Supporting Information

Maslennikov et al. 10.1073/pnas.1001656107

SI Text

SI Materials and Methods. ***ArcB(1-115) Escherichia coli expression and NMR sample preparation.*** An ArcB fragment comprising residues 1-115 was cloned into a Gateway-adapted pHis vector (1), resulting in a construct with a thrombin-cleavable N-terminal His_9 tag: MKHHHHHHHHHGGLESTSLYKKAGSLVPRGSGS, and expressed in *E. coli* BL21 DE3 cells (Invitrogen). Cells obtained from overnight cultures were transferred into a M9 minimal medium and grown at 37 °C. The M9 medium was supplemented with 2 g/L $^{15}NH_4Cl$ and 4 g/L glucose for a uniformly ^{15}N-labeled sample. For ^{15}N-^{13}C- or ^{2}H-^{15}N-^{13}C-labeled samples ^{13}C-glucose or ^{2}H-^{13}C-glucose in 99.9% D_2O was used, respectively. Protein expression was induced with 0.5 mM IPTG at $OD_{600} = 1$, followed by incubation at 18 °C for 16–20 h. Cells were harvested by centrifugation, resuspended in a lysis buffer (20 mM Tris · HCl, pH 8.0, 0.5 mM EDTA) and lysed in M-100L CF microfluidizer (Microfluidics). The pellet from centrifugation (45,000 × *g*, 2 h) was suspended in a solubilization buffer (20 mM Tris · HCl, pH 8.0, 200 mM NaCl, 18 mM FC-12, 4 mM BMe) for membrane extraction and incubated with stirring for 2 h at 4 °C. The extracted protein in the supernatant was separated by centrifugation (45,000 × *g*, 2 h) and purified by Ni-NTA (nitrilotriacetate). In particular, 5 mL of Ni-NTA Agarose (Qiagen) were equilibrated with five column volumes (CV) of a washing buffer (20 mM Tris · HCl, pH 8.0, 200 mM NaCl, 4 mM FC-12) before loading the sample. To improve protein binding to nickel, the beads and the sample were incubated with shaking at 4 °C for 15–20 min. The beads were washed with 8 CV of the wash buffer before elution with 3 CV of an elution buffer (20 mM Tris · HCl, pH 8.0, 200 mM NaCl, 4 mM FC-12, 3 mM BMe, 300 mM Imidazole). For cleaving of the N-terminal tag, elution fractions were concentrated to 2.5 mL in 10 kDa molecular weight cutoff (MWCO) Vivaspin 20 (Sartorius Stedim Biotech, GmbH), desalted in 20 mM Tris · HCl, pH 8.0, 200 mM FC-12, 2 mM $CaCl_2$ using a PD-10 column (GE Healthcare BioSciences Corp.) and cleaved with 10 units of Thrombin/ 1 mg protein (Sigma-Aldrich) overnight at room temperature (RT). The cleaved His_9 tag was removed by incubating the sample with 2 mL of Ni-NTA Agarose, equilibrated with an FPLC buffer (20 mM Tris · HCl, pH 8.0, 200 mM NaCl, 2 mM FC-12, 1 mM DTT) shaken for 15 min at 4 °C, followed by elution with 2 CV of the FPLC buffer. Ni-NTA flow-through was concentrated to 2 mL and purified by size-exclusion FPLC on a 16/60 Superdex™ 200 column (GE Healthcare BioSciences Corp.) equilibrated in the FPLC buffer. To exchange FC-12 with 1-myristoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)] (LMPG; Avanti Polar Lipids; Anatrace), FPLC fractions corresponding to the monomer were concentrated and their pH was changed with 20 mM Tris · HCl, pH 9.0, 1 mM DTT in a 10 kDa MWCO Vivaspin 20 before loading on 2 mL of Q sepharose resin (GE Healthcare BioSciences Corp.) at RT, equilibrated with 20 CV 20 mM Tris · HCl, pH 9.0, 0.2 mM LMPG. Bound protein was washed with 20 CV 20 mM Tris · HCl, pH 9.0, 4 mM LMPG before high salt elution with 30 CV 20 mM Tris · HCl, pH 9.0, 0.5 M NaCl, 1 mM LMPG. For NMR sample preparation, the eluted protein was concentrated and desalted and the sample pH was changed by concentration and washing with 20 mM sodium acetate pH 5.5, 10 mM NaCl, 0.2 mM LMPG using a 10 kDa MWCO Vivaspin 20 concentrator.

Cloning procedures. ArcB(1-115), QseC(1-185), and Kdpd(397-502) for CF expression were amplified from cDNA by standard polymerase chain reaction techniques using Vent DNA-polymerase [New England Biolab (NEB)]. Suitable restriction sites and a C-terminal stop codon were added to the DNA fragments with suitable oligonucleotide primers. Purified PCR fragments were inserted after cleavage into pIVEX2.3 (Roche Applied Science) vectors.

Cysteine residues in ArcB(1-115), QseC(1-185), and Kdpd (397-502), as well as serine residues in KdpD(397-502) for obtaining cysteine-free KdpD-(397-502), were introduced by site-directed mutagenesis. In particular, primers were designed as described elsewhere (2) and quick change reactions were carried out using 1 μL HotStar Polymerase (Qiagen), 1x HotStar Buffer, 2% DMSO, 0.2 μM primers and 3–5 μg/mL template DNA in 50 μL reaction volume. PCR was set up in a thermocyler (Techne Inc) at 95 °C for 0.5 min and cycled 18 times at 95 °C for 0.5 min, 55 °C for 100 s, 68 °C for 10 min with the final extension time of 30 min at 68 °C. Parental DNA was digested with DpnI (NEB) by adding 1 μL enzyme and incubation for 3 h at 37 °C, and subsequently purified by a Nucleotide purification kit (Qiagen) with elution in 30 μL H_2O. Seven microliters DNA were transformed into 25 μL DH10b chemical competent cells (Invitrogen).

