Formation of an Antiparallel, Intermolecular Coiled Coil Is Associated with in Vivo Dimerization of Osmosensor and Osmoprotectant Transporter ProP in *Escherichia coli*†

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ABSTRACT: Membrane transporter ProP from *Escherichia coli* senses extracellular osmolality and responds by mediating the uptake of osmoprotectants such as glycine betaine when osmolality is high. Earlier EPR and NMR studies showed that a peptide replica of the cytoplasmic ProP carboxyl terminus (residues D468−R497) forms a homodimeric, antiparallel, α-helical coiled coil in vitro stabilized by electrostatic interactions involving R488. Amino acid replacement R488I disrupted coiled-coil formation by the ProP peptide, elevated the osmolality at which ProP became active, and rendered the osmolality response of ProP transient. In the present study, either E480 or K473 was replaced with cysteine (Cys) in ProP*, a Cys-less, fully functional, histidine-tagged ProP variant, to use Cys-specific cross-linking approaches to determine if antiparallel coiled-coil formation and dimerization of the intact protein occur in vivo. The Cys at positions 480 would be closer in an antiparallel dimer than those at positions 473. These replacements did not disrupt coiled-coil formation by the ProP peptide. Partial homodimerization of variant ProP*-E480C could be demonstrated in vivo and in membrane preparations via Cys-specific cross-linking with dithiobis(maleimidoethane) or by Cys oxidation to cystine by copper phenanthroline. In contrast, these reagents did not cross-link ProP* with Cys at position 133 or 241. Cross-linking of ProP* with Cys at position 473 was limited and occurred only if ProP was overexpressed, consistent with an antiparallel orientation of the coiled coil in the intact protein in vivo. Although replacement E480C did not alter transporter activity, replacement K473C reduced the extent and elevated the threshold for osmotic activation. K473 may play a role in ProP structure and function that is not reflected in altered coiled-coil formation by the corresponding peptide. Substitution R488I affected the activities of ProP-$(His)_6$, ProP*-E480C, and ProP*-K473C as it affected the activity of ProP. Surprisingly, it did not eliminate cross-linking of Cys at position 480, and it elevated cross-linking at position 473, even when ProP was expressed at physiological levels. This suggested that the R488I substitution may have changed the relative orientation of the C-termini within the dimeric protein from antiparallel to parallel, resulting in only transient osmotic activation. These results suggest that ProP is in monomer−dimer equilibrium in vivo. Dimerization may be mediated by C-terminal coiled-coil formation and/or by interactions between other structural domains, which in turn facilitate C-terminal coiled-coil formation. Antiparallel coiled-coil formation is required for activation of ProP at low osmolality.

Bacteria respond to changes in medium osmolality by modulating their cytoplasmic composition. Osmoregulatory transporters and biosynthetic enzymes mediate solute accumulation as osmolality increases, and mechanosensitive channels mediate solute release as osmolality decreases (*1*−*3*). Although the machinery of osmoregulation has been identified, the mechanism by which cells sense increasing osmolality is not yet understood. That mechanism is being addressed through in-depth study of three systems: H^+-osmoprotectant symporter ProP of *Escherichia coli* (*4*), Na^+-solute symporter BetP of *Corynebacterium glutamicum* (*5*), and ABC transporter[1] OpuA of *Lactococcus lactis* (*6*). Each transporter mediates osmoprotectant uptake at a rate determined by medium osmolality in vivo and after purification

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[1] Abbreviations: ABC transporter, ATP binding cassette transporter; Cu-P, copper(II) *o*-phenanthroline; Cys, cysteine; DMF, *N*,*N*-dimethylformamide; DTME, dithiobis(maleimidoethane); LB, Luria−Bertani medium; MOPS, 4-morpholinopropanesulfonic acid; MTS, methanethiosulfonate; MTSEA, (2-aminoethyl)methanethiosulfonate; MTSET, methanethiosulfonate ethyltrimethylammonium; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.

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and reconstitution in proteoliposomes (*4*−*9*). Transporter ProP of *E. coli* senses extracellular osmolality and mediates the accumulation of osmoprotectants such as proline and glycine betaine (*N*-trimethylglycine) when osmolality is rising or high (*10*).

ProP is a 500 amino acid integral membrane protein and a member of the major facilitator superfamily (*11*). Its structure has been modeled using those of transporters GlpT (*12*) and LacY (*13*) as templates (*14*). LacZ and PhoA fusion analysis and site-directed fluorescent labeling are consistent with that model and with predictions that ProP is comprised of 12 transmembrane helices, with cytoplasmic N- and C-termini (*14*, *15*). The C-terminal sequence of ProP terminates in six to seven of the heptad repeats which characterize α-helical coiled-coil forming proteins (*11*). Studies of synthetic peptides corresponding to residues 456−500 or 468−497 of ProP have shown that residues 468−497 (encompassing four heptads) form an antiparallel, homodimeric α-helical coiled coil of low stability in vitro and constitute the minimal sequence required for coiled-coil formation (*16*, *17*). The coiled-coil structure has been determined by NMR spectroscopy (*18*) (Figure 1).

The presence of basic residues at core heptad "a" positions (H495 and R488) was thought to account for the low stability of the coiled coil in vitro. Unexpectedly, replacement R488I disrupted coiled-coil dimer formation by this peptide replica, providing the first evidence that the orientation of the coiled coil might be antiparallel (*16*). In the NMR structure, the antiparallel orientation appears to be stabilized by electrostatic interactions of R488 with D475 and D478 in adjacent monomers (*18*). A higher osmolality was required to activate the R488I variant of ProP, and it was activated only transiently in response to an osmotic upshift (*16*). Thus the C-terminal coiled coil of ProP is implicated in its osmotic activation. However, some putative ProP orthologues lack the C-terminal coiled-coil domain (*9*). Among them, the ProP orthologue from *C. glutamicum* required a high osmolality for activation, but that activation was sustained (*19*). These results suggest that antiparallel coiled-coil formation by the C-terminal domains of adjacent ProP molecules is required for its activation at low osmolality.

To advance our understanding of key structure−function relationships, we created ProP*, a cysteine- (Cys-) less variant of ProP with a C-terminal six-residue histidine (His) tag. ProP* retains full activity in cells and in proteoliposomes (*15*). Using ProP* as a base, Cys residues can be introduced at specific sites and labeled using thiol-selective reagents such as maleimides or methanethiosulfonates, to which labels may be attached. This approach is yielding a substantial body of structural data for ProP, as for other membrane proteins (*14*, *15*, *20*−*22*). Chemical cross-linking of Cys-containing variants can also be used to determine relative proximities of residues within the protein or to investigate quaternary structure (*23*−*26*).

