OmpR (23). Taken together, CpxA-His\textsubscript{6}-proteoliposomes and His\textsubscript{6}-CpxR catalyzed all known enzymatic activities.

Influence of Various Solutes and pH Variations on the Autophosphorylation Activity of CpxA-His\textsubscript{6} in Proteoliposomes—In vivo data suggested that the Cpx system might be involved in sensing environmental osmolarity (12). To prove this hypothesis we tested the autokinase activity of CpxA-His\textsubscript{6} in proteoliposomes in the presence of various solutes. It was found that this activity of CpxA-His\textsubscript{6} was significantly stimulated in the presence of KCl and RbCl (Fig. 3A). NH\textsubscript{4}Cl also had a stimulatory effect but to a lower extent. In contrast, other osmotic active solutes as NaCl, sucrose, and trehalose did not influence CpxA autokinase activity. Furthermore, the compounds glycine betaine, proline, and glutamate that are accumulated in cells exposed to an osmotic upshift had no stimulatory effect.

We also analyzed the concentration-dependent effect of KCl on CpxA-His\textsubscript{6} autophosphorylation activity using a concentration range between 0.5 and 500 mM KCl. The stimulatory effect of KCl was found to be linear until a concentration of 50 mM, at higher concentrations the stimulation followed a saturation curve (data not shown).

In vivo data showed that the Cpx system is activated by mild alkaline pH (6). Therefore, we analyzed the effect of pH variations on the autophosphorylation activity of CpxA-His\textsubscript{6} proteoliposomes. We observed a stimulation of CpxA autokinase activity in proteoliposomes. CpxR was phosphorylated by CpxA-His\textsubscript{6}-proteoliposomes with [γ\textsuperscript{32P}]ATP (described under “Experimental Procedures”). His\textsubscript{6}-CpxR-P was incubated in the presence or absence of ADP with MgCl\textsubscript{2}, and dephosphorylation was initiated by adding CpxA-containing proteoliposomes (0.5 μM). The reaction was started at time 0 with 2 μM [γ\textsuperscript{32P}]ATP. In all assays, samples were taken at the times indicated, separated by SDS-PAGE and analyzed on a PhosphorImager (upper part). The amounts of [\textsuperscript{32P}]P-phospho-CpxA and [\textsuperscript{32P}]P-phospho-CpxR were determined with a PhosphorImager (lower part) using [γ\textsuperscript{32P}]ATP as standard. Shown are averages ± S.E. from at least three different experiments.
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Inhibition of the Autokinase and Phosphotransfer Activities of CpxA-His<sub>6</sub> in Proteoliposomes by Histidine Kinase Inhibitors—We further characterized the molecular properties of CpxA in more detail by studying the effect of chemicals that are known to inhibit senor kinases. The potential inhibitors used in this study are the salicylanilides Closantel (28) and Tetrachlorosalicylanilid (TCS) (29), the small hydrophobic intercalator-like molecule Ethodin (21), the quinolone Ofloxacin (29), and the phosphate analogue vanadate.

Because all compounds except vanadate were dissolved in Me<sub>2</sub>SO, this solvent was used as an additional control. Interestingly, Me<sub>2</sub>SO alone caused a reduction of the autophosphorylation activity of reconstituted CpxA-His<sub>6</sub> by about 30% in comparison to the control without any additive (Fig. 4).

For CpxA-His<sub>6</sub> in proteoliposomes we observed that the inhibitory effect of Closantel did not differ significantly from the inhibitory effect of the solvent (Fig. 4). Thus, Closantel was without effect. In contrast, the Closantel analogue TCS strongly affected the autophosphorylation activity of CpxA-His<sub>6</sub> (Fig. 4). The IC<sub>50</sub> value was determined to be 150.8 ± 40.9 μM. For Ethodin we observed reduced autophosphorylation activity of CpxA-His<sub>6</sub> (Fig. 4) with a IC<sub>50</sub> value of 219.2 ± 42.4 μM. Ofloxacin slightly inhibited the autophosphorylation activity of CpxA-His<sub>6</sub> (Fig. 4). The proportion of phosphorylated CpxA protein was 87% of the Me<sub>2</sub>SO control reaction.

In addition to the established sensor kinase inhibitors we choose vanadate, a classical inhibitor of P-type ATPases and reconstituted ABC transporters. Vanadate is supposed to inhibit the formation of a phosphorylated intermediate in the case of P-type ATPases (31) and to block the release of ADP in the case of ABC transporters (32, 33). Vanadate caused a slight reduction of the autophosphorylation activity of CpxA-His<sub>6</sub> to 75% of the control without any additive (Fig. 4).

