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The role of the carboxyl terminal α-helical coiled-coil domain in osmosensing by transporter ProP of *Escherichia coli*

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Concentrative uptake of osmoprotectants via transporter ProP contributes to the rehydration of *Escherichia coli* cells that encounter high osmolality media. A member of the major facilitator superfamily, ProP is activated by osmotic upshifts in whole bacteria, in cytoplasmic membrane vesicles and in proteoliposomes prepared with the purified protein. Soluble protein ProQ is also required for full osmotic activation of ProP *in vivo*. ProP is differentiated from structural and functional homologues by its osmotic activation and its *C*-terminal extension, which is predicted to form an α-helical coiled-coil. A synthetic polypeptide corresponding to the *C*-terminus of ProP (ProP-p) formed a dimeric α-helical coiled-coil. A derivative of transporter ProP lacking 26 *C*-terminal amino acids was expressed but inactive. A derivative harbouring amino acid changes K460I, Y467I and H495I (each at the core, coiled-coil '*a*' position) required a larger osmotic upshift for activation than did the wild type transporter. The same changes extended, stabilized and altered the oligomeric state of the coiled-coil formed by ProP-p. Amino acid change R488I (also at the '*a*' position) further increased the magnitude of the osmotic upshift required to activate ProP, reduced the activity attained and rendered ProP activation transient. Unexpectedly, replacement R488I destabilized the coiled-coil formed by ProP-p. The activity and osmotic activation of ProP were even more strongly attenuated by helix-destabilizing change I474P. These data demonstrate that the carboxyl terminal domain of ProP can form a homodimeric α-helical coiled-coil with unusual properties. They implicate the C-terminal domain in the osmotic activation of ProP.

***Keywords:* *Escherichia coli*; osmoregulation; osmosensor; osmoprotectant; ProP; compatible solute; transporter; coiled-coil**

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INTRODUCTION

The ability to osmoregulate (to control cellular hydration, volume and/or turgor pressure) is shared by diverse organisms, including Archaea, Bacteria and Eukarya. Cellular hydration is controlled by modulating the cytosolic content of inorganic and organic solutes (Burg *et al.*, 1997; Joset *et al.*, 1996; Rhodes and Hanson, 1993; Strange, 1994; Varela and Mager, 1996; Wood, 1999). Certain zwitterionic organic solutes, termed 'compatible solutes', are accumulated to raise cytosolic osmolality without impairing cellular functions. Transporter ProP mediates the osmoprotective accumulation of compatible solutes such as proline and glycine betaine (*N*-trimethyl glycine) by *Escherichia coli* (Wood, 1999).

Both genetic (Mellies *et al.*, 1995; Xu and Johnson, 1995, 1997a,b) and biochemical (Racher *et al.*, 1999; Wood, 1999) regulatory mechanisms ensure that ProP activity rises when *E. coli* encounters high osmolality media (Wood, 1999). For bacteria cultivated in low osmolality media, the rate of proline uptake via ProP increases at least 7-fold, with an activation half-time of 1 min, when the bacteria are subjected to an osmotic upshift with NaCl or sucrose (optimum upshift, 0.2 osmolal, Kunte *et al.*, 1999; Milner *et al.*, 1988). In contrast to membrane-impermeant solutes NaCl and sucrose, membrane-permeant solute glycerol effects only transient activation of ProP (Milner *et al.*, 1988). ProP activity is further enhanced when the bacteria are cultivated in high osmolality media because transporter

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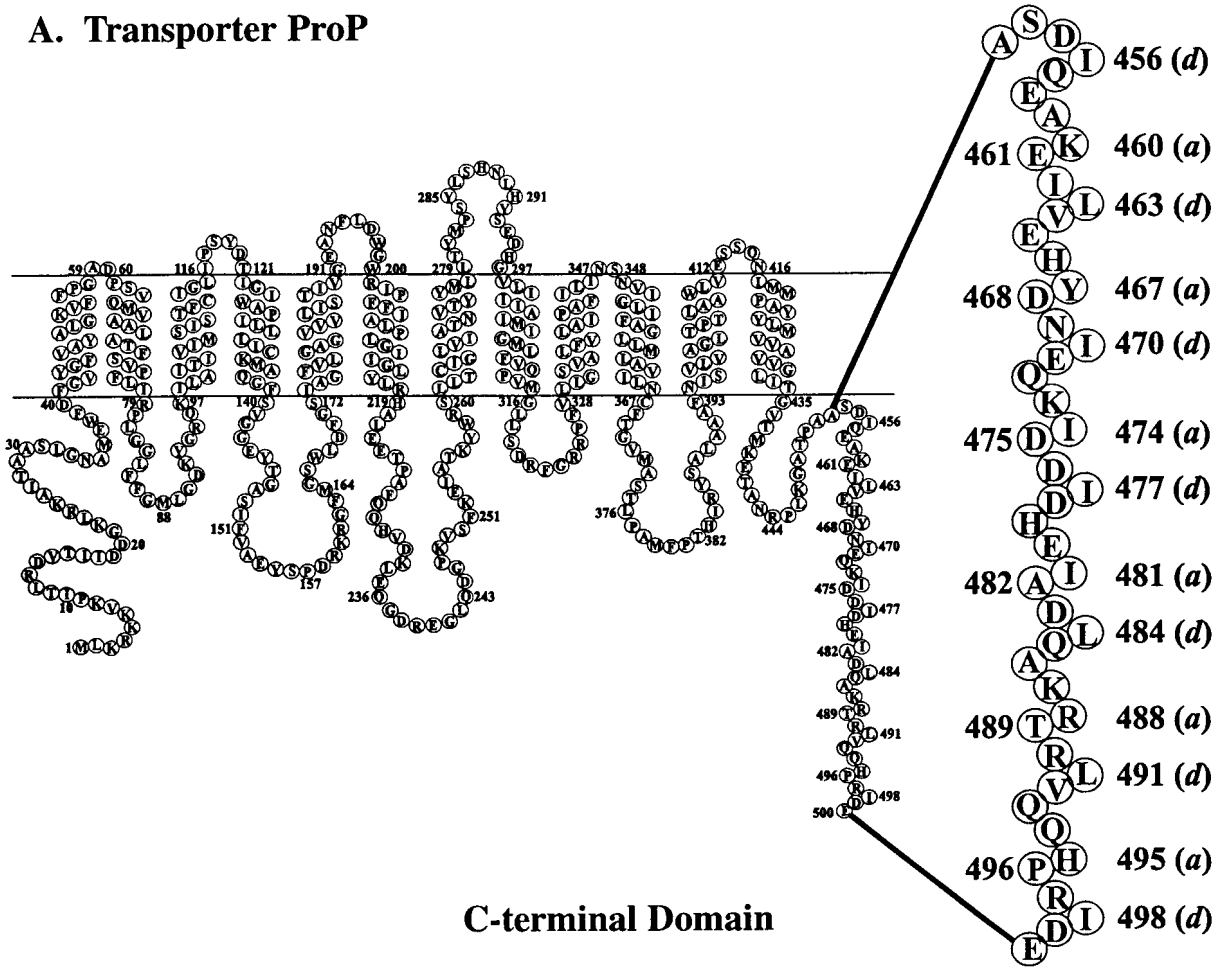
Abbreviations used: CD, circular dichroism; MOPS, 4-morpholino-propanesulfonic acid; PAM, 4-hydroxy-methylphenyl-acetamidomethyl-polystyrene; PCR, polymerase chain reaction; *t*-Boc, *N*-t-butyloxycarbonyl; REN, restriction endonuclease; TFE, trifluoroethanol; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.