This study addresses the hypothesis that dimerization of ProP, mediated by or allowing C-terminal coiled-coil formation, occurs in vivo. We showed previously that Cys replacement and spin labeling at heptad "g" position 473 or 480 did not disrupt coiled-coil formation by ProP peptide D468−R497. Dimerization of that peptide caused more exchange and dipolar broadening of the EPR spectrum originating from a spin label at C480 than from one at C473, as expected of an antiparallel orientation (Figure 1) (*17*). We have now created the corresponding ProP variants, ProP*-E480C and ProP*-K473C, and used cross-linking reagents to investigate whether ProP* can form oligomers in vivo and in vitro. Our results indicate that ProP is dimeric in *E. coli* cells. Dimerization is mediated by or allows cross-linking of the C-terminus in an antiparallel coiled coil. Data obtained using ProP variants containing the R488I substitution, ProP*-E480C-R488I and ProP*-K473C-R488I, also suggest that it does not prevent dimerization of the intact protein but rather may cause reorientation of the dimer strands to parallel.

MATERIALS AND METHODS

Materials. DTME [dithiobis(maleimidoethane)] was obtained from Pierce Biotechnology Inc. (Rockford, IL). MTSEA [(2-aminoethyl)methanethiosulfonate] and MTSET [(2-(trimethylammonium)ethyl)methanethiosulfonate] were products of Toronto Research Chemicals (Toronto, Ontario, Canada). DTME and MTSEA were dissolved in *N*,*N*-dimethylformamide (DMF; Fisher Scientific, Nepean, Ontario, Canada) as stock solutions (64 and 30 mM, respectively) and stored at −20 °C prior to use. 1,10-Phenanthroline (ACS grade) was from ICN Biochemicals Inc. (Aurora, OH). $CuSO_4$ (pentahydrate) was from Anachemia. Ampicillin, L-arabinose, β-mercaptoethanol, dithiothreitol, and phenylmethanesulfonyl fluoride (PMSF) were from Sigma Chemical Co. (St. Louis, MO). All other reagents were of the highest grade available. Buffers were prepared as described by Racher et al. (*27*), and solution osmolalities were measured with a Wescor vapor pressure osmometer (Wescor, Logan, UT).

Bacteria, Plasmids, and Molecular Biological Manipulations. ProP and its variants were expressed from the arabinose- and AraC-controlled P_{BAD} promoter of vector pBAD24 (*28*) in the background of *E. coli* WG350 [F^- trp *lacZ rpsL thi* Δ*(putPA)101* Δ*(proU)600* Δ*(proP-melAB)212*] (*11*). Strains expressing ProP-$(His)_6$ (*27*) and ProP-R488I (*16*) were described previously. Strains expressing ProP* and its variants contained plasmid pDC117 [a derivative of vector pBAD24 encoding ProP* (*15*)] or its derivative. Plasmids encoding ProP* variants and plasmid pDC206 [derived from pDC80 (*27*) and encoding ProP-$(His)_6$-E480C] were created by site-directed mutagenesis as previously described (*15*).

Transport Assays. Bacteria were cultivated in NaCl-free MOPS medium or in MOPS medium supplemented with 0.3 M NaCl (*10*), proline uptake rates were measured and analyzed (*10*), and the expression levels of ProP* and its variants were determined (*16*) as previously described. The levels of ProP, ProP-$(His)_6$, and ProP* attained in bacteria cultivated in arabinose-free MOPS medium are both similar and in the physiological range. Since the levels of some other ProP variants were significantly and reproducibly lower, arabinose was added as required to achieve uniform expression among ProP variants. The appropriate arabinose levels were determined by growing each strain in media supplemented with arabinose at various concentrations and then comparing the transporter expression level with those of appropriate controls by Western blotting using an anti-pentaHis-HRP conjugate (Qiagen Inc., Mississauga, Ontario, Canada) and an ECL-Plus visualization system which has been described previously (*15*, *16*).

