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. Since it is not clear how the Cpx
pathway is able to recognize and correspond 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-6His protein were stimulated by KCl. NaCl also stimulated the
activities but to a lower 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-6His 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 downregulation 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 close relatives
expression of some of the corresponding genes is regulated by the Cpx sensor regulator system
(reviewed in 1).

The Cpx pathway consists of the sensor kinase CpxA, the response regulator CpxR and
the periplasmic CpxP protein (1). 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 cytosolic 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 (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 signalling (14). Indeed, cpx* 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 peptidyl prolyl cis/trans isomerases 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). Since a 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 cpx\(^s\) mutants were constitutively activated (15), it was proposed that the presumed CpxA sensory domain normally functions to maintain the kinase in a downregulated 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 \(\alpha\)-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 in order to study the biochemical properties of the signalling 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 – [\(\gamma\)^32P] ATP, peroxidase conjugated anti mouse and anti rabbit IgG were purchased from GE Healthcare. Ethidin and Ofloxacin were obtained from Sigma. Vanadate was from Strem Chemicals. Clostranl and TCS were gifts of Thomas Schmulling (Free University, Berlin). Ni\(^{2+}\)-NTA resin and His\(_5\) antibody were from Qiagen and Protino-Ni from Macherey & Nagel. Detergents were obtained from Glycon Biochemicals. Purified E. coli lipids were purchased from Avanti Polar lipids. Bio-Beads SM-2 were from Bio-Rad. Trypsin and Trypsin inhibitor were obtained from Roth. Synthetic oligonucleotide primers were from Invitrogen, vectors pIVEX2.3 and pIVEX2.4 from Roche and vector pET15b from Novagen. All other reagents were reagent grade and obtained from commercial source.

Bacterial strains and plasmids – E. coli JM109 [recA1 endA1 gyrA96 thi hsdR17 supE44 \(\lambda\) relA1 Δ(lac-proAB)/F\(^{\prime}\) traD36 proA\(^{B\prime}\) lacf\(^{I}\) lacZΔM15] (Pharmacia) was used as a carrier for the described plasmids. E. coli K-12 strain MG1655 [\(\lambda\) F] (22) was used as parent strain for the PCR-based amplification of the cpxA, cpxR and cpxP genes. E. coli strain BL21(DE3) <pLysS> (Novagen) was used for expression of cpxA, cpxR and cpxP under the control of the T7 promoter.

Construction of cpxA-6His, 6His-cpxR and 6His-cpxP – Construction of p3cpxA was achieved by amplifying the cpxA coding region using primers CpxA5 (5'–ATCATA TGA TAG GCA GCT TAA CCG CG-3') and CpxA3 (5'–ATC CGG GGA CTC CGC TTA TAC AGC GGC AAC C-3'). The resulting fragment was cloned into the NdeI and SmaI sites of pIVEX2.3.

The cpxR coding region was amplified using primers CpxR5 (5'–GCT GAG TAA AAT CCT GTT AGT TGA TG-3') and CpxR3 (5'–CGG GAT TCT CAT GAA GCA GAA ACC ATC AG-3') and cloned into the NcoI and BamHI sites of pIVEX2.4, resulting in pl4cpxR.

Construction of pRF6 was achieved by amplifying the cpxP coding region using primers 5Xhol_EcoCpxP (5'–GAG ACT CGA GGC TGA AGT CGG TTC AGG CGA TAA C-3') and 3BamHI_EcoCpxP (5'–GAG TGG ATC CCT ACT GGG AAC GTG AGT TGC TAT C-3'). The product was digested with XhoI and...
BamHI and cloned into the corresponding sites of pET15b. The resulting constructs were confirmed by sequencing.