We also analyzed the effect of these potential inhibitors on the phosphotransfer activity of CpxA-His<sub>6</sub> in proteoliposomes. No remarkable differences were observed for most of the compounds in comparison to the effect on the autophosphorylation activity. However, CpxA phosphotransfer activity was slightly increased by vanadate to 116% of the control (data not shown).

Closantel Inhibits the Autophosphorylation Activity of the Detergent-solubilized CpxA-His<sub>6</sub> but Not of CpxA-His<sub>6</sub> in Proteoliposomes—TCS and Ethodin were found to be inhibitors for autophosphorylation and phosphotransfer activities of reconstituted CpxA-His<sub>6</sub>. Interestingly and to our surprise, Closantel did not inhibit these two activities when CpxA-His<sub>6</sub> was reconstituted in proteoliposomes. To study this phenomenon further, we compared the effect of Closantel on the autophosphorylation activity of detergent-solubilized with that of reconstituted CpxA-His<sub>6</sub>. In contrast to the effect on the reconstituted CpxA protein, the activity of solubilized protein was reduced to 6% of the control by 0.2 mM Closantel (Fig. 5). To exclude that the hydrophobic component Closantel was not able to inhibit reconstituted CpxA-His<sub>6</sub> because of its dilution in the lipid phase, we tested a higher inhibitor concentration. After addition of 1 mM Closantel, no
activity was observed for the solubilized CpxA-His$_6$ protein and CpxA-His$_6$ in proteoliposomes. Different concentrations of Closantel were tested on the autophosphorylation activity of CpxA-His$_6$ solubilized in detergent (soluble) or CpxA-His$_6$-proteoliposomes (PLS) as described under “Experimental Procedures”. After incubation with Me$_2$SO maximal values of 0.001 nmol of [$^{32}$P-CpxA]/min × mg soluble and [$^{32}$P]-CpxA incorporated into proteoliposomes were obtained. Shown are averages ± S.E. from three different experiments.

FIGURE 6. Inhibition of the autophosphorylation activity of solubilized EnvZ-His$_6$ and EnvZ-His$_6$ in proteoliposomes. The effect of two concentrations of Closantel and TCS was tested on the autophosphorylation activity of purified EnvZ solubilized in detergent (soluble) or incorporated into proteoliposomes (PLS) as described in Ref. 23. Shown is a representative of two independent experiments.

Effect of These Inhibitors on the Autokinase Activity of EnvZ-His$_6$—The inhibitor studies with CpxA-His$_6$ indicated clear differences between the detergent-solubilized and the protein reconstituted in proteoliposomes. To examine whether the difference in susceptibility of the two forms is a unique trait of CpxA or if it is a general property of sensor kinases we analyzed the effect of Closantel and of TCS on the EnvZ sensor kinase of E. coli. Purification and reconstitution of EnvZ was carried out as described before (23). As for the CpxA protein we observed inhibition by the solvent Me$_2$SO alone. The amount of phosphorylated EnvZ protein was reduced for the solubilized protein to 50% and for the reconstituted protein to 68% (Fig. 6). In support of our finding for CpxA only the solubilized EnvZ was inhibited by Closantel but not the reconstituted protein. In contrast, TCS was not able to inhibit the activity of EnvZ-His$_6$ under the tested conditions (Fig. 6).

Taken together, the results of our inhibitor studies indicate that the soluble and reconstituted forms of membrane anchored sensor kinases differ significantly in certain biochemical properties. This clearly underlines the importance of ana-
### A

|        | PLS | PLS + CpxP |
|--------|-----|-----------|
| + ADP  | 15  | 15        |
|        | 30  | 30        |
|        | 45  | 45        |
|        | 60  | 60        |

**Graph**: Level of CpxA−^32^P and CpxR−^32^P over time (min)

- CpxA
- CpxR
- CpxA + CpxP
- CpxR + CpxP

### B

|        | PLS | PLS + CpxP |
|--------|-----|-----------|
| + ADP  | 15  | 15        |
|        | 30  | 30        |
|        | 45  | 45        |
|        | 60  | 60        |

**Graph**: Level of CpxA−^32^P and CpxR−^32^P over time (min)

- CpxA
- CpxR
- CpxA + CpxP
- CpxR + CpxP

Cpx Pathway Reconstitution
lyzing the reconstituted and not the soluble version of a sensor kinase.

**Inhibition of CpxA-His<sub>6</sub> Autophosphorylation Activity by CpxP**—In *vivo* data showed that overproduction of the periplasmic CpxP protein leads to inhibition of the Cpx signaling cascade (17). Our defined *in vitro* system provides an experimental platform to test if this is because of direct protein-protein interaction between the CpxP protein and the CpxA sensor kinase because of the involvement of other factors can be excluded. In addition, our *in vitro* system allows us to analyze which catalytic function of the CpxA protein is affected by CpxP. CpxP might either inhibit the autophosphorylation activity or induce the phosphatase activity of CpxA (17).