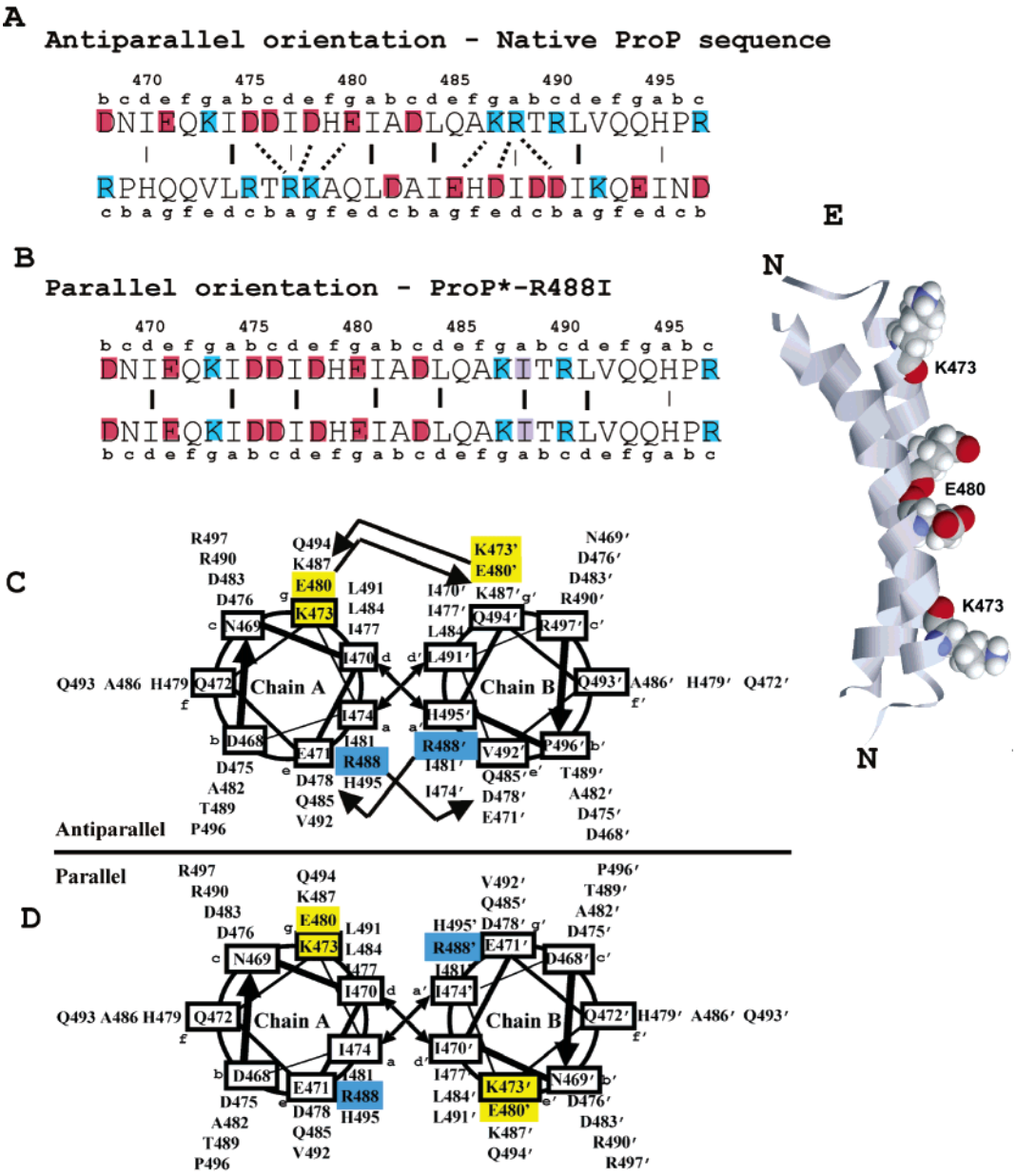

FIGURE 1: Homodimeric α-helical coiled coil formed by residues 468−497 of the ProP C-terminus. (A) Sequence of residues 468−497 of the native ProP C-terminus shown in the antiparallel alignment described by Zoetewey et al. (*18*). (B) Sequence of residues 468−497 of the ProP C-terminus containing the R488I replacement shown in the putative parallel orientation. Heptad a through g positions are indicated. Thick lines indicate a−d and d−a or a−a and d−d interactions across the hydrophobic core. Thin lines indicate interactions between isoleucine and basic residues in the a−d positions in the antiparallel orientation and between His residues in the a positions in the parallel orientation. The R488I substitution is highlighted in lavender. Basic residues are highlighted in blue and acidic residues in red. (C, D) Helical wheels showing the native sequence in the antiparallel and parallel orientations, respectively. R488 is highlighted in blue, and K473 and E480, which were replaced by Cys (both g positions), are highlighted in yellow. These show that, except for R488, the charged residues are not at the dimerization interface. Note that in an antiparallel orientation the g positions of each monomer are on the same side of the coiled coil, while in the parallel orientation they are on opposite sides. The asymmetric arrows represent electrostatic attractions across the dimer interface that may be lost upon replacement of E480 and/or R488 with a nonpolar residue (Ile or Cys). The latter may favor the parallel orientation. However, replacement E480C did not destabilize antiparallel coiled-coil formation by a peptide replica (*17*). (E) NMR solution structure (*18*) of the antiparallel coiled coil formed by a peptide replica of the ProP C-terminal domain, with the N-termini indicated. Residues K473 and E480 (heptad g positions) are shown in CPK. Single Cys residues were placed at the same positions in ProP* variants for cross-linking. Note that Cys at position 473 would be too far apart to be readily cross-linked in the antiparallel orientation.

In Vivo and in Vitro Cross-Linking Experiments. An *E. coli* strain expressing ProP* or its variant was initially grown as a 5 mL overnight culture in LB broth (*29*). One milliliter of the preculture was used to inoculate a 50 mL LB broth. The subcultures were then incubated a further 2−3 h at 37 °C with aeration, and arabinose was added after 1 h if ProP expression was to be induced. For in vitro cross-linking studies, dithiothreitol (15 mM) was normally included in the cell culture at every step until lysis, to prevent spontaneous formation of disulfide linkages between ProP monomers. PMSF (1 mM) was also added during all washes and prior to cell rupture. Cell membranes were prepared by osmotic lysis, as described previously (*15*, *22*). DTME was added directly to the membranes suspended in 20 mM potassium phosphate buffer, pH. 8.0, to give a final concentration of 400 μM or less (<1% solvent concentration). Cross-linking

Table 1: Cross-Linking in Vivo of ProP* Variants Containing Cys at Positions 480 and 473 at Different Expression Levels

	% cross-linked ProP[a]			
	E480C	K473C	E480C-R488I	K473C-R488I
low expression[b]	49 ± 5 ($n = 8$)	0 ± 0 ($n = 7$)	27 ± 8 ($n = 5$)	0 ± 0 ($n = 4$)
moderate expression[c]	51 ± 4 ($n = 5$)	0 ± 0 ($n = 5$)	38 ± 1 ($n = 3$)	24 ± 8 ($n = 5$)
overexpression[d]	65 ± 6 ($n = 7$)	23 ± 4 ($n = 5$)	74 ± 3 ($n = 3$)	55 ± 4 ($n = 4$)

[a] Values represent the mean ± SD of *n* different experiments, each carried out in duplicate. ProP*-E480C was compared to one, two, or all three of the other variants in each experiment. [b] Determined from density analysis of Western blots in experiments such as that shown in Figure 7A. Cells expressing ProP*-E480C or ProP*-K473C were cultivated in LB without arabinose; those expressing ProP*-E480C-R488I and ProP*-K473C-R488I were cultivated in LB with arabinose (33 and 0.7−3.3 μM, respectively) to achieve comparable ProP expression levels. For each variant, the degree of cross-linking did not depend significantly on arabinose concentration in these ranges. [c] Determined from density analysis of Western blots in experiments such as that shown in Figure 7B. Cells expressing ProP*-E480C or ProP*-K473C were cultivated in LB with 0.7−1.7 μM arabinose; those expressing ProP*-E480C-R488I were cultivated in LB with 100−133 μM arabinose, and those expressing ProP*-K473C-R488I were cultivated in LB with 7−17 μM arabinose to achieve comparable ProP expression levels for all variants; the expression level of ProP*-E480C was about 4 times higher than that without induction, determined as described in Materials and Methods. For each variant, the degree of cross-linking did not depend significantly on arabinose concentration in these ranges. [d] Determined from density analysis of Coomassie Blue-stained gels after ProP* purification in experiments such as that shown in Figure 6. Cells expressing ProP* variants were cultivated in LB with 13.3 mM arabinose to overexpress ProP. This resulted in a 75-fold increase in expression of ProP*-E480C, determined as described in Materials and Methods.

proceeded for 10 min at room temperature. (In preliminary experiments, cross-linking by DTME was independent of DTME concentration in the range 10−400 μM and independent of incubation time in the range 3−10 min.) To block free Cys, the membrane-permeant methanethiosulfonate reagent MTSEA or the membrane-impermeant reagent MTSET was added to give a concentration of 0.5−1 mM, as specified in the figure captions, for 10 min at room temperature, immediately before or after incubation with DTME. Three cycles of freeze−thawing in an ethanol/dry ice bath were carried out during MTSET incubations, to ensure penetration into any closed vesicles (*15*). Membranes were then washed via three cycles of resuspension in water and centrifugation. Affinity chromatography using Ni-NTA resin in minicolumns (MicroBioSpin columns; Bio-Rad, Hercules, CA) was used to purify the ProP* variants, as previously described (*15*), but omitting reducing agent from all steps after and including lysis of the cells. Aliquots of purified ProP (approximately 1 μg/μL of protein) were incubated at room temperature for 30 min with or without 1.54 M urea and 0.2 M β-mercaptoethanol. They were then mixed with sample buffer and incubated at 37 °C for 15 min prior to resolution on 12% Tris−glycine precast acrylamide gels (Novex, San Diego, CA) via SDS−PAGE according to the method of Laemmli (*30*). Protein was visualized using Coomassie Brilliant Blue following electrophoresis. Images of gels were obtained using a UVP gel documentation system (DiaMed Inc., Mississauga, Ontario, Canada) with a CCD camera attachment and Macintosh-based data capture and analysis program (UVP-Grab-It). Densitometry was performed using Scion Image (Scion Corp.) software.