B. Alignment of Transporter C-Termini

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                                ↓  ↓    ↓  ↓    ↓    ↓    ↓  ↓    ↓    ↓  ↓  ↓    ↓    ↓
ProPEc  MKETANRPLKGATPA ASDIQEAKEILVEHY DNIEQKIDDIDHEIA DLQAKRTRLVQQHPR IDE 500
OusA    MKETANKPLKGATPA ASDLSEAKEILQEHH DNIEHKIEDITQQIA ELEAKRQLLVAQHPR IND 498
ProPCg  MTESSQKPLLGSFPT VETKSEAVEIVKNQ- D------EDPNIDLS HMPFPDEENVGAE-K QNA 504
KgtP    LHRKG-KGMRL---- --------------- --------------- --------------- --- 432
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Figure 1. Predicted structure and putative orthologues of transporter ProP. (A) Predicted secondary structure and membrane topology. The illustrated, putative membrane topology of ProP is based on the sequence analysis reported by Culham *et al.* (1993). The *C*-terminal heptad repeats are enlarged and arranged to illustrate the series of residues occupying heptad '*a*' and '*d*' positions. (B) Comparison of *C*-terminal sequences for ProP, OusA, ProP*Cg* and KgtP. The *C*-terminal portion of an alignment of the amino acid sequences of transporters ProP (*E. coli*), OusA (*Erwinia chrysanthemi*), ProP*Cg* (*Corynebacterium glutamicum*) and KgtP (*E. coli*) is shown. Arrows designate the positions of the coiled-coil heptad '*a*' and '*d*' positions in ProP and OusA.

activation is sustained and *proP* transcription is enhanced (Grothe *et al.*, 1986; Milner *et al.*, 1988).

A ProP derivative with a *C*-terminal His-tag (six histidine residues) has been overexpressed, purified and reconstituted in proteoliposomes (Racher *et al.*, 1999). Both a membrane potential and an osmotic upshift were required for ProP activity in cytoplasmic membrane vesicles and after its purification and reconstitution in proteoliposomes (Milner *et al.*, 1988; Racher *et al.*, 1999). We would like to understand how ProP senses and responds to osmotic upshifts. Since pure, proteoliposomal ProP undergoes osmotic activation, no cellular constituents other than membrane lipids are essential to that process. At least one additional cellular constituent (ProQ) is required for full osmotic activation of ProP *in vivo*, however (Milner and Wood, 1989). Both the rate and the extent of ProP activation are attenuated in bacteria that lack protein ProQ (Kunte *et al.*, 1999). Thus the response to osmotic shifts that

Table 1. Strains and plasmids

Strain[a] or Plasmid	Genotype	Source or reference
RM2	F^- *trp lacZ rpsL thi* Δ(*putPA*)101	(Wood, 1981)
WG350	RM2 Δ(*proU*)600 Δ(*proP-melAB*)212	(Culham *et al.*, 1993)
WG389	RM2 Δ(*proU*)600 Δ(*proP-melAB*)212 lac^+ Δ(*brnQ phoA proC*)	(Culham *et al.*, 1993)
pDC43	1.9 kB fragment carrying gene *proP* inserted into vector pGEM4	(Culham *et al.*, 1993)
pDC46	pDC43-derived plasmid encoding *C*-terminal deletion derivative ProPΔ26	This work
pBAD24	Expression vector	(Guzman *et al.*, 1995)
pDC79	*proP* under the control of P_{BAD} and AraC in pBAD24	This work

[a] All strains are derivatives of *E. coli* K-12.

originates in ProP is further modulated by a process involving ProQ.

ProP is a member of the major facilitator superfamily (MFS) (Culham *et al.*, 1993) and an osmoprotectant-H^+ symporter (MacMillan *et al.*, 1999, and references cited therein). Members of the MFS exist as monomers integral to the membranes of eukaryotes and prokaryotes (e.g. LacY; Kaback and Wu, 1997). They are believed to span the membrane multiple (often 12) times as α-helical segments that are linked on either side of the membrane by short hydrophilic loops [Fig. 1(A)]. ProP differs from its paralogues [e.g. KgtP (27% sequence identity)] in (1) undergoing osmotic activation, and (2) possessing an extended carboxyl terminus which is predicted to participate in the formation of an α-helical coiled-coil [Culham *et al.*, 1993; Fig. 1(B)]. This observation led us to propose that the *C*-terminal domain of ProP forms an α-helical coiled-coil that is implicated in the osmoregulation of ProP activity (Culham *et al.*, 1993).

Here we show that a peptide replica of the ProP *C*-terminal domain (ProP-p) can form a homomeric α-helical coiled-coil of low stability *in vitro*. Deletion of 26 *C*-terminal amino acids inactivated ProP *in vivo*. Amino acid replacements that perturbed coiled-coil formation by ProP-p *in vitro* increased the magnitude of the osmotic upshift required to activate ProP *in vivo* (the activation threshold), in some cases also reducing the activity attained and the period over which activation was sustained. Thus the carboxyl terminal domain is implicated in the osmotic activation of ProP.

METHODS

Peptide synthesis and analysis

The synthetic *C*-terminal ProP peptides (residues 456–500) were prepared by solid-phase synthesis methodology using 4-hydroxy-methylphenyl-acetamidomethylpolystyrene (PAM) resin with conventional *N-t*-butyloxycarbonyl (*t*-Boc) chemistry on an Applied Biosystems Model 430A peptide synthesizer as described by Serada *et al.* (1993). Peptides were purified by reversed-phase HPLC to homogeneity and characterized by amino acid analysis and electrospray mass spectrometry as described by Kohn *et al.* (1995a,b). Circular dichroism (CD) spectra were recorded on a Jasco J-720 spectropolarimeter (Jasco Inc., Easton, MD) as described by Kohn *et al.* (1995a,b). Each sample was scanned 10 times and noise reduction applied to remove the high frequency before calculating the mean residue molar ellipticity. For wavelength scans, data were collected at 0.05 nm intervals from 190 to 255 nm. For the peptide concentration dependence study, changes in the helical content with concentration of the sample were measured at 220 nm. Peptide concentrations were determined by amino acid analysis. Sedimentation equilibrium experiments were performed as described by Zhu *et al.* (1993). Samples were dialyzed exhaustively against 50 mM potassium phosphate, 100 mM KCl, pH 7, at 4 °C, fringe counts were performed (as described) to determine the initial peptide concentration, a 100 μl aliquot of the sample was loaded into a 12 mm double-sector, charcoal-filled Epon cell and centrifugation proceeded for 48 h with a rotor speed of 26,000 rpm. Peptides were each loaded at initial total peptide concentrations of 184, 386 and 936 μM. The partial specific volume of each peptide was calculated using the program SEQSEE (Wishart *et al.*, 1994). The density of the solvent was calculated to be 1.009 g/ml. Equilibrium photographs were taken at the end of each run and fringe counts measured using a Nikon Model 6C microcomparator. Molecular weight was calculated according to the method described by Chervenka (1969).

Bacterial strains and plasmids

Table 1 contains a description of bacterial strains and plasmids used in this study. Unless otherwise indicated, molecular biological manipulations were carried out as described by Sambrook *et al.* (1989) and restriction endonucleases (REN) were purchased from Gibco BRL (Burlington, Ontario). Plasmid pDC79 was constructed by isolating the 1.6 kB *Nco*I/*Hind*III fragment (including *proP*) from pDC44 (Racher *et al.*, 1999) and ligating it into *Nco*I/*Hind*III digested pBAD24 (Guzman *et al.*, 1995). As a result, *proP* and its variants (see below) were expressed from the P_{BAD} promoter of the arabinose operon under the control of AraC. Bacteria harbouring such plasmids and cultivated as described below (in the absence of arabinose) produced the wild-type ProP protein at levels comparable to those achieved with the intact *proP* promoters under similar conditions (Grothe *et al.*, 1986; Milner *et al.*, 1988; see Results section).

To create a plasmid encoding a truncated ProP variant (plasmid pDC46), a 135 bp DNA fragment (containing the last 17 nucleotides of the *proP* gene and 105 downstream

nucleotides), flanked by *Cla*I and *Hind*III restriction sites, was amplified by the polymerase chain reaction (PCR) (Brown and Wood, 1992) using plasmid pDC15 (Culham *et al.*, 1993) as the template. The amplified DNA was digested with *Cla*I and *Hind*III, ligated into the large *Cla*I/*Hind*III fragment of pDC43 (Culham *et al.*, 1993) and transformed into strain DH5α (Hanahan, 1983). Transformants were selected on LB/ampicillin plates and screened for the presence of the *Hpa*I restriction site (located in the 135 bp insert, but not in pDC43). The resulting *proP* variant, in plasmid pGEM4, retained both of the native *proP* promoters (Culham *et al.*, 1993). Two codons were added during this construction, so that the encoded protein comprised 476 amino acids and terminated in the sequence: $I_{474}A_{475}N_{476}$. It was named ProPΔ26.