CF expression. We established a preparative high-throughput *E.-coli*-based CF expression system that has been optimized and fine-tuned for expression of integral membrane proteins (MPs). Chemicals for CF expression were purchased from Sigma-Aldrich, stable isotope-labeled amino acids and amino acid mixtures were purchased from Cambridge Isotope Laboratories unless otherwise stated. Histidine kinase receptors (HKRs) were produced in an individual continuous exchange CF system according to previously described protocols (3, 4) with further optimizations. In general, CF extracts were prepared from the *E. coli* strain A19 as described in refs. 3 and 4, T7-RNA polymerase was expressed using the pT7-911Q plasmid (5) and purified as described in ref. 6. Preparative scale CF reactions were performed in 20 kDa MWCO Slide-A-Lyzers (Thermo Scientific) using 2 mL of reaction mixture (RM) set with the 1 : 17 volume ratio between RM and the feeding mixture (FM). Slide-A-Lyzers were placed in a suitable plastic box holding the FM and incubated in a shaker (New Brunswick Scientific) for approximately 15 h at 30 °C. The reaction conditions for the CF reaction were as follows. RM and FM, 270 mM potassium acetate; 14.5 mM magnesium acetate; 100 mM Hepes-KOH pH 8.0; 3.5 mM Tris-acetate pH 8.2; 0.2 mM folinic acid; 0.05% sodium azide; 2% polyethyleneglycol 8000; 2 mM Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) (Thermo Scientific); 1.2 mM ATP; 0.8 mM each of CTP, UTP, GTP; 20 mM acetyl phosphate (Fluka); 20 mM phosphoenol pyruvate (AppliChem, GmbH); 1 tablet per 50 mL complete protease inhibitor (Roche Applied Science); 1 mM each amino acid; 40 μg/mL pyruvate kinase (Roche Applied Science); 500 μg/mL *E. coli* tRNA (Roche Applied Science), 0.3 unit/μL RNase Inhibitor (SUPERase-In™, Ambion); 0.5 unit/μL T7-RNA polymerase; 40% S30 extract and 15 μg/mL of pET21a derived plasmid DNA or 7.5 μg/mL of pIVEX2.3 derived plasmid DNA. For CF U-^{15}N labeling, RM and FM were supplemented with 0.5 mM of ^{15}N algal amino acid mixture and 0.5 mM of the ^{15}N amino acids: N, C, Q, and W. For CF U-^{15}N-^{13}C, U-^{2}H-^{15}N, and U-^{2}H-^{15}N-^{13}C labeling, RM and FM were supplemented with 0.5 mM of correspondingly labeled amino acid mixtures. For solid-state NMR measurement U-^{15}N-^{13}C-labeled samples were expressed. For combinatorial labeling of QseC(1-185) and KdpD(397-502) combinations of ^{15}N-labeled A, C, D, E, F, G, I, K, L, M, N, Q, R, S, T, V, W, Y, or 1-^{13}C-labeled A, C, D, E, F, G, I, K, L, M, P, Q, S, V, W, Y,

and nonlabeled amino acids were used (schemes are given in Fig. 4*B* and Table S2). For HKRs prepared in D_2O for deuterium-hydrogen (D-H) exchange experiments, CF expression was carried out in 99% D_2O. In particular, all chemicals where solubilized in D_2O, plasmid DNA was prepared in D_2O, and S30 extract was prepared in D_2O after growing cells in H_2O.

The performance and cost efficiency of this CF system as compared with the standard *E. coli* system is illustrated in Fig. 3. Cost efficiency is estimated in Table S1 by comparing labor ($15/h) and material costs of producing differently uniform isotopically labeled NMR samples of ArcB(1-115) by standard *E. coli* and by an individual CF expression system. Contrary to the widespread belief that CF synthesis is very expensive, the comparison (Table S1) proved that CF expression is 3–4 times less expensive for both nonlabeled and isotopically labeled proteins. In addition, CF enables the NMR sample preparation within 24 h, compared to 5 days by *E. coli* expression, with the additional benefits of reproducible expression and unique labeling possibilities such as combinatorial ^{15}N-^{13}C labeling.

Protein characterization. The Invitrogen gel electrophoresis system (Invitrogen) was used for all SDS-gel analyses following the manufacturer's protocol, using 12% NuPAGE Bis-Tris gels in Mes buffer stained with Coomassie blue or InstantBlue (Expedeon Protein Solutions, Ltd.). The proteins were characterized by SDS-PAGE (Fig. S1*A*), surface enhanced laser desorption/ionization MS analysis, and light scattering coupled with size-exclusion chromatography and refracting index measurements (SEC-UV/LS/RI) (Fig. S1 *B–D*).