To analyze the inhibitory effect of CpxP on CpxA-His<sub>6</sub>, we mixed purified CpxP protein with solubilized CpxA-His<sub>6</sub>. Although CpxP was provided in a 5-fold excess, no inhibition of autophosphorylation activity of the solubilized CpxA-His<sub>6</sub> protein by CpxP was observed (data not shown). To analyze now the effect of CpxP on reconstituted CpxA-His<sub>6</sub>, purified CpxP was incorporated into proteoliposomes to allow access of this protein to the periplasmic loop of CpxA. Although we varied the ratio between CpxP and CpxA-His<sub>6</sub> during the reconstitution process up to 10:1, the CpxP:CpxA ratio found in the proteoliposomes was almost 1:1 (Fig. 7A). Under these conditions CpxP inhibited the autophosphorylation activity of CpxA-His<sub>6</sub> by 51% (Fig. 7B). Interestingly, the His<sub>6</sub>-CpxP variant was unable to inhibit CpxA autophosphorylation activity (Fig. 7B).

**Inhibition of the *in Vitro* CpxRA Signal Transduction Cascade by CpxP**—Next, we examined the effect of CpxP on the CpxR phosphorylation status. Because our experimental setup for monitoring CpxA kinase activity (Fig. 2B) would be influenced by the inhibitory effect of CpxP on CpxA autophosphorylation and because in whole cells a stepwise addition of the components of a signaling pathway is not possible, we established an *in vitro* signal transduction cascade for the CpxARP system. Purified CpxA-His<sub>6</sub> in proteoliposomes and His<sub>6</sub>-CpxR were mixed at a molar ratio of 1:8, and the reaction was started by the addition of [γ-<sup>32</sup>P]ATP. Samples were taken after 15 and 30 min. Then, ADP was added to shift CpxA activities to dephosphorylation, and samples were taken after an additional 15 and 30 min (Fig. 8A, white symbols). As shown before, CpxA has a autokinase and phosphotransfer activities (Fig. 8A, white symbols up to 30 min) and a dephosphorylation activity toward CpxR (Fig. 8A, white squares after 30 min).

This *in vitro* signaling cascade system was now used to analyze the effect of CpxP on the CpxRA system. Proteoliposomes containing CpxA-His<sub>6</sub> and purified CpxP were mixed with His<sub>6</sub>-CpxR at a molar ratio 1:1:8, and the experiment was carried out as described above. As shown below, CpxP caused a decrease in CpxA autophosphorylation (Fig. 8A, black circles). Interestingly, CpxP did not influence the phosphorylation of CpxR (Fig. 8A, black squares) indicating that CpxP did not induce the phosphatase activity of CpxA as speculated by others (17).

As we could not observe an inhibitory effect of CpxP on the CpxR phosphorylation status we speculated if this might be due to an excess of CpxR protein in the reaction mix. Thus we reduced the molar ratio between the CpxP:CpxA:CpxR proteins to 1:1:4. This time, not only the phosphorylation status of the CpxA protein (Fig. 8B, black circles) was reduced in CpxP-containing proteoliposomes but also that of phosphorylated CpxR protein (Fig. 8B, black squares).

Taken together, our data indicate that CpxP inhibits CpxA autokinase activity, but does not induce phosphatase activity. In addition, the balance between the sensor kinase CpxA and the response regulator CpxR seems to be critical for an inhibitory effect mediated by the periplasmic CpxP protein on the complete signaling cascade.

**DISCUSSION**

The aim of the present study was to establish an *in vitro* system to investigate the biochemical properties of the CpxRA envelope stress system and its interaction with the periplasmic CpxP protein. Here, we describe a protocol to overproduce, purify, and reconstitute the full-length membrane-integral CpxA protein of *E. coli* into proteoliposomes as a C-terminal hexa-His fusion protein. The use of a C-terminal His tag fusion is well established for the purification and reconstitution of membrane-anchored histidine kinases such as the *E. coli* KdpD (35) and EnvZ (23) proteins. All known enzymatic activities were detectable for CpxA-His<sub>6</sub>-proteoliposomes under standard phosphorylation conditions, although rates differed somewhat with the purified cytoplasmic histidine kinase domain of the CpxA protein (36). In case of the full-length CpxA protein, transfer of the phosphoryl group to CpxR protein was slower and was not complete after 20 min. In contrast, a very rapid phosphotransfer to CpxR was shown for the cytoplasmic histidine kinase domain of CpxA (36). Thus, our data are in agreement with a suggestion made for the reconstituted KdpD protein that interactions between the domains of sensor kinases may fine-tune their enzymatic activities (35).