Site-directed mutagenesis was carried out essentially as described in the instruction manual for the Stratagene QuikChange™ Site-Directed Mutagenesis Kit. Either *Pfu* (Stratagene, La Jolla, California) or *Pwo* (Boehringer Mannheim, Laval, Quebec) DNA polymerase was used for PCR amplification and *Dpn*I restriction endonuclease was obtained from New England Biolabs (Mississauga, Ontario). No mineral oil overlay was used and amplified DNA was transformed into DH5α competent cells (Hanahan, 1983). The oligonucleotide primers were as follows: for $I_{474}P$, complementary 45-mers (nucleotides 1401–1445 of the *pro*P gene) with the I_{474} codon (ATC) changed to P_{474} (CCG) resulting in the elimination of a *Cla*I site; for $K_{460}I$ $Y_{467}I$, complementary 54-mers (nucleotides 1363–1416) with the K_{460} codon (AAG) changed to I_{460} (ATC) and the Y_{467} codon (TAC) changed to I_{467} (ATC) resulting in the addition of a *Pvu*I site; for $R_{488}I$, complementary 37-mers (nucleotides 1444–1480) with the $K_{487}R_{488}$ (AAA CGT) codons changed to $K_{487}I_{488}$ (AAG ATT) resulting in the addition of an *Mbo*II site; for $H_{495}I$, complementary 44-mers (nucleotides 1452–1495) with the R_{488} codon (CGT) changed to I_{488} (ATT) and the H_{495} codon (CAT) changed to I_{495} (ATT) resulting in the addition of an *Apo*I site. DNA extracted from transformants recovered using this last set of primers included the $H_{495}I$ mutation but not the expected $R_{488}I$ change. Transformants were selected on LB/ampicillin plates and extracted DNA was screened for the appropriate REN site alterations. In each case, the sequence of the entire *pro*P gene of the site-directed mutant was determined by GenAlyTiC (University of Guelph, Guelph, Ontario) and appropriate plasmids were introduced to *E. coli* WG350 [Δ(*putPA*)*101* Δ(*proU*)*600* Δ(*proP-melAB*)*212*] for subsequent experimentation.

Media and growth conditions

Bacteria were grown in LB medium (Miller, 1972) for genetic constructions or DNA preparation. For transport assays bacteria were cultured in 4-morpholinopropanesulphonic acid (MOPS)-based minimal medium (Neidhardt *et al.*, 1974) supplemented with 9.5 mM NH_4Cl as nitrogen source and 0.4% (v/v) glycerol as carbon source. L-Tryptophan (245 μM), thiamine hydrochloride (1 μg/ml) and L-proline (25 μM or 25 mM) were added when necessary to meet auxotrophic requirements. Osmotic stress was imposed by including 0.3 M NaCl in the growth medium unless otherwise indicated. If necessary, antibiotics were included at the following concentrations: ampicillin 100 μg/ml; and streptomycin 100 μg/ml. Bacteria were grown at 37 °C under aerobic conditions to an optical density at 600 nm of 1 (Fig. 4) or 0.8 (all other experiments).

Transport assays

Cells were harvested, washed, and transport measurements were performed as described by Milner *et al.* (1988). Samples were analyzed at least in triplicate, and each experiment was performed at least twice. Protein concentrations were determined by the bicinchoninic acid assay (Smith *et al.*, 1985) using the BCA kit from Pierce (Rockford, IL) with bovine serum albumin (BSA) as standard.

ProP protein levels

To determine ProP protein levels, everted membrane vesicles (prepared as described by Racher *et al.* (1999)) (Fig. 4 only) or whole cells were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting as described by Racher *et al.* (1999). ProP-specific bands were visualized after Western blotting as described by Racher *et al.* using the BioRad HRP 4CN colour development reagent (Fig. 4) or the Amersham (Oakville, Ontario) Enhanced Chemiluminescence reagents and pre-flashed Amersham Hyperfilm (Fig. 5). The film was pre-flashed as described by Sambrook *et al.* (1989). The level of each ProP protein variant was calculated as the amount of ProP protein divided by the amount of total cell protein loaded. Amounts of ProP protein were determined from Western blots as the density of the ProP-specific band in each sample relative to that of a ProP-$(His)_6$ standard prepared as described by Racher *et al.* (1999). The corresponding background value obtained with extracts of *E. coli* strain WG708 (WG350 pBAD24) was subtracted. Amounts of total cell protein were determined from densitometry of a Coomassie Brilliant Blue-stained SDS polyacrylamide gel loaded equivalently to the Western blot. The ProP protein level of each strain is reported here relative to that of the wild-type protein expressed in strain WG709 (WG350 pDC79). Densitometric analysis of Western blots and Coomassie Brilliant Blue-stained SDS–polyacrylamide gels was performed using the Scion Image for Windows 95 (Version Beta-3b) densitometry software (from http://www.scioncorp.com).

RESULTS

A synthetic polypeptide corresponding to the carboxyl terminus of ProP (ProP-p) forms a homodimeric α-helical coiled-coil *in vitro*

A 45-amino-acid polypeptide corresponding to amino acids 456–500 of the ProP protein (ProP-p) showed a CD spectrum with ellipticity minima at 222 and 207 nm (Fig. 2, middle) that is typical of α-helical proteins (Chen *et al.*, 1972; Gans *et al.*, 1991). When present at a concentration of

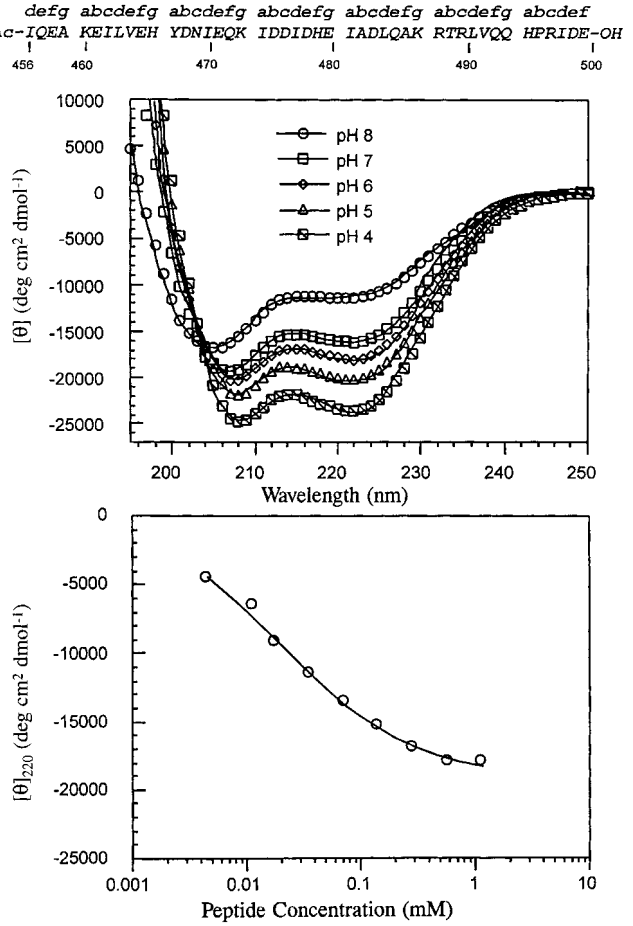

Figure 2. *In vitro* self-association of a synthetic polypeptide analogous to the ProP *C*-terminus. *Top*: amino acid sequence of the synthetic ProP C-terminal analogue used in this study (ProP-p). Ac denotes *N*-α-acetyl; -OH denotes the *C*-terminal α-carboxyl group. The repeating heptad positions designated *a–g* are shown above the sequence. *Middle*: circular dichroism spectra of ProP-p under varying conditions of pH. Spectra were recorded at 25 °C in a 50 mM phosphate, 100 mM KCl buffer prepared from KH_2PO_4 or K_2HPO_4 and adjusted with KOH or HCl to the desired pH. Peptide concentrations were 163 μM. The α-helical content was pH dependent [pH 8 (32%), pH 7 (45%), pH 6 (50%), pH 5 (56%) and pH 4 (66%)]. *Bottom*: the dependence of mean residue molar ellipticity $[\theta]_{220}$ on concentration of ProP-p. Spectra were recorded at 25 °C in a 50 mM potassium phosphate, 100 mM KCl, pH 7 buffer.