**Expression of proteins and preparation of cytosolic fraction and membrane vesicles – E. coli strain BL21(DE3) <pLysS> was transformed either with pI3cpxA, pI4cpxR or pRF6, respectively, and grown at 30 °C with aeration in LB medium supplemented with 100 µg/ml ampicillin and 30 µg/ml chloramphenicol. Gene expression was induced with 0.5 mM IPTG for 3-4 h. 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 PMSF). 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 6His-CpxR** – 6His-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 PMSF). Bound 6His-CpxR was eluted with an imidazole concentration of 150 mM in buffer R. 6His-CpxR-containing fractions were mixed and further purified with a Protino Ni 2000 prepacked column kit according to the manufactures instructions (Macherey & Nagel). Purified 6His-CpxR protein was passed through a PD10 column (GE Healthcare) to remove imidazole and stored at –80 °C until use.

**Purification of 6His-CpxP** - 6His-CpxP fusion protein was purified by means of Ni-affinity chromatography in batch. Binding of the protein (600 mg cytosolic protein / 3 ml Ni-NTA resin) 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 cocktail (Roche)). After washing with buffer P containing 20 mM imidazole 6His-CpxP was eluted by increasing the imidazole concentration to 150 mM. 6His-CpxP-containing fractions were passed through a PD10 column to remove imidazole and further purified by an SP-sephadex column (Pharmacia). Bound 6His-CpxP was washed extensively with buffer P2 (50 mM MOPS/K⁺ pH 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 6His-Fusion from the N-terminus of CpxP was cleaved by thrombin using the THROMBIN CleanCleaveKit essentially as described (Sigma).

**Purification of CpxA-6His** – For detergent selection following detergents were tested: 1 % Triton X-100, 1 % lauryldimethylamine oxide (LDAO); 1 % decylmaltoside (DM); 1 % dodecylmaltoside (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-6His solubilization (%) is used as measure for the distribution of the amounts of CpxA-6His between the soluble and the membrane fraction after treatment of membrane vesicles with detergent.

1 ml of 10 x DM was added to 9 ml of membrane vesicles (~ 10 mg/ml) prepared from BL21(DE3) <pLys/pI3cpxA>. Samples were incubated on ice for 1 h and centrifugated at 200,000 x 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 DTT, 0.1 mM PMSF). Binding of CpxA-6His was carried out by incubating the solubilized fraction and Ni²⁺-NTA resin at 4°C for 1 h. The protein-resin complex was passed into a column (GE Healthcare) 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-6His 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-6His 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 (v/v) 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 x g. The pellet was resuspended in 50 mM Tris/HCl, pH 7.5, 10% glycerol (v/v) and 2 mM DTT. 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 to 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 DTT, 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 x SDS sample buffer. To analyze phosphorylation, purified 6His-CpxR was added to this mixture after 10 min, additional samples were taken and reaction was stopped by the addition of 5 x SDS sample buffer.

To test dephosphorylation, purified 6His-CpxR was phosphorylated as described above with CpxA-6His-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 6His-CpxR-P was initiated by the addition of fresh CpxA-6His in proteoliposomes. At the indicated times, aliquots were taken, and the reaction was stopped as described above.

To test the whole signalling cascade in vitro, CpxA-6His-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 x SDS sample buffer. To analyze CpxA-6His 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 PhosphorImager system (Molecular Imager Fx, and software Quantity one, BioRad) using [γ-32P] ATP as a standard.

**Inhibitor studies** – Inhibitors (except vanadate) were dissolved in DMSO. Vanadate solutions were prepared in water at pH10 as described (25). The phosphorylation assays were performed as described above in the presence of the indicated inhibitors. To reflect the presence of DMSO 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-6His 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 (BioRad) and protein bands were quantified with Quantity one (BioRad).

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-6His protein was comparable to the native protein: Both autokinase and kinase activities in membrane fractions were only slightly lower than in authentic CpxA (data not shown). Thus, CpxA-6His was used for further in vitro analysis.