SEC-UV/LS/RI analysis of HKRs-LMPG complexes was performed by measuring the relative refractive index signal (Optilab rEX, Wyatt Technology Corp.), static light scattering (LS) signals from three angles (45°, 90°, 135°) (miniDAWN TREOS, Wyatt Technology Corporation), and UV extinction at 280 nm (Waters™ 996 Photoiode Array Detector, Millipore Corp.) during HPLC (Waters™ 626 Pump, 600S Controller, Millipore Corp.) size-exclusion chromatography with polymer column (Shodexfi Protein KW-803). HKRs were analyzed by injecting 50 μL of 200 μM IMP solubilized in LMPG into HPLC buffer (20 mM Mes-BisTris pH 6.0, 150 mM NaCl, 0.01% LMPG) at 0.8 mL/ min. The fractions, containing target proteins, were concentrated in 5 kDa MWCO Vivaspin 2 concentrators (Sartorius Stedim Biotech, GmbH) to 50 μL and reinjected. The data were collected and analyzed using the Astra V 5.3.2.12 Software (Wyatt Technology Corp.). The average molar weights of the protein-detergent complex, of the protein, and of the detergent fraction in the complex (Fig. S1 *B–D*) were calculated by applying the Protein Conjugate module of the Astra program.

NMR sample preparation. All HKRs were expressed as precipitate (p-CF) in the absence of detergents (3). Precipitated recombinant proteins were removed from the RM by centrifugation at $20,000 \times g$ for 15 min and washed in two steps. First, in order to remove coprecipitated RNA, precipitates were suspended in 50% volume equal to the RM volume in 20 mM Mes-BisTris buffer pH 6.0, 0.01 mg/mL RNase A and shaken at 900 rpm and 37 °C for 30 min. After incubation, precipitates were harvested by centrifugation at $20,000 \times g$ for 10 min and suspended in 100% volume equal to the RM volume in NMR buffer [20 mM Mes-BisTris pH 5.5 for ArcB(1-115) and 20 mM Mes-BisTris pH 6.0 for QseC(1-185) and KdpD(397-502)]. NMR samples were prepared from washed precipitate of 1 mL RM by solubilization in 300 μL 5% (wt/vol) LMPG in NMR buffer. The suspension was sonicated in a water bath sonicator (Bransonic) for 1 min and subsequently incubated for 15 min with shaking at 900 rpm and 37 °C, followed by centrifugation at $20,000 \times g$ for 10 min. NMR samples were pH-adjusted, supplemented with 5% D_2O and 0.5 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid and treated with five freeze-thaw cycles using liquid nitrogen flash freezing followed by 37 °C water bath incubation. Shigemi NMR tubes (Shigemi) were used for solution NMR measurements. "Fingerprint" spectra of the CF-expressed proteins are shown in Fig. S1 *E–G*. For hydrogen-deuterium (H-D) exchange experiments samples prepared in H_2O or D_2O were washed in H_2O or D_2O, respectively. H_2O and D_2O samples were solubilized in 5% LMPG in D_2O-NMR and H_2O-NMR buffers, respectively. H-D exchange samples were measured instantly after 1 min water bath sonication. For solid-state NMR measurements, the pellet produced in 2 mL RM was washed as described above using the same buffers and loaded into a 4 mm magic angle spinning (MAS) rotor. The solid-state NMR sample of ArcB(1-115) solubilized with 5% LMPG was prepared from a solution NMR sample by lyophilization.

Three single cysteine mutants of ArcB(1-115) (positions F23, S52, and Q79) and seven single cysteine mutants of QseC(1-185) (S9, Q36, T93, M156, Q164, A171, and M179) were prepared for paramagnetic relaxation enhancement (PRE) experiments. Because KdpD(397-502) has two cysteines (positions 402 and 409), they were mutated to serine and five single cysteine mutations were introduced at the positions Q398, A425, S448, T469, and Q501. Additionally, we used C402S mutant, which has a native cysteine at position 409. NMR samples with single cysteine mutants obtained from 1 mL CF RM were prepared in 400 μL in order to measure PRE in a standard NMR tube. The samples were measured consequently before spin labeling, spin labeled in oxidized and in reduced states and after removing the spin label. Spin-labeling samples were supplemented with 5 mM 1-Oxyl-(2,2,5,5-tetramethyl-Δ^3-pyrroline-3-methyl)methanethiosulfonate (MTSL) (Toronto Research Chemicals, Inc.), solubilized in Acetonitrile. After overnight incubation at RT, the excess of MTSL was removed by 24 h dialysis at RT against 3×500 mL NMR buffer in Ettan™ minidialysers (GE Healthcare BioSciences Corp.). Spin label was reduced with 5 mM ascorbic acid using a 200 mM stock solution adjusted to pH 6.5. Finally, MTSL was removed from the protein by an addition of 50 mM TCEP (Thermo Scientific) and 4 h incubation at RT before overnight dialysis against 500 mL NMR buffer.

NMR experiments. Solid state NMR, 2D ^{13}C-DARR, experiments (7) were performed on Bruker AVANCE 850 spectrometer (213.765 MHz for ^{13}C) using a 4 mm MAS-DVT probe at 273 K and the 14 KHz spinning rate (Centre for Biomolecular Magnetic Resonance). Two milligrams of precipitant was loaded into a 4 mm MAS rotor. The ^1H radio frequency field strength was matched to the MAS speed during the mixing period. A DARR experiment with ArcB(1-115) was recorded using 100 ms mixing time, 256 increments of 320 scans each. The SPINAL-64 pulse with the field strength of 62.5 KHz was applied during acquisition. A DARR experiment with KdpD(397-502) was recorded using 30 ms mixing time, 128 increments of 320 scans each. The SPINAL-64 pulse with the field strength of 71 KHz was applied during acquisition.