163 μM in benign buffer conditions at pH 7, the peptide had an α-helix content of 45% based upon the theoretical mean residue molar ellipticity at 222 nm of −35,900 deg cm^2 dmol^{-1} for a completely helical peptide of 45 residues (Chen *et al.*, 1974; Su *et al.*, 1994). The addition of 50% TFE did not substantially increase the α-helical content (58%), suggesting that the peptide cannot be induced to form a fully α-helical molecule with this helix inducing solvent (Lau *et al.*, 1984; Sonnichsen *et al.*, 1992). Although TFE disrupts the tertiary interactions of coiled-coil structures, it stabilizes peptides as single-stranded α-helices (Lau *et al.*, 1984; Zhou *et al.*, 1992a,b).

The increase in magnitude of the mean residue molar ellipticity at 220 nm observed with increasing concentration of this peptide was characteristic of the transition from a monomeric peptide to a homostranded oligomeric α-helical coiled-coil (Fig. 2, bottom; Zhou *et al.*, 1992a,b; Zhu *et al.*, 1993). A higher peptide concentration was required to maximize the magnitude of the mean residue molar ellipticity for ProP-p (approximately 1 mM), than for synthetic model peptides designed to form more stable α-helical coiled-coils (approximately 0.1 mM; Zhou *et al.*, 1992a,b).

To determine the oligomerization state of the putative homostranded α-helical coiled-coil, its molecular weight was determined by sedimentation equilibrium ultracentrifugation analyses performed at a variety of peptide concentrations (at 184, 386, 936 and 1425 μM). Although equilibrium peptide distributions characteristic of mixed monomeric and dimeric species were observed when the lower peptide concentrations were examined, at the highest concentration the peptide migrated as a single species with an estimated molecular weight of 10,184 Da (data not shown). (The calculated dimer molecular weight is 10,892 Da.) On the basis of these data, peptide ProP-p may form coiled-coils which are parallel, antiparallel or both. These results also indicated a very weak association, which correlated with the concentration-dependent α-helical content (Fig. 2, bottom). The α-helix content of the peptide was pH dependent, with increasing ellipticity as the pH was lowered from pH 8 to pH 4 (Fig. 2, middle).

Protein design studies have yielded extensive sequence–structure–stability correlations for homomeric parallel (Hodges, 1996), but not for heteromeric or antiparallel coiled-coils. Although it includes more than six of the heptad repeats characteristic of coiled-coil forming proteins, the sequence of the ProP C-terminus also differs significantly from those of proteins which form stable, homostranded, parallel coiled-coils (Adamson *et al.*, 1993; Alber, 1992; Baxevanis and Vinson 1993; Hodges, 1992, 1996). Homostranded, parallel oligomerization of ProP-p (illustrated in Fig. 3) is expected to be weak because:

(1) Destabilizing residues (K460, Y467, R488 and H495) occur in four of six *a* positions of the hydrophobic core, located at the *N*- and *C*-termini of the peptide. The α-helical content in coiled-coils can be substantially decreased from theoretical values due to end effects of such destabilizing residues (Zhou *et al.*, 1992a,b).
(2) β-Branched isoleucine residues occur at six of 13 hydrophobic core positions, four of them in the *d* position at which the presence of isoleucine can have a significant effect on the oligomerization state of coiled-coils (Harbury *et al.*, 1993, 1994).
(3) Proline, which has been shown to disrupt α-helical structures (Zhou *et al.*, 1994a,b) is present in this sequence at position 496. This residue may be partially responsible for the limited α-helical content observed by CD spectroscopy, even in the presence of TFE (Zhou *et al.*, 1994a,b) or at peptide concentrations which support coiled-coil formation (Fig. 2).
(4) The homostranded, parallel coil would contain a number of intrachain electrostatic repulsions between *i* and *i* + 3 or *i* and *i* + 4 positions.

The pH dependence of the peptide's α-helix content (Fig. 2, middle) is consistent with a need to form stabilizing salt bridges or to otherwise overcome such repulsions. The

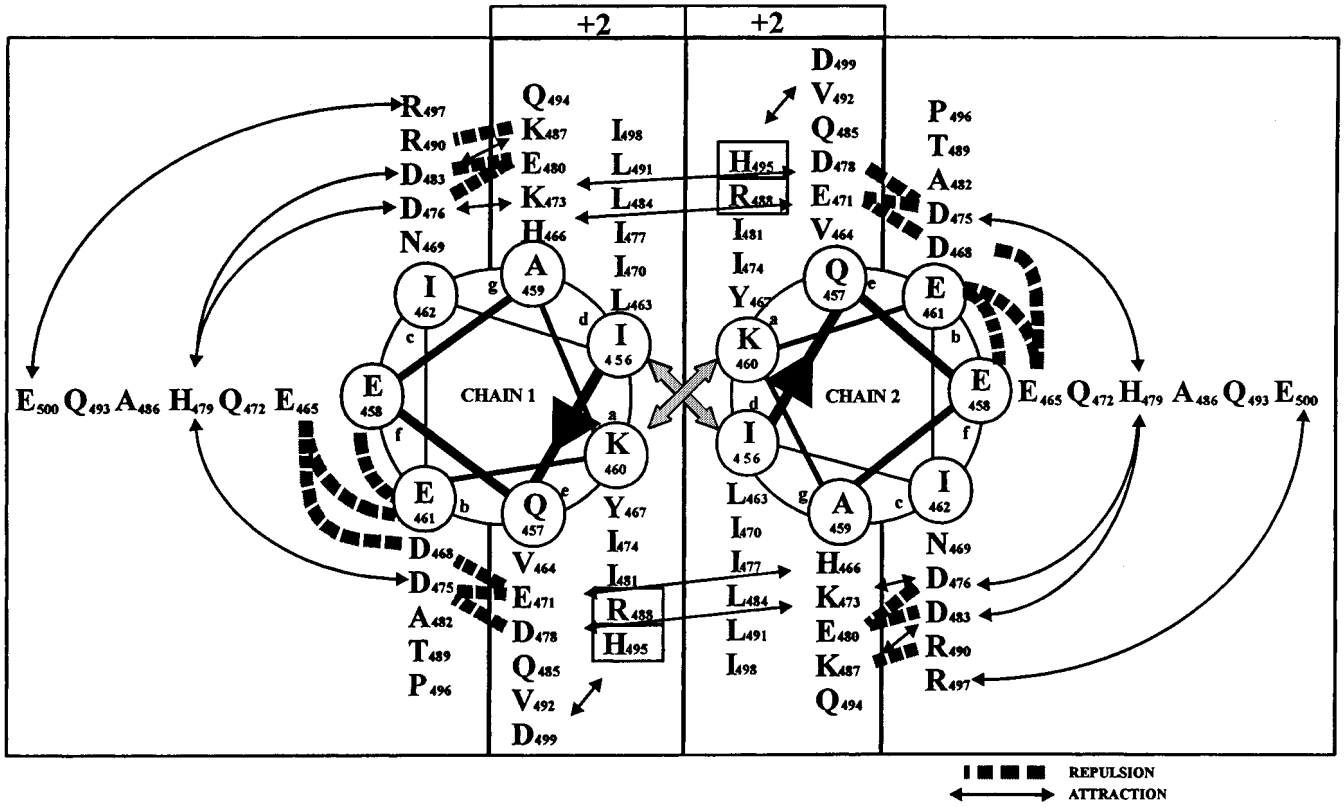

Figure 3. Predicted structure of a homodimeric α-helical coiled-coil composed of parallel ProP carboxyl termini. A helical wheel representation of a homodimeric α-helical coiled-coil comprising two parallel ProP C-terminal sequences (residues 456–500) illustrating a hydrophobic core which includes over six heptad repeats but is destabilized by residues K460, Y467, R488 and H495. Interchain and intrachain electrostatic interactions are indicated by solid lines (attractions) and dashed lines (repulsions). Each α-helix bears a net charge of +2 for the positions *a, d, e* and *g* involved in the dimer interface interactions (including histidine ionization). The +2 interface net charge is suggestive of non-specific charge–charge repulsion between the α-helices which would be destabilizing.

importance of salt bridges and repulsions in stabilizing or destabilizing two-stranded α-helical coiled-coils has been well documented (Kohn *et al.*, 1995a,b; Krylov *et al.*, 1994; Yu *et al.*, 1996; Zhou *et al.*, 1994a,b). An increase in hydrophobicity upon protonation (Hodges *et al.*, 1994) would also stabilize the structure through increased hydrophobic interactions with hydrophobic interface residues at positions *a* and *d*. These observations offer a guide for further exploration of the role of coiled-coil formation in ProP structure and function (see below).