As a prerequisite for CpxA-6His purification by affinity chromatography the protein had to be efficiently solubilized. Since the yield of solubilized CpxA-6His was found to be 80 % when decylmaltoside was used as detergent (69 % n-dodecylmaltoside, 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-6His significantly (data not shown). The highest degree of purified CpxA-6His was achieved when the protein was bound to the Ni²⁺-NT-agarose in the presence of 50 mM imidazol and 0.15 M NaCl. In a typical experiment about 1 mg CpxA-6His was obtained from 1 L culture. As demonstrated by Coomassie blue staining (Fig. 1A), the purity of CpxA-6His 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 6His-CpxR protein was purified by the use of two affinity chromatography steps. This procedure yielded 95 % pure 6His-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 \textit{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 \textit{degP} strain (data not shown). Therefore, the protein was overproduced and purified from the cytosol. After purification by Ni-chelate 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). Since DnaK was not found in proteoliposomes after reconstitution (Fig. 7A lane 3) the preparation was not purified further.

Incorporation of CpxA-6His into proteoliposomes – Purified CpxA-6His protein was incorporated into \textit{E. coli} phospholipids using the detergent-mediated method as described (26). The efficiency of CpxA-6His incorporation into liposomes was approximately 40 % (data not shown).

To analyze the orientation of CpxA-6His 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. Since 82 % of the CpxA-6His 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-6His protein in proteoliposomes is in the inside-out orientation (Fig. S1). As a control for the integrity of the proteoliposomes, soluble MalK ATPase was incorporated along with CpxA-6His. 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-6His activities in proteoliposomes – CpxA-6His proteoliposomes were autophosphorylated in the presence of [\textgamma³P] ATP at room temperature. The autophosphorylation increased almost linearly for 10 min (Fig. 2A). Autokinase activity of CpxA-6His proteoliposomes was 1.5 times higher compared to that of CpxA-6His in detergent (data not shown).

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

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

To test now the dephosphorylation of 6-His-CpxR$^{32}$P by CpxA, CpxA-6His-containing proteoliposomes were added and dephosphorylation was monitored over a period of 60 min. The half-life of 6His-CpxR$^{32}$P 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 6His-CpxR$^{32}$P 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-6His-proteoliposomes and 6His-CpxR catalyzed all known enzymatic activities.

We also analyzed the concentration-dependent effect of KCl on CpxA-6His autophosphorylation activity using a concentration range between 0.5 mM and 500 mM KCl. The stimulatory effect of KCl was found to be linear till 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-6His proteoliposomes. We observed a stimulation of CpxA autokinase activity by mild alkaline pH but not by neutral or mild acetic pH (Fig. 3B).

Inhibition of the autokinase and phosphotransfer activities of CpxA-6His in proteoliposomes by inhibitors of two-component systems – We further characterized the molecular properties of CpxA in more detail by studying the effect of chemicals that are known to inhibit sensor kinases. The potential inhibitors used in this study are the salicylanilides Closantel (28) and Tetrachlorosylicylanilid (TCS) (29), the small hydrophobic intercalator-like molecule Ethodin (21), the quinolone Ofloxacin (29) and the phosphate analogue vanadate.

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

For CpxA-6His 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-6His (Fig. 4). The IC$_{50}$ value was determined to be 150.8 ± 40.9 µM.

For Ethodin we observed reduced autophosphorylation activity of CpxA-6His (Fig. 4) with a IC$_{50}$ value of 219.2 ± 42.4 µM.

Ofloxacin slightly inhibited the autophosphorylation activity of CpxA-6His (Fig. 4). The proportion of phosphorylated CpxA protein was 87 % of the DMSO 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-6His 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-6His 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 but not of CpxA-6His in proteoliposomes – TCS and Ethodin were found to be inhibitors for autophosphorylation and phosphotransfer activities of reconstituted CpxA-6His. Interestingly and to our surprise, Closantel did not inhibit these two activities when CpxA-6His 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-6His. 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-6His 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-6His but the activity of the protein in proteoliposomes was not inhibited (Fig. 5).

Effect of these inhibitors on the autokinase activity of EnvZ-6His – The inhibitor studies with CpxA-6His 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 DMSO 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-6His 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 analyzing the reconstituted and not the soluble version of a sensor kinase.