For in vivo cross-linking studies, 10 mL aliquots of cells grown in LB medium with no or low concentrations of arabinose were transferred to 30 mL Corex tubes, and 12 mL aliquots of cells grown in LB medium with high arabinose concentrations were transferred to 50 mL Falcon tubes, in all cases prewarmed to 37 °C. DTME was added to give a final concentration of 400 μM. The samples were incubated at 37 °C for 10 min with shaking, followed by addition of 1 mM MTSEA and 10 min further incubation. Cells grown with no or low arabinose concentrations were then washed once with LB medium. Cells grown with high arabinose concentrations were washed three times with 50 mM potassium phosphate buffer containing 100 mM K_2SO_4 at pH 8.

For cross-linking procedures using copper(II) *o*-phenanthroline (Cu-P), aliquots of either a 60 mM stock solution of Cu-P prepared according to Lee et al. (*31*), to give a final concentration of 300 μM Cu-P, or an equal volume of 100 mM sodium phosphate buffer, pH 7.4, was added directly to 12 mL aliquots of cells in which ProP expression had been maximally induced, followed by incubation with gentle agitation for a period of 10 min at 37 °C. Reactions were terminated by adding 1.0 mL of 0.5 M EDTA, pH 8.0, to each sample.

Following cross-linking, cells in which ProP expression had been maximally induced with 13.3 mM arabinose (ProP overexpression) were harvested by centrifugation, processed according to the microscale purification procedure, and analyzed via SDS−PAGE, as detailed above. Cells containing lower levels of ProP were pelleted by centrifugation and resuspended in small volumes (0.5−2.0 mL) in 100 mM potassium phosphate buffer, pH 7.4. Protein concentrations of these suspensions were determined using a BCA assay kit (Pierce, Rockford, IL), using bovine serum albumin (2 mg/mL) as the standard. Cell samples were mixed with sample loading buffer without β-mercaptoethanol, incubated for 15 min at 37 °C, and passed through 30 gauge needles ($^1/_2$ CC insulin syringes; Becton Dickinson, Mississauga, Ontario, Canada) several times. Protein (7−30 μg as specified in the text) was loaded onto 12% or 14% Tris−glycine precast acrylamide gels and resolved via SDS−PAGE, and the proteins were detected via Western blotting as above. The amount of monomer and dimer was quantified by densitometry of stained gels or Western blots. Since dithiothreitol was usually not added when whole cells were used for in vivo cross-linking, the percent dimer formed spontaneously in the absence of DTME (usually less than 10%) was subtracted from that formed in the presence of DTME.

To estimate ProP*-E480C expression in bacteria cultured in LB medium without arabinose (low expression), with 0.7 μM arabinose (moderate expression), or with 13.3 mM arabinose (overexpression) for experiments shown in Table 1, cell samples were analyzed by SDS−PAGE as described above. Samples from cells grown with different arabinose concentrations were all loaded on the same gel (0.1−1 μg of protein for overexpressed cells and 10 μg of protein for

low to moderately expressed ProP). Western blots were done as described above, and the relative amounts of ProP were quantified by densitometry. Low arabinose (0.7 μM) increased ProP expression about 4 times and high arabinose (13.3 mM) increased ProP expression about 75 times, relative to that obtained with no arabinose.

It proved difficult to achieve completely uniform levels of ProP expression among the four strains compared in Table 1, over the limited range of arabinose concentrations used for each strain for moderate induction. However, the degree of cross-linking also did not vary much over that range. Therefore, the degree of cross-linking of the four strains was compared in each experiment even though there were sometimes as much as 3-fold differences in expression among them in a single experiment.

RESULTS

Impacts of Amino Acid Replacements on ProP Function. To facilitate this work, selected residues were replaced with Cys in the background of the Cys-less ProP variant, ProP*. Neither replacement A133C nor replacement G241C affects the activity or expression level of ProP* (*15*). Residue 133 is in transmembrane helix IV and residue 241 is in the central, cytoplasmic loop of ProP (*14*, *15*). In a previous study (*17*) we detected stronger spin−spin interactions between spin labels located on C480 than on C473 of a synthetic peptide corresponding to residues D468−R497 of ProP (Figure 1). The data indicated that the spin labels on residues E480 were located within 10 Å of each other in an antiparallel homodimeric α-helical coiled-coil structure and, thus, close enough for oligomerization of intact ProP to be detected by cross-linking (*17*). We therefore constructed ProP variants ProP*-E480C and ProP*-K473C, anticipating that neither replacement would affect ProP* activity and that the former protein would undergo cross-linking in vivo to a greater extent than the latter.

The osmotic activation profile of ProP*-E480C (Figure 2), like that of ProP-$(His)_6$-E480C (not shown), was similar to that of ProP* (*15*). Analysis of the osmotic activation curves as described by Culham et al. (*10*) yielded osmolalities required for half-maximal activity of 0.230 ± 0.006 and 0.227 ± 0.007 mol/kg for ProP* and ProP*-E480C, respectively. [The corresponding value for ProP-$(His)_6$ is 0.222 ± 0.005 mol/kg (*10*).] The amplitudes of osmotic activation for the E480C variants were similar to those of the parent transporters once a small reduction in expression level due to the Cys replacement was taken into account (compare, for example, the expression levels of ProP* and ProP*-E480C, Figure 2). In contrast, replacement K473C reduced ProP* activity more than 2-fold (Figure 2) and significantly elevated the activation threshold (osmolality at half-maximal activity, 0.300 ± 0.009 mol/kg). Although little alteration in molecular weight would be associated with the K473C replacement, that change visibly altered the electrophoretic mobility of the protein (Figure 2) and hence must have altered the protein structure retained even after SDS treatment. These observations suggest that residue K473 plays an important role in the structure and function of ProP* even though replacement K473C does not alter the propensity of peptide D468−R497 to form a coiled coil (*17*).

We also constructed ProP*-E480C-R488I and ProP*-K473C-R488I, anticipating that they would not be cross-linked, since replacement R488I disrupted coiled-coil formation by the peptide ProP-I456−E500. The same replacement elevated the threshold for osmotic activation of wild-type ProP and rendered that activation transient (*16*). Replacement R488I also had these effects on other transporter variants relevant to this study (Figures 2 and 3). As a result, the activities of the R488I variants after cultivation in NaCl-free medium and assay in NaCl-supplemented medium were reduced approximately 2-fold (Figure 3, black bars). Even

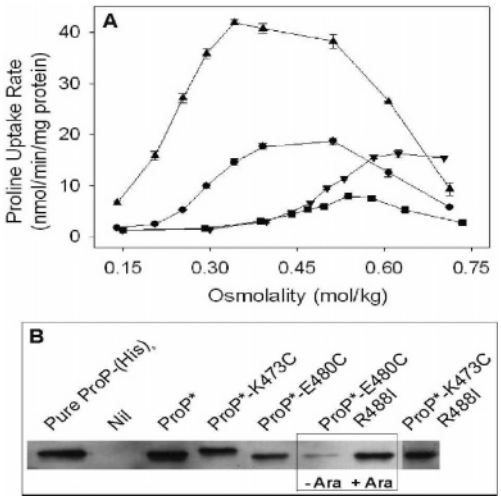

FIGURE 2: Osmotic activation profiles. The proline uptake activities and expression levels of the ProP* variants (panels A and B, respectively) were measured as described in Materials and Methods using assay mixtures adjusted to the indicated osmolalities with NaCl. With the exception of those expressing variants ProP*-E480C-R488I and ProP*-K473C-R488I (induction with 100 and 17 μM arabinose, respectively, to compensate for particularly low levels of expression), bacteria were cultivated in the absence of arabinose. Panel A: triangles, ProP*-E480C; circles, ProP*-K473C; squares, ProP*-E480C-R488I; inverted triangles, ProP*-K473C-R488I.