Cytoplasmic potassium and glutamate levels are known to rise dramatically, approaching concentrations as high as 0.76 M (Cayley *et al.*, 1991) and 0.3 M (Cayley *et al.*, 1992), respectively, as *E. coli* responds to media of high osmolality. Indeed, potassium ions and potassium glutamate have been cited as possible osmoregulatory second messengers (Csonka and Epstein, 1996). We therefore investigated the effects of KCl and potassium glutamate on the ellipticity of ProP-p. The CD results showed no change in the observed ellipticity with increasing KCl or potassium glutamate concentration, with the final scans taken at a KCl concentration of 1 M or a potassium glutamate concentration of 0.5 M (the maximum level compatible with CD analysis, data not shown). These results suggest that if ProP is affected by potassium or glutamate, such effects would be mediated by more than coiled-coil formation by its *C*-terminal domain.

Carboxyl terminal deletions inactivate ProP

Before continuing to explore the structure of the ProP C-terminus, we established that the *C*-terminal domain is essential to ProP activity. Two *C*-terminal deletion derivatives of ProP were constructed. In the first (ProPΔ26) residues A475 N476 replaced 26 *C*-terminal amino acids (residues D475–E500, including approximately one-half of the putative coiled-coil forming amino acid sequence of the native protein) and in the second (ProPΔ45) six histidine residues replaced the *C*-terminal domain (45 amino acids, residues I456–E500, the entire putative coiled-coil forming sequence of the native protein). [Note that the addition of six histidine residues to the *C*-terminus of the intact protein does not alter its activity or activation (Racher *et al.*, 1999).] Derivative ProPΔ26 was expressed but had little activity, regardless of whether cells were grown with or without osmotic stress and assayed under low or high osmolarity conditions (Fig. 4). Derivative ProPΔ45 was also inactive under all conditions tested but it was expressed at less than 10% of the level of the wild-type transporter (data not shown). An intact *C*-terminal domain appeared to be essential for ProP activity, *in vivo*. However these data did not reveal whether truncation or elimination of the *C*-terminal domain impaired transporter activation, substrate translocation or both.

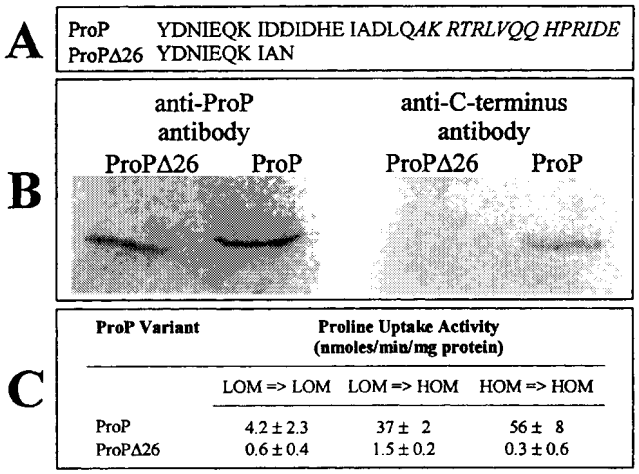

Figure 4. Expression and activity of ProPΔ26. *Top:* comparison of the *C*-terminal sequences of transporters ProP and ProPΔ26, the former italicized to illustrate the 15 amino acid peptide used to raise the anti-*C*-terminus antibody (Culham *et al.*, 1994). *Middle:* membrane vesicles prepared from *E. coli* strains WG389 pDC46 (*proPΔ26*) and WG389 pDC43 (*proP*) were analyzed by SDS–PAGE and Western blotting as described in the Methods section. *Bottom:* bacteria expressing ProP (strain WG389 pDC43) or ProPΔ26 (strain WG389 pDC46) were cultivated in low or high osmolality medium (LOM or HOM, MOPS medium that was not or was supplemented with 0.3 M NaCl, respectively) and initial rates of proline uptake were measured in assay media that were not (LOM) or were (HOM) supplemented with 0.3 M NaCl (see Methods section). For example, LOM = > LOM refers to bacteria cultivated in and transport activity measured in low osmolality medium. Proline uptake activities are given as the means of three determinations plus or minus standard errors.

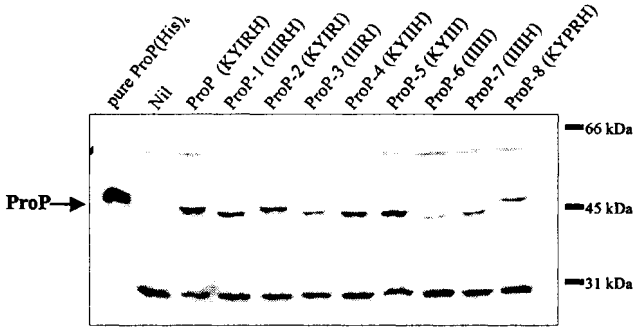

Figure 5. *C*-terminal mutations influence the cellular levels of ProP and its derivatives. The levels of the ProP protein present in cell extracts were determined by SDS–PAGE and Western blotting as determined in the Methods section. A representative Western blot is shown here.

Osmotic activation of ProP C-terminal variants

The relationship between the amino acid sequence of the carboxyl terminal domain and ProP activity was explored. The transporters were encoded in plasmids based on vector pBAD24 (Guzman *et al.*, 1995) to achieve expression of *proP* and its variants that was independent of growth medium osmolality but comparable to that observed in wild-type bacteria. The observed transport activities were corrected for effects of mutations on ProP protein levels as detected by Western blotting (e.g. Fig. 5).

As discussed above, replacements K460I, Y467I, R488I and/or H495I, each at the core '*a*' position of a coiled-coil heptad, would be expected to promote homodimeric, parallel coiled-coil formation by the carboxyl terminus of ProP. The proline uptake activities of bacteria expressing ProP variants with these amino acid replacements are reported in Table 2. Mutation H495I alone was without effect on ProP activity under these conditions (transporter ProP-2). Changes K460I Y467I, alone (ProP-1) or in combination with change H495I (ProP-3), reduced ProP activity in bacteria cultivated and assayed in either low or high osmolality medium (LOM → LOM or HOM → HOM). They had no effect on the activity attained when the bacteria were subjected to an osmotic upshift immediately before the transport assay (LOM → HOM). Transporter ProP R488I (ProP-4) was reduced in activity under all conditions, but change H495I attenuated its effect (ProP-5). Further combinations of amino acid changes that included R488I eliminated transporter activity (ProP-6, ProP-7). Amino acid change I474P was designed to introduce a kink in the polypeptide chain near the mid-point of the putative coiled coil. Proline has been shown to totally disrupt the coiled-coil structure when substituted in this position (Wagschal *et al.*, 1999a,b). Like change R488I, change I474P (ProP-8) reduced ProP activity under all conditions tested. This transporter attained even lower proline uptake activity after an osmotic upshift, however. The impact of single amino acid change I474P was comparable to that of *C*-terminal deletion ProPΔ26 (Fig. 4).

The activation of transporters ProP-3 (K460I Y467I H495I), ProP-4 (R488I) and ProP-8 (I474P) was examined further. The magnitude of the osmotic upshift required to activate these transporters (the activation threshold) increased in the order listed [wild-type ProP < ProP-3 < ProP-4 < ProP-8, Fig. 6(A)]. Thus small changes in amino acid sequence of the *C*-terminal domain modulated the sensitivity of ProP to osmotic upshifts. The activities of the wild-type transporter and of variants ProP-3 and ProP-4 decreased in a similar way at very large osmotic upshifts (to osmolalities greater than approximately 0.5 osmols/kg). Thus, in that range, the activation of ProP appears to be limited by an effect that is not modulated by these *C*-terminal amino acid changes.