Inhibition of CpxA-6His autophosphorylation activity by CpxP – In vivo data showed that overproduction of the periplasmic CpxP protein leads to inhibition of the Cpx signalling cascade (17). Our defined in vitro system provides an experimental platform to test if this is due to direct protein-protein interaction between the CpxP protein and the CpxA sensor kinase since 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-6His, we mixed purified CpxP protein with solubilized CpxA-6His. Although CpxP was provided in a five fold excess, no inhibition of autophosphorylation activity of the solubilized CpxA-6His protein by CpxP was observed (data not shown). To analyze now the effect of CpxP on reconstituted CpxA-6His, 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-6His during the reconstitution process up to 10:1, the CpxP:CpxA ratio found in the proteoliposomes was always 1:1 (Fig. 7A). Under these conditions CpxP inhibited the autophosphorylation activity of CpxA-6His by
Inhibition of the in vitro CpxRA signal transduction cascade by CpxP: Next, we examined the effect of CpxP on the CpxR phosphorylation status. Since our experimental setup for monitoring CpxA kinase activity (Fig. 2B) would be influenced by the inhibitory effect of CpxP on CpxA autophosphorylation and since in whole cells a stepwise addition of the components of a signalling pathway is not possible, we established an in vitro signal transduction cascade for the CpxARP system. Purified CpxA-6His in proteoliposomes and 6His-CpxR were mixed at a molar ratio of 1:8, and the reaction was started by the addition of [γ32P] ATP. Samples were taken after 15 and 30 min. Then, ADP was added to shift CpxA activities to dephosphorylation and samples were taken after 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 towards CpxR (Fig. 8A, white squares after 30 min).

This in vitro signalling cascade system was now used to analyze the effect of CpxP on the CpxRA system. Proteoliposomes containing CpxA-6His and purified CpxP were mixed with 6His-CpxR at a molar ratio 1:1:8, and the experiment was carried out as described above. As shown before, 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 it 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 signalling 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 *Escherichia 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-6His-proteoliposomes under standard phosphorylation conditions, although rates differed somewhat compared 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, NH₄Cl and NaCl also mediated activation of CpxA activities although to a lower extent compared to KCl. Since sucrose and trehalose did not show any effect on CpxA activities the data also imply that stimulation is not due to 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-6His 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-6His protein. Ofloxacin and vanadate did not inhibit any activity of the CpxA protein. Interestingly, Cloantel inhibited only the activities of solubilized CpxA-6His protein but not of CpxA-6His 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 Cloantel.

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 Cloantel 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 signalling 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 57%. 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 signalling 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 signalling 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.
REFERENCES


**FOOTNOTES**

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1 The abbreviations used are: Closantel, N-[5-chloro-4-[(R,S)-(4-chloro-phenyl)cyanomethyl]-2-methylphenyl]-2-hydroxy-3,5-diodobenzamide; DMSO, dimethylsulfoxide; Ethodin, 6,9-diamino-2oxyethyl acridine lactate; IPTG, isopropyl-β-D-thiogalactopyranoside; LDAO, N,N-dimethyldodecylamine N-oxide; MBP, maltose binding protein; MOPS, 3-(N-Morpholino)-propanesulfonic acid; NTA, nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; TCS 3,3’,4’-5-Tetrachlorosalicylanilide; vanadate, Na-orthoVanadate-decahydrat
Fig. 1: Purification of CpxA, CpxR and CpxP. Samples from different stages of the purification procedure were separated on 12.5% SDS-PAGE: A, solubilization and purification of CpxA: Lanes: 1, everted membrane vesicles; 2, decylmaltoside-solubilized protein; 3, membrane vesicles after solubilization; 4, purified CpxA-6His after elution from Ni-NTA agarose with imidazole (3.5 µg of protein). B, purification of CpxR: Lanes: 1, cytoplasmic fraction; 2, 6His-CpxR after elution from Ni-NTA-agarose with imidazole; 3, purified 6His-CpxR after elution from Protino-Ni-agarose with imidazole (4 µg of protein). C, purification of CpxP: Lanes: 1, cytoplasmic fraction; 2, 6His-CpxP after elution from Ni-NTA-agarose with imidazole; 3, purified CpxP after thrombin cleavage (4 µg of protein). The proteins were stained with Coomassie-blue. The molecular mass markers are shown on the left.