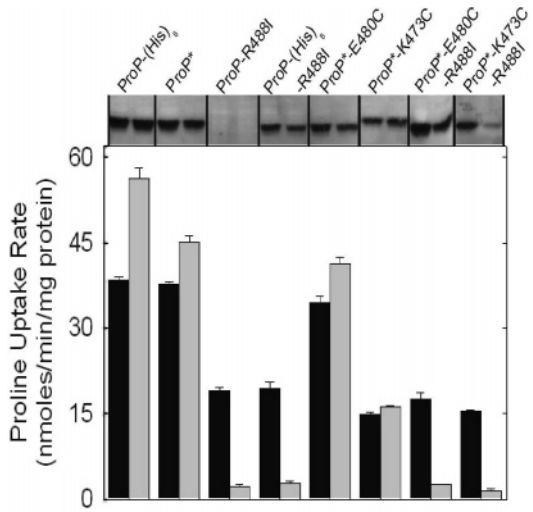

FIGURE 3: Expression levels and activities of ProP variants. The expression levels and proline uptake activities of the ProP* variants (top and bottom, respectively) were measured as described in Materials and Methods. Bacteria were cultivated in NaCl-free MOPS medium (black bars) or in MOPS medium supplemented with 0.3 M NaCl (gray bars), and initial rates of proline uptake were determined in the MOPS medium supplemented with 0.3 M NaCl. Bacteria expressing variants ProP*-E480C-R488I and ProP*-K473C-R488I were cultured with 100 and 17 μM arabinose, respectively, to induce protein expression. Note that ProP-R488I lacks the six-histidine tag detected by Western blotting.

this activation was transient, as illustrated by the fact that very little transport activity could be detected when cells expressing the same variants were cultivated and transport activity was measured in NaCl-supplemented medium (Figure 3, gray bars). Note that, for the wild-type transporter (*16*), ProP-$(His)_6$, ProP*, ProP*-E480C, and ProP*-K473C, osmotic activation was sustained indefinitely (the gray bars were comparable to or taller than the black bars in Figure 3). Introduction of replacement R488I significantly reduced transporter expression in all backgrounds tested (e.g., variant ProP*-E480C-R488I, Figure 2B). However, culturing with arabinose at empirically determined concentrations increased expression of those variants to similar levels as those of their appropriate comparators (Figures 2B and 3). Unexpectedly, variant ProP*-K473C-R488I was routinely expressed at a lower level when bacteria were cultured in NaCl-supplemented MOPS medium than when they were cultured in NaCl-free MOPS medium at the same arabinose concentration (17 μM). This contributed to the particularly low activity of that variant in bacteria cultivated at high osmolality (Figure 3, gray bar).

Chemical Cross-Linking Occurs in Membrane Preparations and Is Specific to ProP-E480C*. For chemical cross-linking, we chose the Cys-reactive, homobifunctional, and cleavable reagent dithiobis(maleimidoethane) (DTME). DTME has a maximal cross-linking span of just over 13 Å and contains an internal disulfide which may be cleaved upon treatment with reducing agents. Interestingly, DTME has also recently been used as a membrane-permeant cross-linking reagent in isolated mitochondria and in mammalian cells (*32*, *33*), suggesting the potential utility of this reagent as an in vivo cross-linker for bacterial cells.

For our initial cross-linking experiments, bacteria were induced to overexpress ProP*-E480C by cultivating them in LB medium supplemented with 13.3 mM arabinose. Membranes obtained via osmotic lysis were treated with DTME, the transporter was recovered by small-scale affinity purification, and it was analyzed via SDS−PAGE with or without β-mercaptoethanol (Figure 4). Like other integral membrane proteins, ProP has an electrophoretic mobility (that of a 40−45 kDa protein) that is greater than expected from its calculated molecular mass (54.9 kDa) (*4*, *11*). In the absence of reductant, the protein recovered from DTME-treated membranes migrated exclusively with the electrophoretic mobility of a 90 kDa protein (Figure 4B, left lane). The protein from the corresponding solvent-treated control samples migrated as a mixture of proteins with apparent molecular masses of 40 and 90 kDa (Figure 4A, left lane). In the presence of β-mercaptoethanol, most of the protein from the DTME-treated and solvent control samples had an apparent molecular mass of 40 kDa (Figure 4, right lanes), indicating that the protein with an apparent molecular mass of 90 kDa was a dimeric form of ProP*-E480C.

To determine if Cys outside of the C-terminal domain could also be cross-linked, we also determined whether other Cys-containing variants could be cross-linked by DTME. Cys residue 133, also present in wild-type ProP, is predicted to occur in transmembrane helix IV. In contrast to ProP*-E480C, ProP*-A133C remained monomeric after DTME treatment of membranes (Figure 5A). Note that dimerization of ProP*-E480C in the absence of DTME and reducing agent (Figure 4A, lane 1, and Figure 5A, lane 3) was higher than

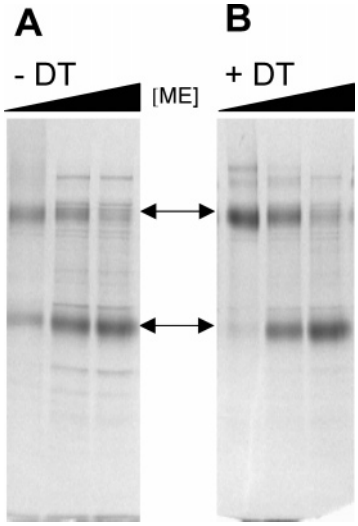

FIGURE 4: DTME-mediated cross-linking of ProP*-E480C in *E. coli* membranes. Bacteria were cultivated in LB, and overexpression of ProP*-E480C was induced by arabinose (13.3 mM). Cell lysis, membrane isolation, DTME cross-linking, membrane solubilization, and microscale purification of ProP*-E480C were as described in Materials and Methods. Membranes were treated with the cross-linker DTME [+DT (0.4 mM)] or with an equal volume of DMF solvent (−DT). Protein samples (approximately 20 μg per well) were loaded on SDS−PAGE gels in sample buffer containing 0, 14, or 140 mM β-mercaptoethanol (ME, increasing concentration indicated by increase in width of wedges). The arrows indicate the positions of 40 and 90 kDa molecular mass markers.