The kinetics of ProP activation were also examined. The wild-type transporter, ProP-3 and ProP-4 all increased in activity at similar rates after an osmotic upshift [Fig. 6(B)]. Whereas the wild-type transporter remained fully activated for an indefinite period [Table 2 and Fig. 6(B)], ProP-3 showed a gradual decline in activity towards a lower steady-state level [Table 2 and Fig. 6(B)]. The osmotic activation of ProP-4 was even more transient. The activity of this transporter failed to reach that of wild-type ProP before it decreased rapidly, attaining a low, steady-state level within approximately 10 min after the osmotic upshift [Fig. 6(B)]. The latter observation explained the low proline uptake activity of bacteria that expressed transporter ProP-4 while growing in a high osmolality environment (Table 2, Fig. 6A). Thus although ProP-4 could be activated, its ability to remain in an active conformation was dramatically reduced by replacement R488I. Again, subtle changes in *C*-terminal amino acid sequence profoundly affected the osmotic activation of ProP.

Table 2. Proline uptake activities of bacteria expressing ProP and its derivatives

	Variable residue(s) (residue number)					Proline uptake activity[c] (nmoles min^{-1} mg $protein^{-1}$)			Proline uptake activity[d] (corrected for ProP expression level)		
Transporter[a]	460	467	474	488	495	LOM → LOM	LOM → HOM	HOM → HOM	LOM → LOM	LOM → HOM	HOM → HOM
Nil[b]	—	—	—	—	—	−0.01 ± 0.6	−1.1 ± 0.6	−0.2 ± 0.2	—	—	—
ProP	K	Y	I	R	H	16.0 ± 1.9	31.1 ± 1.9	70.4 ± 0.5	16.0 ± 1.9	31.1 ± 1.9	70.4 ± 0.5
ProP-1	**I**	**I**	I	R	H	1.6 ± 0.7	20.3 ± 0.7	39.4 ± 1.1	2.5 ± 1.1	31.7 ± 1.1	57.1 ± 1.6
ProP-2	K	Y	I	R	**I**	10.9 ± 1.7	22.5 ± 1.7	90.1 ± 1.8	14.9 ± 2.3	30.8 ± 2.3	74.5 ± 1.5
ProP-3	**I**	**I**	I	R	**I**	1.1 ± 0.6	18.4 ± 0.6	22.5 ± 0.8	2.3 ± 0.6	30.1 ± 1.2	44.1 ± 1.6
ProP-4	K	Y	I	**I**	H	−0.5 ± 1.7	16.1 ± 1.7	3.2 ± 0.1	−0.8 ± 2.7	25.6 ± 2.7	4.8 ± 0.2
ProP-5	K	Y	I	**I**	**I**	1.5 ± 0.7	27.1 ± 0.7	7.4 ± 0.6	2.2 ± 1.0	39.3 ± 1.0	17.2 ± 1.4
ProP-6	**I**	**I**	I	**I**	**I**	0.9 ± 1.4	0.4 ± 1.4	0.1 ± 2.2	[e]	[e]	[e]
ProP-7	**I**	**I**	I	**I**	H	0.5 ± 0.6	0.2 ± 0.6	0.6 ± 0.5	[e]	[e]	[e]
ProP-8	K	Y	**P**	R	H	0.6 ± 0.4	3.0 ± 0.4	1.3 ± 0.9	1.7 ± 1.1	8.3 ± 1.1	5.0 ± 3.5

[a] Plasmids expressing the listed ProP variants, constructed by modifying plasmid pDC79, as described in the Methods section, were expressed in *E. coli* WG350 (*proP*$^-$) (see Table 1). Amino acid replacements are indicated by letters that are bold and underlined (i.e. **I** or **P**).
[b] Vector control (plasmid pBAD24). No ProP present.
[c] The bacteria were cultivated in low or high osmolality medium (LOM or HOM, MOPS medium that was not or was supplemented with 0.3 M NaCl, respectively) and initial rates of proline uptake were determined 3 min after their introduction to transport assay media that were not (LOM) or were (HOM) supplemented with 0.3 M NaCl (see the Methods section).
[d] Proline uptake activities corrected for protein expression by dividing the uptake rate (nmol min^{-1} mg cell $protein^{-1}$) by the level of the ProP protein variant relative to that of wild-type ProP. Protein levels were determined by SDS–PAGE of extracts of the cells used to determine the corresponding transport activity, Western blotting and densitometry as described in the Methods section and

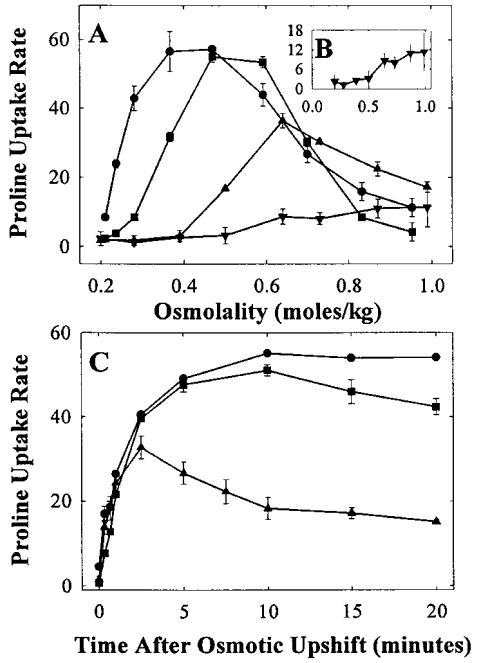

Figure 6. Osmotic activation of ProP, ProP K460I Y467I H495I, ProP-R488I and ProP-I474P. (**A**) The activation threshold: bacteria expressing wild-type ProP (circles), ProP K460I Y467I H495I (ProP-3, squares), ProP-R488I (ProP-4, triangles) or ProP-I474P (ProP-8, inverted triangles) were cultivated in low osmolality medium (LOM) and proline uptake assays were initiated 3 min after their introduction to an NaCl-supplemented transport assay medium of higher osmolality. Initial rates of proline uptake are plotted versus the osmolality of the transport assay medium. The inset (**B**) provides an expanded view of the activation of ProP I474P (ProP-8). The initial rates of proline uptake (nmol min^{-1} mg cell $protein^{-1}$) have been adjusted to compensate for the expression levels of proteins ProP-K460I Y467I H495I (0.83), ProP-R488I (0.81) and ProP-I474P (0.28) relative to that of wild-type ProP (assigned a value of 1.0) as determined by Western blotting (see Methods section) (**C**) The activation time course: bacteria expressing wild-type ProP (circles), ProP-K460I Y467I H495I (ProP-3, squares) or ProP-R488I (Prop-4, triangles) were cultivated in low osmolality medium (LOM) and proline uptake assays were initiated at a series of times after their introduction to an NaCl (0.24 M)-supplemented transport assay medium (0.63 mol kg^{-1}). Initial rates of proline uptake are plotted versus the time post-osmotic upshift at which the transport assay was initiated. Proline uptake activities (nmol min^{-1} mg cell $protein^{-1}$) have been adjusted to compensate for the expression levels of proteins ProP-K460I, Y467I, H495I (1.0) and ProP-R488I (0.75) relative to that of wild-type ProP (assigned a value of 1) as determined by Western blotting (see the Method section). The $t_{1/2}$ for the activation of ProP, determined as described by Milner *et al.* (1988), was 1.4 min.