Our studies on the effect of solutes and pH conditions support *in vivo* data of Cpx pathway activation by mild alkaline pH (6). In addition, our data revealed the importance of KCl for CpxA autophosphorylation and phosphotransfer activities. Other solutes such as RbCl, NH<sub>4</sub>Cl, and NaCl also mediated activation of CpxA activities although to a lower extent compared with KCl. Because sucrose and trehalose did not show any effect on CpxA activities the data also imply that stimulation is not caused by an increase of osmolality *per se* as suggested by recent *in vivo* data (12). Furthermore, the compounds proline or glycine betaine, which are might to be accumulated.
in osmotically stressed cells did not exhibit a stimulatory effect on CpxA autokinase activity.

Both the solubilized and reconstituted CpxA-His₆ were further characterized for susceptibility to known sensor kinase inhibitors. To the best of our knowledge, this is the first time that inhibitors of two-component systems have been tested on a membrane-anchored sensor kinase that was purified as a full-length protein and reconstituted into proteoliposomes. Until now, soluble histidine kinases have received the most attention as potential targets of new antimicrobial agents. Examples are the B. subtilis KinA kinase that is essential for sporulation (37), the E. coli NRII (GlNL) kinase involved in nitrogen regulation (21) and the AlgR2 kinase essential for alginate production of mucoid strains of Pseudomonas aeruginosa (38). Although the effect of sensor kinase inhibitors on some membrane-integral histidine kinases was also studied, these proteins were never analyzed after purification and reconstitution. Either artificially truncated, soluble forms, as in the case of Thermotoga maritima HpkA kinase (29), or membrane preparations, as in the case of the VanS kinase of Enterococcus faecium (39) were used.

We investigated TCS and Ethodin as inhibitors for autokinase and phosphotransfer activities of both the soluble and the reconstituted CpxA-His₆ protein. Ofloxacin and vanadate did not inhibit any activities of the CpxA protein.

Interestingly, Closantel inhibited only the activities of solubilized CpxA-His₆ protein but not of CpxA-His₆ in proteoliposomes. This observation was confirmed by the use of the reconstituted EnvZ protein of E. coli. EnvZ, like CpxA, is an inner membrane protein with a short cytosolic N terminus, a 114-amino acid periplasmic domain flanked by two membrane-spanning domains and a cytoplasmic C-terminal domain (40). As observed for CpxA, only the soluble EnvZ was inhibited by Closantel.

Taken together, our results clearly indicate that purified membrane-integral histidine kinases mimic their natural activities only after reconstitution into proteoliposomes. Our observations for the histidine kinase inhibitor agent Closantel underscore the importance of studying the full-length membrane protein.

The availability of purified components allowed the reconstruction of the whole CpxRAP signal transduction cascade in vitro to analyze in more detail the in vivo observation that the periplasmic protein CpxP inhibits the Cpx signaling cascade (1). In particular we addressed the question, whether a direct interaction between CpxP and CpxA is involved. A CpxP:CpxA ratio of 1:1 was sufficient to inhibit the autophosphorylation activity of the sensor kinase CpxA up to 51%. In contrast, a CpxP variant that was fused to an N-terminal His tag reduced the autophosphorylation activity of CpxA only by 20%. This finding supports recent in vivo data showing that the N-terminal part of the CpxP protein is critical for its inhibitory activity on the Cpx signaling cascade (20). Interestingly, an inhibitory effect on the phosphorylation status of the response regulator CpxR was not observed indicating that neither the phosphotransfer nor the phosphatase activities of CpxA are influenced by CpxP. Therefore, we suggest that the CpxA-mediated phosphotransfer reaction is much faster than autophosphorylation. In other words, phosphorylated CpxA immediately transfers the phosphoryl group to CpxR. Concomitantly, we found that the balance between the sensor protein CpxA and its cognate regulator CpxR is critical for the signaling cascade. CpxP decreased significantly the amount of phosphorylated CpxR when the molar ratio of CpxP:CpxA:CpxR was adjusted to 1:1:4.

In summary, we demonstrated that CpxA catalyzes several reactions: autophosphorylation, the transfer of the phosphoryl group to CpxR and the dephosphorylation of CpxR—P. In addition, by establishing an in vitro signal transduction cascade for the whole CpxRAP system we were able to show that the periplasmic CpxP protein inhibits only the autokinase activity of CpxA and does not stimulate its phosphatase activity as speculated before (16). As the in vitro system excludes the involvement of other factors, this is the first biochemical indication for direct protein-protein interaction between these two proteins. Thus, we suggest that CpxP intervenes at the initial step of signal transduction, keeping the pathway in a resting state.

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