High-resolution NMR spectra of ArcB(1-115) expressed in *E. coli* were recorded at 45 °C on a Bruker 900 MHz spectrometer (Korea Basic Science Institute). NMR spectra of transmembrane (TM) domains of ArcB, QseC, and KdpD expressed in the CF system were recorded at 45° and 37 °C on a Bruker 700 MHz spectrometer (Salk Institute). Both spectrometers are equipped with four radio-frequency channels and a triple-resonance cryoprobe with a shielded z-gradient coil. [^{15}N,^1H] TROSY and TROSY-based (8) HNCO experiments were measured for each selectively [^{15}N,^{13}C]-labeled sample for combinatorial assignment. TROSY-based experiments HNCA, HNCO (9), HNCACB, HNCOCA, HNCOCACB, and HNCACO (10), as well as 3D ^{15}N-resolved TROSY-[^1H,^1H]-NOESY (mixing time 120 ms) were used for traditional assignment of backbone ^1H, ^{15}N, and

^{13}C resonances. Partial side chain assignment was performed using a 3D ^{15}N-resolved TROSY-[^{1}H,^{1}H]-NOESY experiment. Torsion angle restraints were defined from the $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ chemical shift deviations from the "random coil" values (11, 12). Distance constraints for structure calculation were obtained from a 3D ^{15}N-resolved TROSY-[^{1}H,^{1}H]-NOESY experiment collected with the mixing time of 120 ms.

Measurement of the PRE effect was performed as described (13, 14). [^{15}N,^{1}H] TROSY spectra were measured consequently with all cysteine mutants before spin labeling, after the labeling in oxidized and reduced states, and after removal of the spin label. In order to evaluate a possible intermolecular PRE effect, additional [^{15}N,^{1}H] TROSY spectra were measured with the mixed samples containing a 1:1 mixture of uniformly ^{15}N-labeled protein with the "cold" (not labeled with stable isotopes) spin-labeled protein. All the spectra were transformed identically, and their integral intensities were calibrated against the intensities in the spectra of the reduced samples using 8–12 cross-peaks with the minimal relative signal decrease. Distance constraints were derived from the measured PRE effect according to the procedure described in ref. 14.

Solution NMR analysis of H-D exchange.
The forming of the secondary structure of the TM domains of ArcB and KdpD, which were expressed in the p-CF mode, was studied by exchange of backbone labile protons to solvent deuterons. The ^{15}N-labeled proteins, ArcB(1-115) and [C402,409S]-KdpD(397-502) were expressed in the p-CF mode in 99% D_2O or 100% H_2O and solubilized by 5% LMPG in 100% D_2O or H_2O. A comparison of the [^{15}N,^{1}H]-TROSY-HSQC spectra shows significant differences in numbers and integral intensities of the cross-peaks depending on the history of the sample (Figs. S2 *B* and *C* and S3).

The samples, which were expressed, washed, and solubilized in the buffers with the same isotopic composition, showed 100% of the expected TROSY cross-peaks (in H_2O, Figs. S2*B* and S3*A*) or none (in D_2O, Fig. S3*D*), and were used as "positive" and "negative" controls, respectively. When we subsequently used the D_2O solubilization buffer for the sample expressed in H_2O, we detected cross-peaks for only those H-N groups which correspond to α-helical TM regions (Fig. S3*C* and S4*C*, red). Conversely, the protein expressed in D_2O after solubilization in the H_2O buffer showed mostly cross-peaks assigned to the H-N groups from loop and tail regions (Figs. S2*C* and S3*B*, blue), with intensities similar to the positive control spectrum. For the same sample, cross-peaks for the H-N groups located in TM helices were either absent or lost >60% of their intensity as compared to the positive control. Analysis of localization of the HN protons, demonstrating slow exchange to solvent deuterons (Fig. S4), showed that the majority of backbone amide hydrogens located in the TM helices participated in stable hydrogen bonds, which are already preformed in a precipitated protein.