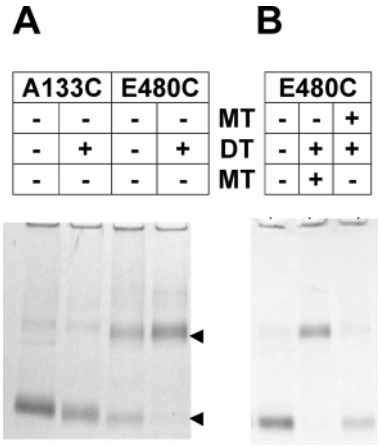

FIGURE 5: Cross-linking is not apparent for ProP*-C133 or MTSET-blocked ProP*-E480C. Cross-linking experiments were carried out on membranes obtained from arabinose-induced cells as described in the legend to Figure 4 and Materials and Methods. (A) DTME cross-linking of ProP*-E480C compared to that of ProP*-A133C. The images of nonreducing SDS−PAGE gels (no β-mercaptoethanol) show ProP* from membranes treated with DTME [+DT (0.4 mM)] or DMF solvent (−DT). (B) DTME cross-linking of ProP*-E480C is blocked by prior addition of MTSET (MT). Dithiothreitol (15 mM) was added to the cell culture, as well as at every step until cell lysis, to prevent spontaneous formation of disulfide linkages between ProP monomers. The images of nonreducing SDS−PAGE gels show ProP*-E480C as in (A). Lanes are labeled with treatments and orders of addition for MTSET (MT) (0.8 mM) and DTME (DT) (0.4 mM). Arrows indicate the positions of protein standards at 40 and 90 kDa for both gels.

for wild-type ProP or ProP* (not shown) or for other Cys-containing variants such as ProP*-A133C (Figure 5A, lane 1). To prevent spontaneous disulfide bond formation by ProP*-E480C in all subsequent experiments on membranes, dithiothreitol was added to the cultures immediately before

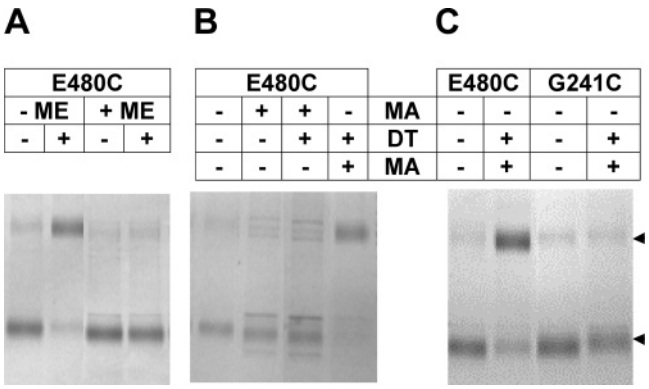

FIGURE 6: DTME-mediated in vivo cross-linking of ProP*-E480C in intact cells. Cross-linking experiments were carried out on arabinose-induced cells as described in the legend to Figure 4 and Materials and Methods. (A) Bacteria were incubated in the growth medium with DTME (DT) [0.4 mM (+)] or DMF alone (−). SDS−PAGE was performed in the presence (+) or absence (−) of β-mercaptoethanol (ME). (B) Bacteria were incubated in the presence of MTSEA (MA) (1 mM) alone, prior to or after incubation with DTME (DT) (0.4 mM), in the order indicated. Protein samples were analyzed via nonreducing SDS−PAGE. (C) Comparison of in vivo DTME cross-linking efficiency for ProP*-E480C and for ProP*-G241C. Bacteria were incubated with either DTME (DT) or solvent, as indicated, followed by MTSEA (MA). Protein samples were analyzed via nonreducing SDS−PAGE. Arrows indicate the positions of protein standards at 40 and 90 kDa for each gel.

harvest, as well as in all steps preceding cross-linking. This significantly reduced the amount of dimer observed in the absence of DTME (Figure 5B, lane 1).

To ensure that Cys residues were being selectively cross-linked, we preincubated membranes containing ProP*-E480C with the negatively charged, Cys-specific methanethiosulfonate reagent MTSET. Subsequent treatment with DTME failed to cause cross-linking (Figure 5B, rightmost lane). MTSET added after cross-linking had no effect (Figure 5B, center lane).

Residue-Specific Chemical Cross-Linking Also Occurs in Vivo. Cross-linking of ProP*-E480C in situ in membrane preparations suggested that ProP would also be dimeric in vivo. To confirm this, intact cells overexpressing ProP*-E480C were treated with DTME in LB medium. Almost all of the protein recovered from these cells was dimeric, indicating that DTME penetrated both membranes of the cells and that ProP was dimeric in vivo under these conditions (Figure 6A). After subtraction of the amount of spontaneously cross-linked dimer, DTME caused cross-linking of an additional 65% of ProP from cells in which it was overexpressed (Table 1, overexpression). Addition of β-mercaptoethanol (ME) caused reduction of the 90 kDa dimer to 40 kDa monomers (Figure 6A). No other bands of differing M_r were observed, indicating that cross-linked ProP*-E480C is a homodimer.

To show that cross-linking did not result from disulfide adduct formation after DTME addition and during processing of the cells and membranes, excess MTSEA was added to cells in growth medium before or after DTME treatment. MTSEA is a methanethiosulfonate reagent which modifies Cys side chains and is membrane permeant in its uncharged form. Prior addition of MTSEA blocked DTME cross-linking under these conditions (Figure 6B). If MTSEA was added following DTME treatment, the cross-linked dimer still formed (Figure 6B, rightmost lane). Thus the observed dimers did not arise from spontaneous formation of disulfide linkages during the processing of samples.

To further test the specificity of cross-linking, we also determined if ProP*-A133C or ProP*-G241C could be cross-linked in vivo. The Cys in the latter variant occurs in the large, central cytosolic loop of ProP (*15*). When cells containing either variant were treated with DTME followed by MTSEA, both ProP variants were monomeric on SDS gels (shown only for G241C in Figure 6C). In contrast, 23% of ProP*-K473C from cells overexpressing this variant was cross-linked under these conditions, significantly less than for ProP*-E480C (Table 1, overexpression).

Cross-Linking of ProP-E480C Occurs under Physiological Conditions, and the Coiled-Coil Orientation Is Antiparallel.* Further experiments were performed to determine whether dimerization occurred under more physiological conditions and whether the observed cross-linking reflected antiparallel, homodimeric α-helical coiled-coil formation by the C-terminal domains of adjacent ProP* molecules. In this experimental system, *proP* is expressed from vector pBAD24 under the control of the P_{BAD} promoter. When the bacteria are cultivated in LB without arabinose, the ProP level is similar to that observed if *proP* is expressed from its own promoter (data not shown). DTME cross-linking analysis was performed using *E. coli* cells cultivated in LB medium without arabinose, thereby avoiding overexpression of the transporter. After cross-linking, the cells were solubilized, and the electrophoretic mobilities of the ProP* variants were analyzed by Western blotting. Under these conditions, the expression of ProP*-E480C was approximately 75 times less than when cells were induced with 13.3 mM arabinose (data not shown). Densitometric analysis indicated that approximately half of ProP*-E480C was dimeric (Table 1, low expression; Figure 7A). Thus dimerization decreased somewhat with a large decrease in ProP concentration in the membrane. ProP-$(His)_6$-E480C was cross-linked to a similar extent (not shown), indicating that the Cys-less background of ProP*-E480C did not influence dimerization and cross-linking.