Fig. 2: Enzymatic activities of purified CpxA-6His in proteoliposomes. A, time-dependent autophosphorylation activity of CpxA-His in proteoliposomes. CpxA (1 µM) was incubated with [γ-32P]ATP in buffer containing 50 mM KCl. B, phosphotransfer activity of CpxA in proteoliposomes. CpxA was phosphorylated under standard conditions, at time point 0 min 3 µM purified CpxR was added to initiate phosphotransfer activity. C, phosphatase activity of CpxA in proteoliposomes. CpxR was phosphorylated by CpxA-6His-proteoliposomes with [γ-32P]ATP (described under experimental procedures). 6His-CpxR~P was incubated in the presence or absence of ADP with MgCl2 and dephosphorylation was initiated by adding CpxA-containing proteoliposomes (0.5 µM). The reaction was started at time 0 with 3 µM [32P-CpxR]. In all assays samples were taken at the times indicated, separated by SDS-PAGE and analyzed on a PhosphorImager (upper part). The amounts of [32P] phospho-CpxA and [32P] phospho-CpxR were determined with a PhosphorImager (lower part) using [γ-32P] ATP as standard. Shown are averages ± SEM from at least three different experiments.

Fig. 3: Influence of different solutes and pH variations on the autophosphorylation activity of CpxA. Autokinase activity of CpxA-6His in proteoliposomes (1 µM) was probed in buffers containing various solutes (A). Shown are the results obtained with various solutes tested at a low (0.5 mM) or a high (0.5 M) concentration. B, CpxA-6His containing proteoliposomes (1 µM) were prepared in the presence of indicated pH, collected and resuspended in phosphorylation buffer containing 50 mM KCl, with either a constant pH of 7.5 (black bars) or with indicated pH (white bars). For both experiments samples were taken after 10 min. The averages ± SEM from three different experiments are shown.

Fig. 4: Inhibition of the autokinase activity of CpxA-6His-proteoliposomes by histidine kinase inhibitors. Purified CpxA was incorporated into liposomes and the effect of different inhibitors (200 µM) on autokinase activity was tested. The phosphorylation assay was performed as described in experimental procedures with CpxA containing proteoliposomes (0.5µM) in buffer containing 50 mM KCl. The relative amount of [32P] phospho-CpxA after incubation the CpxA containing proteoliposomes (1µM) with different inhibitors as indicated. Shown are averages ± SEM from two different experiments with two duplicates each. For CpxA without any inhibitor a phosphorylation level of 1 nmol [32P-CpxA] / mg was obtained after 30 min.

Fig. 5: Effect of Closantel on solubilized CpxA-6His protein and CpxA-6His in proteoliposomes. Different concentrations of Closantel were tested on the autophosphorylation activity of CpxA-6His solubilized in detergent (soluble) or CpxA-6His-proteoliposomes (PLS) as described in experimental procedures. After incubation with DMSO maximal values of 0.001 nmol [32P-CpxA] / min x mg soluble and [32P-CpxA] incorporated into proteoliposomes were obtained. Shown are averages ± SEM from three different experiments.
Fig. 6: Inhibition of the autophosphorylation activity of solubilized EnvZ-6His and EnvZ-6His 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 (23). Shown is a representative of two independent experiments.

Fig. 7: Inhibition of autophosphorylation activity of CpxA-6His by CpxP. Proteoliposomes containing purified CpxA-6His and CpxP proteins were prepared and tested for autophosphorylation as described under experimental procedures. A, CpxA-6His containing proteoliposomes loaded with CpxP or buffer were separated on a 12.5 % - SDS-gel, and stained with Coomassie blue. Protein was quantified in the scanned gels with Quantity one software. B, Autophosphorylation activity of CpxA-His in proteoliposomes loaded with purified CpxP (1 µM), 6His-CpxP (5 µM) or buffer. CpxA (1 µM ) was incubated with $[^{32}\text{P}]$ ATP (40 µM) in buffer containing 50 mM KCl. Samples were separated by SDS-PAGE and analyzed with a PhosphorImager (upper part). The amounts of $^{32}$P-CpxA were determined on a PhosphorImager (lower part) using $[^{32}\text{P}]$ ATP as a standard. In the absence of CpxP an autophosphorylation rate of 0.04 ± 0.007 nmol /min x mg was calculated. Shown are averages ± SEM from three different experiments.