1. Kefala G, Kwiatkowski W, Esquivies L, Maslennikov I, Choe S (2007) Application of Mistic to improving the expression and membrane integration of histidine kinase receptors from *Escherichia coli*. *J Struct Funct Genom* 8(4):167–172.
2. Zheng L, Baumann U, Reymond JL (2004) An efficient one-step site-directed and site-saturation mutagenesis protocol. *Nucleic Acids Res* 32(14):e115.
3. Klammt C, et al. (2004) High level cell-free expression and specific labeling of integral membrane proteins. *Eur J Biochem* 271(3):568–580.
4. Klammt C, Schwarz D, Dotsch V, Bernhard F (2007) Cell-free production of integral membrane proteins on a preparative scale. *Method Mol Biol* 375:57–78.
5. Ichetovkin IE, Abramochkin G, Shrader TE (1997) Substrate recognition by the leucyl/phenylalanyl-tRNA-protein transferase. Conservation within the enzyme family and localization to the trypsin-resistant domain. *J Biol Chem* 272(52):33009–33014.
6. Savage DF, Anderson CL, Robles-Colmenares Y, Newby ZE, Stroud RM (2007) Cell-free complements in vivo expression of the *E. coli* membrane proteome. *Protein Sci* 16(5):966–976.
7. Takegoshi K, Nakamura S, Terao T (2001) 13C–1H dipolar-assisted rotational resonance in magic-angle spinning NMR. *Chem Phys Lett* 344(5–6):631–637.
8. Pervushin K, Riek R, Wider G, Wuthrich K (1997) Attenuated T2 relaxation by mutual cancellation of dipole-dipole coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution. *Proc Natl Acad Sci USA* 94(23):12366–12371.
9. Salzmann M, Pervushin K, Wider G, Senn H, Wuthrich K (1998) TROSY in triple-resonance experiments: new perspectives for sequential NMR assignment of large proteins. *Proc Natl Acad Sci USA* 95(23):13585–13590.
10. Salzmann M, Wider G, Pervushin K, Senn H, Wuthrich K (1999) TROSY-type Triple-Resonance Experiments for Sequential NMR Assignments of Large Proteins. *J Am Chem Soc* 121(4):844–848.
11. Wishart DS, Sykes BD (1994) The 13C chemical-shift index: A simple method for the identification of protein secondary structure using 13C chemical-shift data. *J Biomol NMR* 4(2):171–180.
12. Luginbuhl P, Szyperski T, Wuthrich K (1995) Statistical basis for the use of 13C alpha chemical shifts in protein structure determination. *J Magn Reson Ser B* 109:229–233.
13. Battiste JL, Wagner G (2000) Utilization of site-directed spin labeling and high-resolution heteronuclear nuclear magnetic resonance for global fold determination of large proteins with limited nuclear overhauser effect data. *Biochemistry* 39(18):5355–5365.
14. Roosild TP, et al. (2005) NMR structure of Mistic, a membrane-integrating protein for membrane protein expression. *Science* 307(5713):1317–1321.
15. Dalton JA, Michalopoulos I, Westhead DR (2003) Calculation of helix packing angles in protein structures. *Bioinformatics* 19(10):1298–1299.

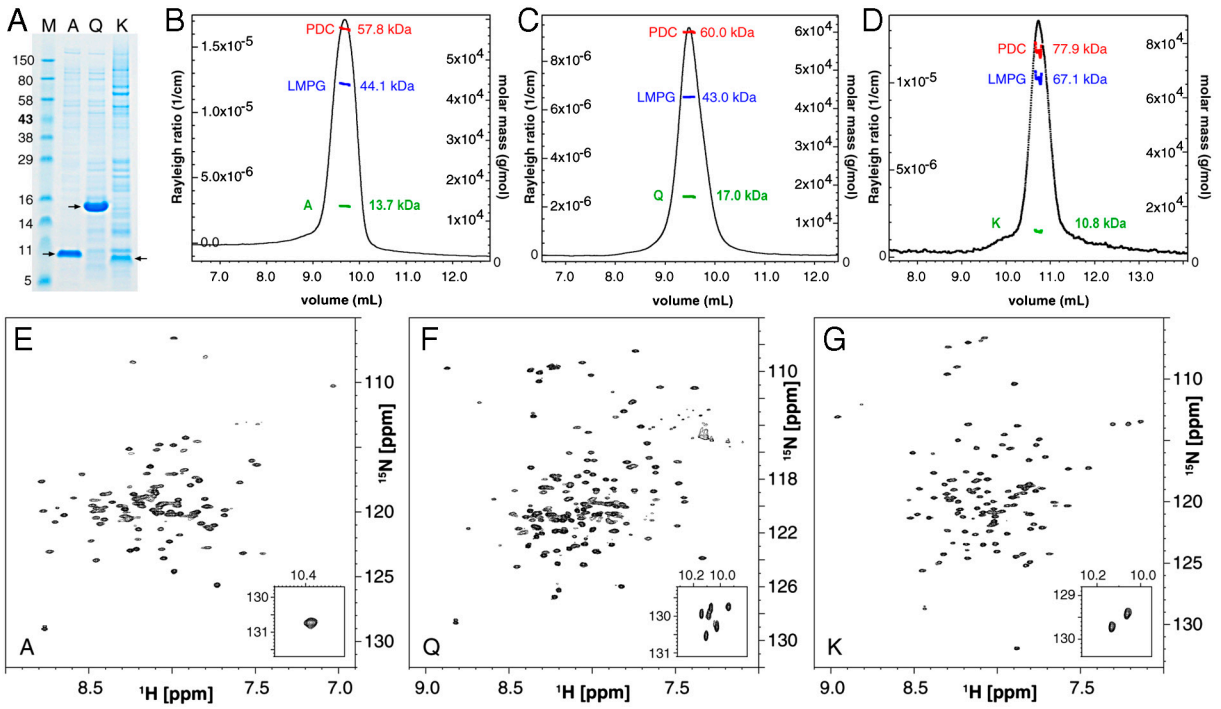

Fig. S1. Characterization of the ArcB(1-115), QseC(1-185), and KdpD(397-502) expressed in the CF system. (*A*) SDS-PAGE analysis of 0.5 μL NMR samples from 1 mL CF precipitate solubilized in 1-myristoyl-2-hydroxy-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (LMPG): Marker (lane M), ArcB(1-115) (lane A), QseC(1-185) (lane Q), and KdpD(397-502) (lane K). Proteins of interest are indicated by arrows. (*B*–*G*) Chromatograms and spectra for ArcB(1-115) (*B* and *E*), QseC(1-185) (*C* and *F*), and KdpD(397-502) (*D* and *G*). (*B*–*D*) Analysis of protein-detergent complexes (PDC) performed by LS coupled with size-exclusion gel chromatography and refracting index measurements. Black lines correspond to the LS signal; red, blue, and green lines show average molar masses of the complexes of the detergent component and of the protein component in PDC, respectively. (*E*–*G*) [^{15}N,^{1}H]-TROSY-HSQC spectra of HKR's TM domains, expressed by CF synthesis and solubilized in 5% LMPG.