No dimerization was observed for variant ProP*-K473C under these conditions (cross-linked dimers could not be detected even when a greater amount of cell extract was loaded on the SDS gel as in Figure 7A) (Table 1, low expression). Lower cross-linking was expected for ProP*-K473C since, in the antiparallel coiled coil, the distance between C473 residues (approximately 27 Å) exceeds the span of DTME (13.3 Å). Spin labels attached to C473 in a peptide replica of the ProP C-terminus interacted by spin−spin interactions much less than spin labels bound to C480 (*17*). The 23% cross-linking of ProP*-K473C when overexpressed probably reflects transient contacts of the C-termini when the ProP concentration in the cells is high.

The R488I Substitution May Cause Reorientation of the C-Termini from Antiparallel to Parallel. Replacement R488I disrupted the coiled coil formed by ProP peptide I456−E500 (*16*). We investigated the impact of this mutation on cross-linking at different ProP expression levels by adjusting the arabinose concentration during cell culture. When the ProP variants were expressed at low levels, mutation R488I decreased cross-linking of ProP*-E480C from 49% to 27% (Table 1, low expression; Figure 7A). Even this degree of

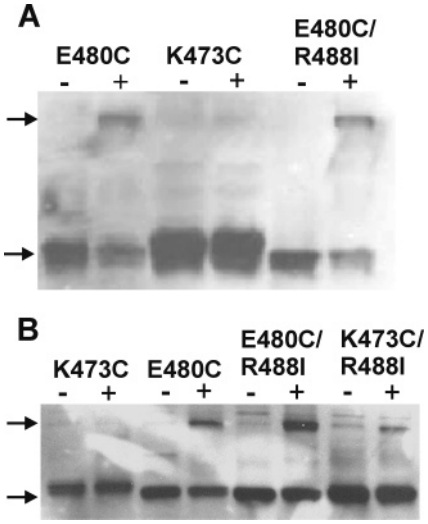

FIGURE 7: (A) Cross-linking of variants ProP*-E480C-R488I and ProP*-K473C compared to ProP*-E480C at low expression levels in intact cells. Cells expressing ProP*-E480C or ProP*-K473C were cultivated in LB without arabinose; those expressing ProP*-E480C-R488I were cultivated in LB with 100 μM arabinose to achieve comparable ProP expression levels. Cells were incubated in the presence (+) of DTME (0.4 mM) or solvent control (−) as indicated, followed by addition of MTSEA (0.5 mM) in all cases. Whole cell extracts (15 μg of protein per well for ProP*-E480C, 7 μg per well for ProP*-E480C-R488I, and 30 μg per well for ProP*-K473C) were analyzed by nonreducing SDS−PAGE on 12% Tris−glycine gels. Western blotting was performed as described in Materials and Methods. The position of the 40 and 90 kDa standards is indicated by arrows. (B) Cross-linking of variants ProP*-E480C-R488I, ProP*-K473C-R488I, and ProP*-K473C compared to ProP*-E480C at moderate expression levels in intact cells. Cells expressing ProP*-E480C or ProP*-K473C were cultivated in LB with 0.7 μM arabinose, those expressing ProP*-E480C-R488I were cultivated in LB with 100 μM arabinose, and those expressing ProP*-K473C-R488I were cultivated in LB with 17 μM arabinose to achieve comparable ProP expression levels for all variants; for ProP*-E480C the expression level was about 4 times higher than that without induction. Cells were incubated in the presence (+) of DTME (0.4 mM) or solvent control (−) as indicated, followed by addition of 0.5 mM MTSEA in all cases. Whole cell extracts (10 μg of protein in each case) were analyzed by nonreducing SDS−PAGE on 14% Tris−glycine gels. Western blotting was performed as described in Materials and Methods. The position of the 40 and 90 kDa standards is indicated by arrows.

cross-linking of ProP*-E480C-R488I was unexpected, since replacement R488I in the ProP C-terminal peptide powerfully disrupted coiled-coil dimer formation (*16*). Neither ProP*-K473C nor ProP*-K473C-R488I was cross-linked under these conditions (Table 1, low expression).

Since ProP overexpression affected cross-linking of ProP*-E480C and ProP*-K473C, the effect of overexpression on cross-linking of ProP*-E480C-R488I and ProP*-K473C-R488I was determined. Both variants were then highly cross-linked, similar to ProP*-E480C, and much more than ProP*-K473C under these conditions (Table 1, overexpression).

To determine the effect of more moderate elevations of ProP expression on cross-linking, lower concentrations of arabinose (adjusted for each strain as described in Materials and Methods) were used. For ProP*-E480C, this resulted in approximately 4-fold higher expression than was obtained in the absence of arabinose. This moderate increase in expression resulted in cross-linking of 24% of ProP*-K473C-R488I compared to 38% for ProP*-E480C-R488I and 51% for ProP*-E480C, whereas ProP*-K473C was still not cross-linked under these conditions (Table 1, moderate expression; Figure 7B). The persistence of cross-linking of E480C despite the R488I substitution and the increased cross-linking of ProP*-K473C-R488I compared to ProP*-K473C suggested that the R488I replacement increased the tendency of the C-terminal strands to orient parallel to each other rather than antiparallel, thus allowing cross-linking of Cys located at both the 473 and 480 positions.

Oxidative Cross-Linking Was Also Specific to C480. Chemical cross-linking can overestimate protein oligomerization by trapping proteins in an oligomeric state, particularly as the length and flexibility of the bifunctional reagent increase. Therefore, a copper(II) *o*-phenanthroline (Cu-P) complex was also used to cross-link ProP*-E480C in vivo. This reagent produces oxidizing conditions in vivo and in vitro, promoting interhelical cross-linking within membrane proteins containing two Cys within close proximity (*31*, *34*). We treated bacteria expressing ProP*-E480C or ProP*-A133C with Cu-P. Cu-P caused disulfide cross-linking of approximately 25% of the ProP*-E480C in arabinose-induced cells (results not shown), compared to the non Cu-P treated controls. The proportion of the protein that was dimeric following reducing SDS−PAGE was nearly the same as for the buffer control, indicating cleavage of the disulfide formed. Therefore, the efficiency of dimer formation via this method was much lower than that observed for DTME-treated cells and membranes. By comparison, protein isolated from cells expressing ProP*-A133C showed no significant change in dimer/monomer ratio when treated with Cu-P and analyzed under reducing or nonreducing conditions. Again, cross-linking was specific to residues brought into close proximity within the coiled-coil structure.

DISCUSSION

Investigations of osmosensing and osmoregulation by ProP have until recently been limited to measurements of proline uptake activity in cells and in proteoliposomes in response to osmotic upshifts or downshifts. Construction of ProP*, a Cys-less platform for structural studies of the protein, is allowing us to test structural models of ProP via Cys replacements and Cys-specific labeling (*14*, *15*). In this study, Cys replacements were used to investigate the oligomerization state of ProP and assess the ability of the C-termini to form an antiparallel coiled-coil dimer in vivo.