Effects of amino acid sequence changes on coiled-coil formation by peptide ProP-p

The amino acid replacements shown above to influence ProP transporter activity *in vivo* were also found to modulate coiled-coil formation by the peptide replica (ProP-p) *in vitro*. As expected, amino acid replacements K460I, Y467I and H495I, singly or in combination, stabilized the coiled-coil formed by ProP-p (Table 3 and Fig. 7, peptides ProP-p1, ProP-p2 and ProP-p3). The relative magnitudes of the ellipticities and α-helix content observed in benign medium were higher for these peptides than for native ProP-p (Table 3), indicating more extensive coiled-coil formation by the modified peptides. The high propensity of peptides ProP-p1, ProP-p2 and ProP-p3 for α-helical coiled-coil formation was also reflected in their enhanced stabilities (Table 3, Fig. 7). The double substitution at the N-terminal of the sequence (K460I, Y467I) and the single substitution at the *C*-terminal of the sequence (H495I) each induced similar increases in α-helix content and stability (ProP-p1 and ProP-p2, Table 3). The peptide ProP-p3 which contains both *N*- and *C*-terminal substitutions had a large increase in α-helical content and a large increase in stability suggesting that the coiled-coil now extended from amino acid 456 to amino acid 494 (Table 3). These observations suggest that α-helical coiled-coil formation by native ProP-p may be confined to a core peptide region (approximately residues 470–491). In contrast to ProP-p and ProP-p1, peptides with replacement H495I, alone or in concert with those included in ProP-p1, formed higher order oligomers (e.g ProP-p2 and ProP-p3). This observation suggested that H495 played a particular role in determining the oligomeric state of the coiled-coil formed by ProP-p.

Amino acid change R488I had unexpected effects on ProP-p structure. The absolute magnitude of the ellipticity observed in a benign medium (though not in TFE) and the ellipticity ratio ($[\theta]_{222}/[\theta]_{208}$) were much lower for ProP-p4 (R488I) than for native ProP-p (Table 3), indicating much less extensive coiled-coil formation by the modified peptide. Although sedimentation equilibrium analysis indicated that this peptide could self-associate, a monomer–trimer equilibrium replaced the monomer–dimer equilibrium characteristic of native ProP-p and the resulting trimers were acutely unstable (Table 3, Fig. 7). The coiled-coil *instability* caused by the R488I replacement was unexpected. Isoleucine (I) is one of the most stabilizing amino acids at position '*a*' in parallel, homodimeric coiled-coils. Compared to an arginine (R) substitution, I increased coiled-coil stability in a model peptide by 4.7 kcal mol^{-1} ($[urea]_{1/2}$ values for peptides including I and R were 7.4 and 1.5 M, respectively; Wagschal *et al.*, 1999a,b). These unusual results could be explained if ProP-p formed a coiled-coil with an antiparallel orientation, stabilized by interchain electrostatic interactions, and if substitution R488I changed the oligomeric state and orientation of ProP-p, with no increase in stability. However, further investigations are required to document an antiparallel orientation of ProP-p. When amino acid replacement R488I was combined with other changes, mixed results were obtained. Coiled-coil stabilizing replacement H495I mitigated the effects of replacement R488I on coiled-coil formation (compare ProP-p4 and ProP-p5, Table 3) as it did for proline uptake activity (compare ProP-4 and ProP-5, Table 2). Like ProP-p2 and ProP-p3, each of which also includes the H495I change, ProP-p5 (R488I H495I) formed higher order oligomers that were comparable in stability to native ProP-p (Table 3, Fig. 7). This observation reinforced the earlier indication that residue H495 controls the oligomeric state of coiled-coils formed from this polypeptide. Like peptide ProP-p4 (R488I), peptide ProP-p6 (K460I, Y467I, R488I, H495I) formed extremely unstable trimers (Fig. 7). Clearly the upstream isoleucine replacements did not exert the same

Table 3. Ellipticities and stabilities for coiled-coils formed by peptide ProP-p and its derivatives

Peptide name[a]	Variable residue(s)[b] (residue number)				$[\theta]_{222}$[c] (deg cm^2 dmol^{-1})		Helical content[d]		$[\theta]_{222}/[\theta]_{208}$[e]	$[\text{Urea}]_{1/2}$[f] (M)		Sedimentation equilibrium[g]
	460	467	488	495	Benign	50%TFE	(%)	(no.)		4 °C	25 °C	
ProP-p	K	Y	R	H	−14,140	−20,540	39	18	0.86	2.0	0.3	M/D
ProP-p1	**I**	**I**	R	H	−17,260	−26,108	48	22	0.86	2.8	1.0	M/D
ProP-p2	K	Y	R	**I**	−16,590	−20,370	46	21	0.89	2.9	1.3	*
ProP-p3	**I**	**I**	R	**I**	−30,790	−25,867	86	39	1.00	3.4	2.9	*
ProP-p4	K	Y	**I**	H	−8,660	−20,170	24	11	0.66	<0	<0	M/T
ProP-p5	K	Y	**I**	**I**	−12,790	−25,360	36	16	0.75	2.0	0.2	*
ProP-p6	**I**	**I**	**I**	**I**	−7,670	−19,842	21	10	0.60	<0	<0	M/T

[a] Names of the ProP peptides.
[b] Amino acid positions substituted in this study. Residues existing in each of these positions for each peptide are shown with the isoleucine replacements indicated in bold and underlined (**I**). For the complete amino acid sequence of ProP-p peptide see Fig. 2.
[c] The mean residue molar ellipticities at 222 nm were measured at 25 °C in benign buffer (0.1 M KCl, 0.05 M potassium phosphate, pH 7). For samples containing TFE, the above buffer was diluted 1:1 (v/v) with TFE. Peptide concentrations were 100 μM.
[d] The helical content in benign buffer (%) was calculated from the ratio of the observed $[\theta]_{222}$ value divided by the predicted molar ellipticity × 100. The predicted molar ellipticity was calculated from the equation $[\theta]_{222} = [\theta]^{\infty}(1-k/N)$ for the chain length dependence of an α-helix (Zhou *et al.*, 1992b), where $[\theta]^{\infty} = 40 \times 10^3$ for a helix of infinite length, k the wavelength dependent constant at 222 nm = 4.6, and N is the number of residues in the peptide chain. For a 45-residue peptide the predicted value for a 100% α-helix is −35,900. The number of helical residues was calculated by multiplying the percentage helical content by the total number of residues in the peptide.
[e] The ratio of the mean residue molar ellipticities of 222 nm to 208 nm.
[f] $[\text{Urea}]_{1/2}$ is the transition midpoint, the concentration of urea (M) required to give a 50% decrease in molar ellipticity at 222 nm between the folded and unfolded states. Urea denaturations were carried out at 4 °C and 25 °C as described in the Methods section.
[g] Oligomer equilibria of the ProP peptides determined by sedimentation equilibrium analysis. M/D denotes data which was best fit to a monomer to dimer association model, M/T denotes data which was best fit to a monomer to trimer associative model,* denotes data which indicated peptide associations which formed oligomeric species from monomer to tetramer and above.