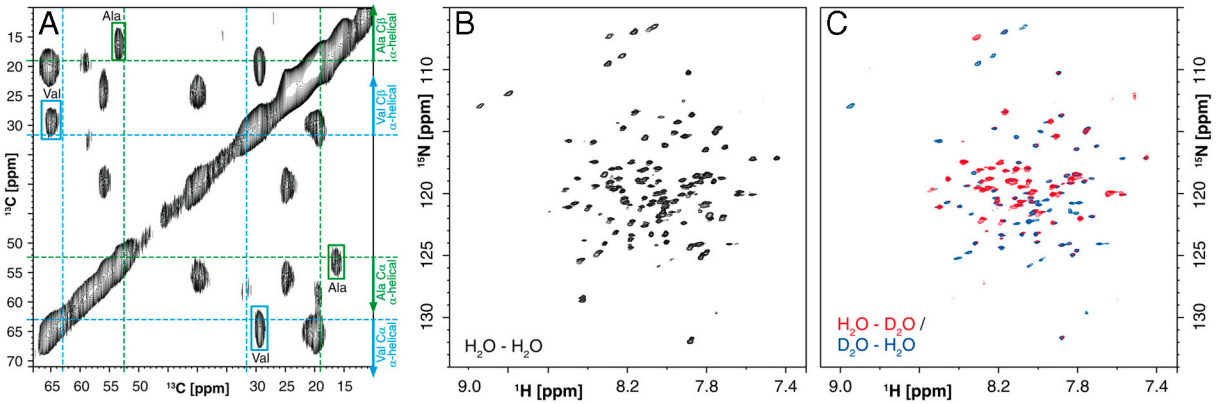

Fig. S2. Analysis of the secondary structure of the [C402, 409S]-KdpD(397-502) precipitated during p-CF synthesis. (*A*) Two-dimensional ^{13}C DARR-NMR spectrum: The p-CF reaction pellet (2 mg) was washed with 20 mM Mes-BisTris buffer, pH 6.0, and was loaded into a 4 mm MAS rotor. The spectrum was acquired on a Bruker Avance 850 spectrometer (213.765 MHz for ^{13}C) using a 4 mm MAS-DVT probe at 273 K and the 14 KHz spinning rate. The green and cyan lines correspond to the random coil $^{13}C^{\alpha}$, $^{13}C^{\beta}$ chemical shift values for valine (Val) and alanine (Ala), respectively; arrows show the regions corresponding to α-helical conformation. (*B* and *C*) [^{15}N,^{1}H]-TROSY-HSQC spectra of [C402, 409S]-KdpD(397-502). The protein was expressed and precipitated during CF reaction and solubilized with 5% LMPG (1-myristoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)]) in 20 mM Mes-BisTris buffer, pH 6.0. (*B*) The spectrum of protein expressed and solubilized in H_2O; (*C*) The overlay of spectra of the proteins expressed/solubilized in H_2O/D_2O (red) or D_2O/H_2O (blue). The protein concentration was 0.3 mM in all samples. The spectra were measured at 45 °C on a 700 MHz Bruker NMR instrument with 400 increments and eight scans per increment. The measurements were started at 10 min after solubilization of the protein.