When membranes derived from *E. coli* cells overexpressing ProP*-E480C were treated with the homobifunctional cleavable cross-linker, DTME, a band corresponding to a dimer was identified on SDS−PAGE under nonreducing conditions (Figure 4). Nearly 100% of the recovered ProP variant was cross-linked under the conditions tested. Similar cross-linking results were obtained when DTME was added to *E. coli* cells overexpressing ProP*-E480C (Figure 6). No other bands of M_r differing from those of monomeric or dimeric ProP were observed before or after reduction, indicating that no other protein was cross-linked to ProP. In contrast, these reagents did not cross-link other ProP* variants with a single Cys located outside the C-terminal coiled coil [e.g., ProP*-A133C (Figure 5), ProP*-G241C (Figure 6)], indicating that cross-linking is not due to random collisions. CuP was less efficient at cross-linking the Cys at position 480 presumably because the distance between them in an antiparallel dimer is greater than 5 Å.

DTME treatment also yielded cross-linked dimers under conditions in which ProP*-E480C was present in the membrane at physiological levels. A smaller proportion of the protein was cross-linked than for induced cells, suggesting that dimerization increases as ProP concentration in the membrane increases. However, the results show that the C-termini of some molecules of ProP are in close proximity in vivo at physiological levels.

Variants ProP*-K473C, ProP*-E480C-R488I, and ProP*-K473C-R488I were also examined to further test the hypothesis that dimerization of ProP is associated with formation of an antiparallel, intermolecular coiled coil. Failure of variant ProP*-K473C to cross-link, except when overexpressed (Figure 7), was consistent with the postulated, antiparallel orientation of the C-terminal coiled coil. Unexpectedly, substitution R488I reduced but did not eliminate cross-linking of ProP*-E480C, and it enhanced cross-linking of ProP*-K473C (Table 1). It has been suggested that the antiparallel orientation of the strands in the native ProP coiled coil is favored by the overall dipolar nature of residues 468−497 and by specific electrostatic interactions of R488 (at a heptad "a" position) with aspartates on the opposing strand (*18*) (Figure 1A). Replacement R488I could favor parallel coiled-coil formation by diminishing those electrostatic interactions while extending the continuous array of paired, core hydrophobes (hydrophobic residues in heptad "a" and "d" positions) in the parallel orientation from 5 to 7 (Figure 1B). Thus placement of I at position 488 may have improved the propensity of the structure to form the parallel coiled coil as originally predicted by Culham et al. (*16*).

For the I488 variants, formation of a parallel coiled coil and cross-linking at C473 or C480 may seem unlikely because the I488 variant of peptide ProP-I456−E500 did not form a coiled-coil structure at low concentration (*16*) and the available Cys would be on opposite sides of a parallel coiled-coil dimer (Figure 1D). However, association of the C-termini would be favored more in the intact protein, where freedom of motion is restricted because ProP is integrated in the membrane, than in the isolated peptides, particularly if the dimer interface extends beyond the coiled-coil domain. Analytical ultracentrifugation showed the I488 variant of peptide ProP-I456−E500 to be in a monomer−trimer equilibrium at high concentration (*16*). In the intact protein, two additional coiled-coil heptads (I456−N469) are found upstream from the sequence that forms an antiparallel coiled coil in vitro. These may contribute to parallel coiled-coil formation by the I488 variants. The adjacent, highly acidic sequences D468−D483 would be expected to repel each other in a parallel homodimer (Figure 1D), and this end of the coiled coil may be partly unraveled. The resulting unraveling may introduce flexibility that allows the Cys of each monomer to transiently come together closely enough to be cross-linked. Once cross-linked, the monomers remain covalently dimerized. The repulsion of the dimer strands would increase upon replacement of K473 with Cys (Figure 1B). This may account for the lower degree of cross-linking of ProP*-K473C-R488I relative to ProP*-E480C-R488I (Table 1). Alternatively, the greater cross-linking of K473C in the R488I background could be due to structural perturbations in the C-terminal domain or to altered alignment of the dimer strands while still in the antiparallel orientation, due to loss of R488 interactions with D475 and D478 in the adjacent monomer. These possibilities can be tested through structural study of the relevant peptides. They are important because association of a phenotype (transient ProP activation) with orientational switching of a coiled coil would be an unusual biological phenomenon.

The cross-linking approach cannot be used to quantify the amount of monomer and dimer present. It can overestimate dimerization following transient association as discussed above. Cross-linking with DTME and CuP might also underestimate the amount of dimer due to cleavage of the cross-linked dimer in the reducing environment of the cytoplasm. However, the fact that both monomer and dimer are detected under most conditions and the fact that the amount of dimer increases with ProP concentration in the membrane, following induction, suggest that ProP is in a monomer−dimer equilibrium in the cytoplasmic membranes of *E. coli* cells. Dimerization may be mediated by coiled-coil formation by the C-terminus, or it may be mediated by interaction between other structural domains, which in turn allows C-terminal coiled-coil formation. Monomer−monomer interactions within the dimer may not be strong, however, as cross-linking controls and previous experience with large-scale protein purifications have indicated that ProP migrates mostly as monomers on nonreducing and reducing SDS gels. Cross-linking of ProP with the R488I substitution shows that this substitution does not prevent ProP dimerization.

A number of techniques, including cross-linking (*35*−*37*), have been used to show that some secondary transport proteins form dimers or higher order oligomers [reviewed by Veenhoff et al. (*23*)]. In many cases oligomerization affects transporter function even if each individual subunit seems to contain a complete translocation pathway. The crystal structures of OxlT, GlpT, and LacY and other data indicate that large conformational changes occur in order for substrates to be translocated across the membrane (*38*, *39*). Although other transporters appear to function as monomers, cooperation between monomers may play a role in reorienting the empty binding site of two oligomeric sugar transporters, LacS and GLUT1 (*37*). For other systems, including NhaA (*36*) and Band 3 (*40*), oligomerization is associated with regulation of transporter activity rather than with the catalytic cycle, per se. Mutation R488I renders osmotic activation of ProP transient. Perhaps antiparallel coiled-coil formation by a ProP dimer stabilizes it in an active conformation, whereas lack of or parallel coiled-coil formation precludes formation of the correct structure for ProP. Some ProP orthologues lack the C-terminal coiled-coil sequence, and variants have been constructed in which an existing ProP coiled coil is disrupted by mutation. Those variants with a coiled-coil structure activate at lower extracellular osmolalities than those without it (*16*, *19*; Y. Tsatskis and J. M. Wood, unpublished data). Thus the antiparallel coiled coil modulates the sensitivity of ProP to osmotic upshifts rather than acting as an osmosensor, per se.

While ProP acts alone as both an osmosensor and an osmoregulator in proteoliposomes, cytoplasmic protein ProQ amplifies the activity attained by ProP in osmotically stressed cells (*41*). Elimination of ProQ does not affect *proP* transcription or cellular levels of the ProP protein (*41*, *42*). Thus ProQ may further stabilize ProP in its osmotically activated conformation, perhaps by associating with the

C-terminal domain (*42*). Replacement K473C clearly impaired the osmotic activation of ProP (Figures 2 and 3A) even though it did not impair coiled-coil formation by a replica of the ProP C-terminus (*17*). K473 may be involved in a regulatory interaction of the ProP C-terminus with ProQ or with ProP sequences outside the coiled-coil domain.

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