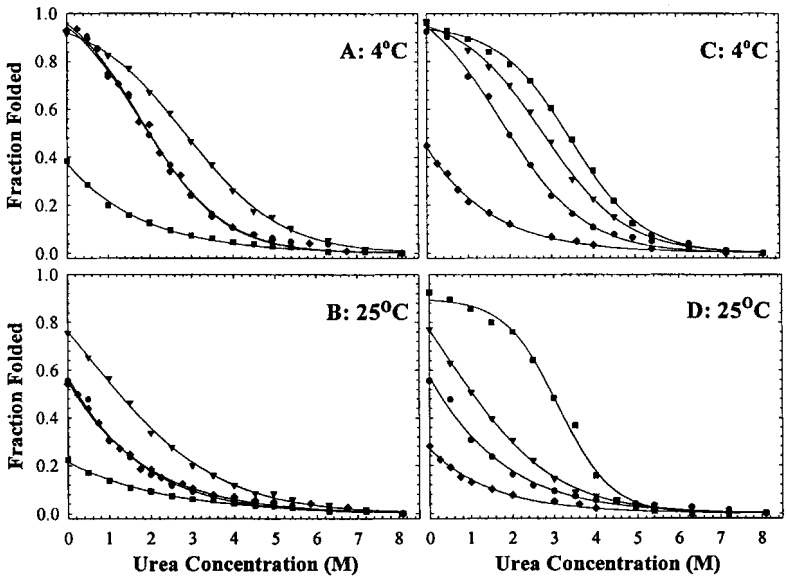

Figure 7. Stabilities of α-helical coiled coils formed by ProP-p and its derivatives. Urea denaturation profiles of the ProP-p peptides (for structures see Table 2). A and B. Urea denaturation profiles of peptides ProP-p (wild-type, circles), ProP-p2 (H495I, inverted triangles), ProP-p4 (R488I, squares) and ProP-p5 (H495I R488I, diamonds). Denaturation profiles were recorded at 4 °C (A) and 25 °C (B) in 0.1 M KCl, 0.05 M potassium phosphate, pH 7 buffer with various urea denaturant concentration. The fraction folded (f_f) of each peptide was calculated as $f_f = ([\theta]-[\theta]_u)/([\theta]_n-[\theta]_u)$, where $[\theta]$ is the observed mean residue ellipticity at 222 nm at any particular denaturant concentration and $[\theta]_n$ and $[\theta]_u$ are the mean residue ellipticities at 222 nm of the native 'folded' and 'unfolded' states, respectively. Peptide concentrations were 100 mM. (C and D) Urea denaturation profiles of ProP-p peptides ProP-p (wild-type, circles), ProP-p1(K460I, Y467I, inverted triangles), ProP-p3 (K460I, Y467I, H495I, squares) and ProP-p6 (K460I, Y467I, R488I, H495I, diamonds). Denaturation profiles were recorded at 4 °C (C) and 25 °C (D) as described above.

mitigating effect on structure and function for peptides containing the R488I replacement as did replacement H495I.

DISCUSSION

Transporter ProP possesses an extended, cytoplasmic, *C*-terminal domain. The *C*-terminal extension of ProP includes heptad sequence repeats characteristic of proteins which participate in the formation of α-helical coiled-coils (Figs 1 and 3) and ProP is activated by osmotic upshifts (Culham *et al.*, 1993). These observations led Culham *et al.* (1993) to propose that a *C*-terminal α-helical coiled-coil may be implicated in osmosensing by ProP. Data presented above show that the *C*-terminal domain of ProP can form an α-helical coiled-coil and that the same domain is involved in osmosensing.

A synthetic peptide corresponding to the ProP C-terminus (ProP-p) forms a relatively unstable, homostranded dimeric α-helical coiled-coil *in vitro*. That tendency is documented by the concentration dependence of both its α-helicity (CD analysis, Fig. 2) and its oligomeric state (sedimentation equilibrium analysis, data not shown). Amino acid replacements K460I, Y467I and/or H495I, each at the heptad '*a*' position, stabilized and extended the coiled-coil formed by ProP-p (compare the behaviour of peptide ProP-p with that of peptides ProP-p1, ProP-p2 and ProP-p3, Table 3 and Fig. 7). This behaviour was expected on the basis of known sequence–structure relationships for homodimeric, parallel α-helical coiled-coils. In contrast, replacement R488I, also at a heptad '*a*' position, dramatically destabilized this coiled-coil. This observation was not expected and it suggests that ProP-p may form an antiparallel, homodimeric α-helical coiled-coil. Such a structure could be readily accommodated by the intact, integral membrane protein. Alternative coiled-coil arrangements assumed by isolated ProP-p variants (e.g. parallel vs antiparallel, higher order oligomeric vs dimeric) may not be accessible to the *C*-terminal domains of the corresponding membrane-integral ProP variants. Further analysis will be required to fully delineate the coiled-coil structure (or structures) accessible to ProP-p *in vitro*, and to determine what structure(s) is assumed by the *C*-terminal domain of ProP (and its sequence variants) *in vivo* and/or *in vitro*.

Truncation of the *C*-terminus dramatically reduced transporter activity (Fig. 4). However, this observation did not reveal whether the *C*-terminal domain was essential for osmotic activation and/or for substrate translocation. Single (R488I or I474P) and multiple (K460I, Y467I, H495I) amino acid replacements at the critical coiled-coil '*a*' position, each dramatically perturbing α-helical coiled-coil

formation by the corresponding peptide (Table 3, Fig. 7 and Wagschal *et al.*, 1999a,b), yielded transporters with elevated activation thresholds. These transporters could be activated only transiently and, in some cases, to lower levels than the wild-type system (Fig. 6). These data clearly indicate that the structure of the *C*-terminal domain determines the sensitivity of ProP to osmotic upshifts and the stability of its active conformation.

Is a *C*-terminal coiled-coil domain essential for osmosensing? Two apparent orthologues of ProP have now been identified: OusA of *Erwinia chrysanthemi* (80% sequence identity; Gouesbet *et al.*, 1996) and ProP of *Corynebacterium glutamicum* (ProP*Cg*; 37% sequence identity; Peter *et al.*, 1998a,b). Like ProP, OusA and ProP*Cg* have *C*-terminal extensions. The *C*-termini of ProP and OusA are similar in sequence [Fig. 1(B)] and are predicted with similar, high probabilities (Lupas *et al.*, 1991) to form α-helical coiled-coils. After expression in *E. coli*, the response of OusA to osmotic upshifts imposed with NaCl is similar to that of ProP (Gouesbet *et al.*, 1996; M. Jebbar and C. Blanco, Personal Communication). The *C*-terminal extension of ProP*Cg* diverges in sequence from those of ProP and OusA and does not include the heptad repeats characteristic of coiled-coil forming proteins [Fig. 1(B)]. Nevertheless ProP*Cg* does undergo osmotic activation after expression in either *C. glutamicum* or *E. coli* (Peter *et al.*, 1998a,b). In addition, sodium-osmoprotectant symporters OpuD of *Bacillus subtilis* (Kappes *et al.*, 1996) and BetP of *Corynebacterium glutamicum* (Peter *et al.*, 1996) undergo osmotic activation, yet neither possesses a *C*-terminal coiled-coil sequence. Truncation of the hydrophilic, *N*-terminal domain of transporter BetP (or of both its *N*- and *C*-terminal domains) also influenced the sensitivity of that transporter to osmotic upshifts *in vivo* (Peter *et al.*, 1998a,b). Neither the absolute activities of BetP variants nor the stabilities of their activated states were determined, however. Thus a coiled-coil domain is not essential for the osmotic activation of all secondary transporters. It is possible that functions assumed by a coiled-coil domain in ProP are fulfilled by other structures in osmotically activated secondary transporters that lack coiled-coils (including ProP*Cg*). Of course the most obvious function of a coiled-coil domain would be to effect transporter homo- or hetero-oligomerization.

Coiled-coils of relatively low stability (dynamic coiled-coils) have been implicated in a number of biological regulatory phenomena (Oas and Endow, 1994). Particularly interesting are coiled-coil-forming, *trans*-acting transcription factors, including those implicated in yeast mating type selection (e.g. Ho *et al.*, 1994) and oncogene expression (e.g. Busch *et al.*, 1990), whose distribution among homo- and heterodimeric complexes is correlated with variations in gene expression. Given its location on the cytoplasmic surface of the cytoplasmic membrane [Fig. 1(A)], the *C*-terminus of ProP may participate in the formation of homomeric or heteromeric coiled-coils, the latter with cytoplasmic proteins or the cytoplasmic domains of membrane-associated proteins, or it may associate with the membrane surface. Since ProP can sense and respond to osmotic shifts after purification and reconstitution in proteoliposomes (Racher *et al.*, 1999), heteromeric coiled-coil formation is not essential to its osmosensory function or catalytic activity. The formation of heteromers could nevertheless modulate the osmosensory function of ProP or link it to other cellular functions. Mutation *proQ*::Tn*5* eliminates and reduces the ProP activity of *E. coli* cells cultivated in low and high osmolality media, respectively (Milner and Wood, 1989). It does not influence *proP* transcription (Milner and Wood, 1989) or ProP protein levels, but it does reduce the rate and extent of ProP activation in response to osmotic upshifts (Kunte *et al.*, 1999). The ProQ protein does not participate directly in coiled-coil formation as its predicted amino acid sequence includes no heptad repeats (Kunte *et al.*, 1999). ProQ could associate with the *C*-terminus of ProP, however, influencing the stability of a *C*-terminal coiled-coil and of the active ProP conformation. Indeed, in 1991 Csonka and Hanson predicted the existence of a stabilizer for activated ProP (Csonka and Hanson, 1991).

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