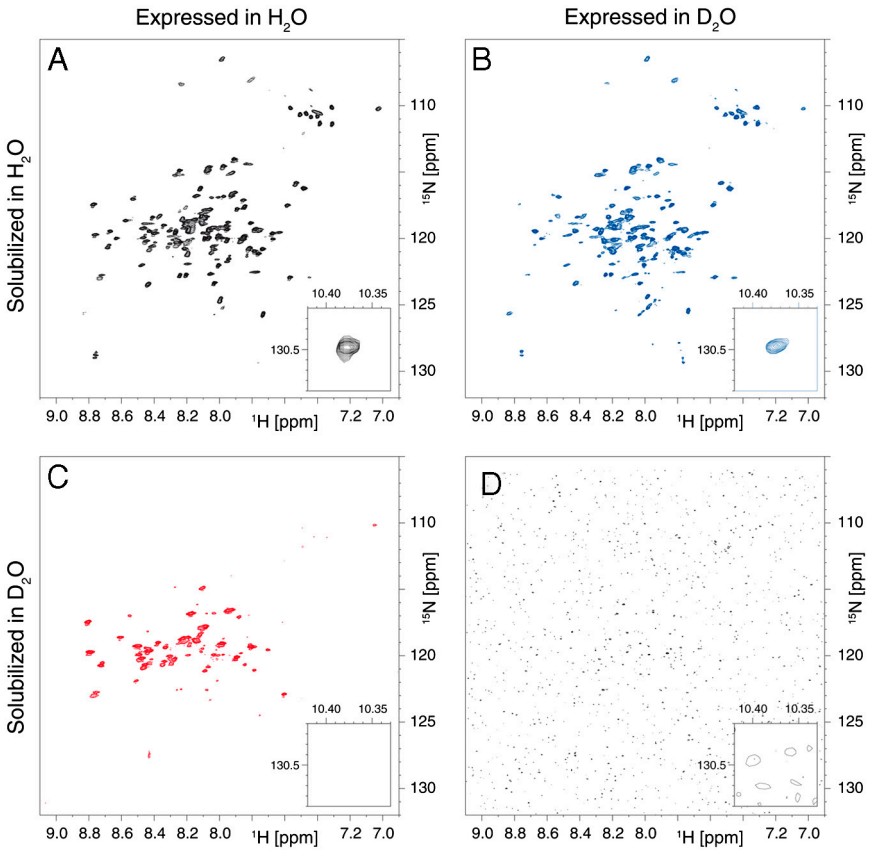

Fig. S3. [^{15}N,^{1}H]-TROSY-HSQC spectra of ArcB(1-115). Protein was expressed and precipitated during CF reaction and solubilized with 5% LMPG (1-myristoyl-2-hydroxy-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]) in 20 mM Bis-Tris buffer, pH 6.0. Protein was expressed/solubilized in (*A*) H_2O/H_2O, (*B*) D_2O/H_2O, (*C*) H_2O/D_2O, and (*D*) D_2O/D_2O. Protein concentration was 0.3 mM in all samples. The spectra were measured at 45 °C on a 700 MHz Bruker NMR instrument with 320 increments and eight scans per increment. The measurements were started 10 min after solubilization of the protein.

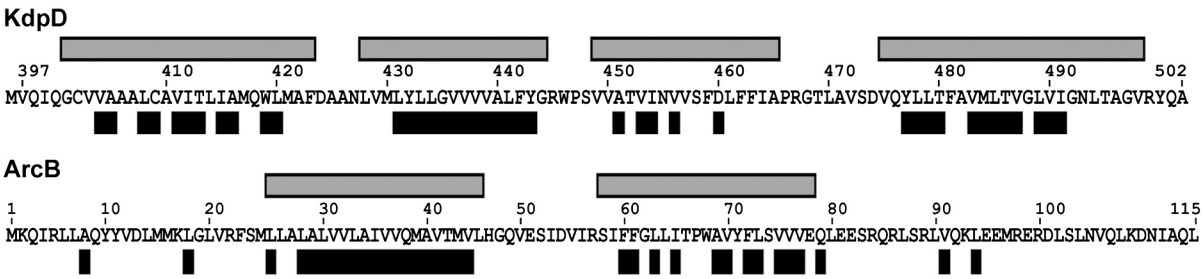

Fig. S4. Regions with slow H-D exchange of backbone amide groups of [C402, 409S]-KdpD(397-502) and ArcB(1-115). TM helical regions are shown as gray bars above the amino acid sequences. Residues with H^N protons demonstrating slow exchange with solvent are marked with black bars below the sequence. The exchange rates were estimated by calculating the ratio between integral intensities of the cross-peaks in [^{15}N,^{1}H]-TROSY-HSQC spectra of samples solubilized in H_2O and in 100% D_2O. The ^{15}N-labeled proteins were expressed and precipitated during CF reaction in H_2O and solubilized with 5% LMPG in 20 mM Mes-Bis-Tris, pH 6.0, H_2O or 100% D_2O buffer. The measurements were started 10 min after protein solubilization.

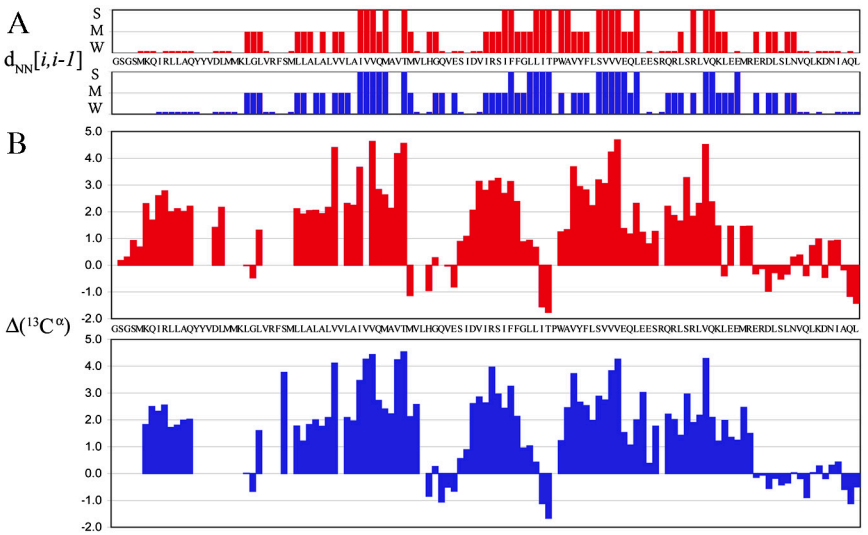

Fig. S5. Summary of structural NMR data collected for ArcB(1-115) expressed in the *E. coli* (red) and in the CF system (blue): (*A*) backbone NOEs, and (*B*) deviation of $^{13}C^{\alpha}$ chemical shifts from the random coil values.