Fig. 8: Effect of CpxP on the Cpx signalling cascade in vitro. Phosphorylation assay for the CpxA/CpxR signal transduction cascade was performed as described under experimental procedures. A, CpxA-6His-containing proteoliposomes (0.5µM) and 6His-CpxR (4 µM) were mixed in reaction buffer containing 50 mM KCl. The reaction was started by the addition of 100 µM $[^{32}\text{P}]$ ATP and samples were taken at the indicated times. After 30 min ADP (1mM) was added, and further samples were taken. The experiment was repeated with CpxP (0.5 µM) loaded in proteoliposomes. B, Effect of CpxP on the whole Cpx signalling cascade containing a reduced amount of CpxR. 2 µM 6His-CpxR protein was added to CpxA-6His or CpxA-6His/CpxP containing proteoliposomes. For both sets of experiments samples were separated by SDS-PAGE and analyzed on a PhosphorImager. The amounts of $^{32}$P-CpxA and $^{32}$P-CpxR were quantified on a PhosphorImager using $[^{32}\text{P}]$ ATP as a standard. Shown are averages ± SEM from three different experiments.

Figure S1: Tryptic digestion of CpxA-6His incorporated into proteoliposomes. A, Proteoliposomes were incubated with trypsin (15 ng/µl) at a protease/protein ratio of 1:100 at room temperature. At the indicated times, the reaction was stopped by trypsin inhibitor (2 µg/µl), the proteins were subjected to Western Blot analysis and immunoblots were probed with antiserum to penta-His fusion (mouse) to visualize full length CpxA-6His or with antiserum to MalK (rabbit). MalK protein of Salmonella typhimurium was incorporated into proteoliposomes to control their impermeability for trypsin. B, model of proteoliposome composition. During reconstitution process CpxA-6His can be incorporated into liposomes in the in-side out or the right-side out orientation. 50 % of the adjusted MalK during proteoliposome preparation is membrane associated. Since all membrane-associated MalK was degraded after 1.5 min and the amount of remaining MalK was stable, time point 1.5 min was taken to calculate the orientation of CpxA-6His in proteoliposomes.
Fig. 1

A

B

C

kDa 1 2 3 4

CpxA-6His

kDa 1 2 3

6His-CpxR

kDa 1 2 3

DnaK 6His-CpxP CpxP
**Fig. 2**

A

![Graph showing CpxA\(^{32}P\) level over time](image)

- **Y-axis:** CpxA\(^{32}P\) level (pmol)
- **X-axis:** Time (min)

B

![Graph showing CpxA\(^{32}P\) and CpxR\(^{32}P\) over time](image)

- **Y-axis:** pmol
- **X-axis:** Time (min)

- **CpxA\(^{32}P\):** Open circle line
- **CpxR\(^{32}P\):** Solid square line
Fig. 2

C

![Graph showing the effect of ADP, PLS, and PLS + ADP on CpxR-P levels over time.](image)
Fig. 3

A

![Bar chart showing relative CpxA-P level (%) for different compounds at low and high levels.]

B

![Bar chart showing relative CpxA-P level (%) for different pH values, comparing pH in and pH in = pH out.]

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Fig. 4

[Bar chart showing relative CpxA-P levels for different treatments: 
- Sine
- DMSO
- TSC
- Ofloxacin
- Closantel
- Ethidium
- Vanadate]
Fig. 7

A

B

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min

CpxA~\(^{32}\)P

relative CpxA-P level (%)
Fig. 8

### A

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![Image](image1.png)

**Fig. 8A**

- CpxA-\(^{32}\)P
- CpxR-\(^{32}\)P

### B

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![Image](image2.png)

**Fig. 8B**

- CpxA-\(^{32}\)P
- CpxR-\(^{32}\)P

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