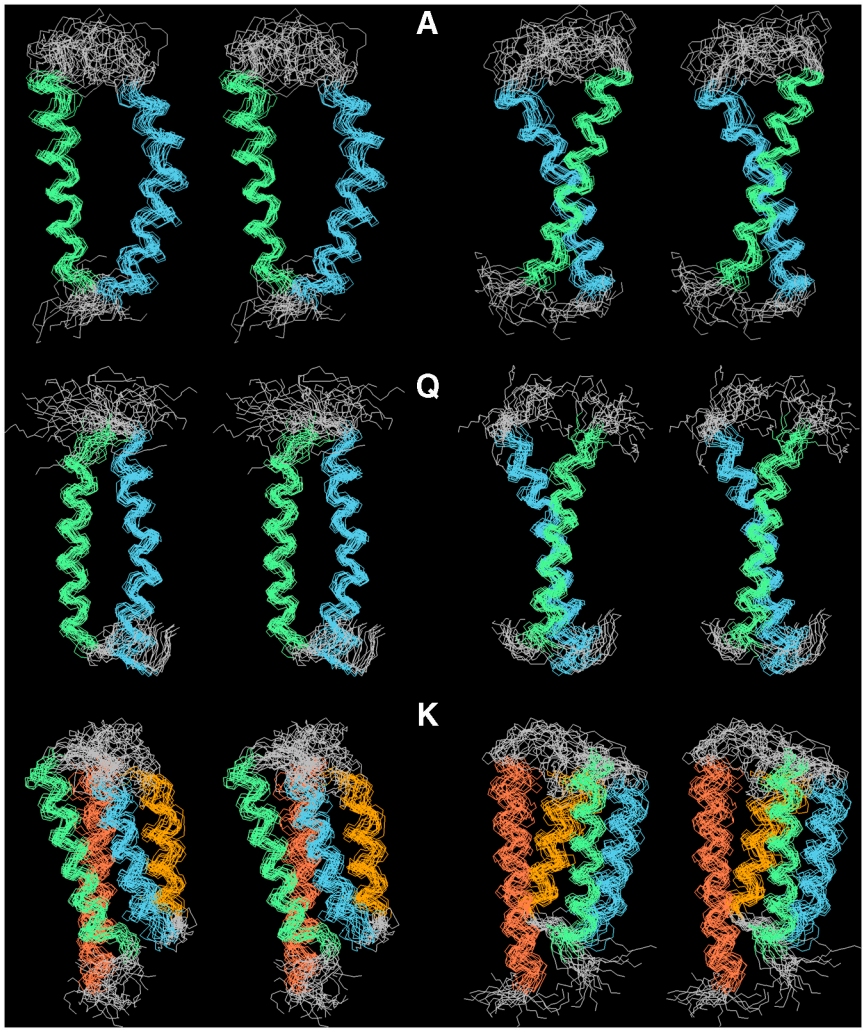

Fig. S6. A stereo view of 20 superimposed structures of (A) ArcB(1-115), (Q) QseC(1-185), and (K) KdpD(397-502). Backbones are shown for the stable regions: ArcB(1-115), residues 20-83; QseC(1-185), residues 10-38 and 156-185; and KdpD(397-502), residues 397-502. Consecutive TM helices are colored in the following order: green, blue, orange, and coral. Structures in the stereo pairs on the right are rotated 90° vertically.

Table S1. Comparison of cost efficiency of the CF system with the standard *E. coli* system

	E. coli, 0.5 L	CF, 1 mL RM
Media / reagents	$2	$42
Materials / concentrators / dialyzers	$5	$4
In-lab T7-polymerase, S30 extract, plasmid prep.	—	$1.3
Labor fermentation / CF setup	$30 (2 h)	$30 (2 h)
Thrombin / detergents / LMPG	$90	$3
labor purification / solubilization	$300 (20 h)	$30 (2 h)
Price per NMR sample (1–3 mg of MP):		
Unlabeled	$427	$108
^{15}N-labeled	$445	$125
^{15}N, ^{13}C-labeled	$515	$150
^{2}H, ^{15}N-labeled	$595	$155
^{2}H, ^{15}N, ^{13}C-labeled	$770	$210

Table S2. Combinatorial selective ^{15}N, ^{13}C labeling scheme for QseC(1-185)

	A	D	E	F	G	I	K	L	M	N	P	Q	R	S	T	V	W	Y
I	C	N	C	C	N	N	C	N	C	N	C	N	N	N	N	C	C	C
II	N	C	C	N	C	C	C	N	C	N	C	N	N	C		N	N	C
III	N	N	C	C	N	N	N	N	C					C	N	C	N	N
IV	N	N	N	C	N	C	C	N	C	N	C	N		N		N	C	C
V	N	N	C	C	N	C	N	C	N		C	N	N	N	N	N	C	C
VI	N	N	N	N	C	N	N	C	N		C		N	C	N	C	C	C
VII	C	C	N	N	N	N	N	C	N					C	N	C	N	N

For each combinatorially labeled sample (I–VII): N denotes ^{15}N labeling, C - 1-^{13}C labeling, and a blank cell means that the amino acid was not labeled in the sample.

Table S3. Packing of TM helical domains

	TM helices	Bend/kink angle	Packing angle	Distance
ArcB	25-45	8.17 ± 2.88	142.0 ± 6.5	11.09 ± 0.98
(1-115)	58-77	22.38 ± 2.15		
QseC	14-34	9.99 ± 2.19	156.5 ± 4.19	11.64 ± 1.04
(1-185)	159-180	25.78 ± 3.37		
KdpD	400-421	9.19 ± 2.17	−168.2 ± 3.6; 21.8 ± 5.3; −156.6 ± 4.1	7.50 ± 2.16 (1-2)
(397-502)	428-445	10.19 ± 2.66	−164.7 ± 5.6; 24.5 ± 4.9	9.36 ± 0.93 (2-3)
	449-464	9.19 ± 3.90	−154.1 ± 5.7	10.26 ± 0.48 (3-4)
	476-497	9.61 ± 3.19		8.81 ± 1.93 (1-4)

Parameters of helix—helix packing were calculated for the final sets of structures using the helix-pairs program (15).