Aspartate 55 in the Na^+/Proline Permease of *Escherichia coli* Is Essential for Na^+-Coupled Proline Uptake†

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Received December 13, 1996; Revised Manuscript Received February 13, 1997⊗

ABSTRACT: Four acidic residues in the N-terminal domain of Na^+/proline permease of *Escherichia coli* (Asp33, Asp34, and Asp55 in putative loop 2, Glu75 in putative transmembrane domain II) were individually replaced with neutral or charged amino acid residues. Replacement of Glu75, the only residue in the permease presumed to be in the middle of a transmembrane domain, Asp33, or Asp34 had little or no influence on the kinetics of Na^+-coupled proline transport. In contrast, removal of the carboxylate at position 55 (Asp55 → Asn or Asp55 → Cys permease) impaired proline uptake completely while lengthening of the side chain at this position by one methylene group (Asp55 → Glu permease) allowed transport at a reduced initial rate. Importantly, all permease molecules were present in the membrane at concentrations comparable to the wild-type protein. Kinetic analysis of Na^+-coupled proline transport catalyzed by Asp55 → Glu permease revealed a 5-fold increase of the K_m for proline and a 30-fold decrease of the V_{max} compared to wild-type. Remarkably, replacement of Asp55 by Glu led to a 50-fold decrease of the apparent affinity of the permease for Na^+. Furthermore, replacement of Asp55 with Cys or Asn blocked proline-induced Na^+ uptake whereas significant Na^+ transport was observed with Asp55 → Glu permease. In addition, transport of proline down its concentration gradient was not detectable with deenergized cells containing Asp55→Glu permease at low Na^+ concentrations. However, downhill transport activity was observed in the presence of high Na^+ concentrations. Replacement of Asp55 with Asn or Cys impaired downhill transport under all conditions tested. The observations demonstrate that a carboxylate at position 55 of proline permease is essential for Na^+-coupled proline transport. It is suggested that Asp55 may be involved in binding of the coupling ion.

Secondary transport systems utilize electrochemical ion gradients to drive accumulation of substrates in cells of bacteria and higher organisms. However, the molecular mechanism by which energy stored in these gradients is transduced into work is only poorly understood. In order to gain new insights into the mechanism of Na^+-coupled transport, we are focusing on the Na^+/proline permease of *Escherichia coli* which is an integral protein of the cytoplasmic membrane and catalyzes the coupled translocation of proline and Na^+ (Li^+) ions [Chairney et al., 1984; Chen et al., 1985; Stewart & Booth, 1983; Tsuchiya et al., 1984; see also Yamato and Anraku (1993) for a review]. The *putP* gene encoding the Na^+/proline permease has been cloned and sequenced (Nakao et al., 1987; Wood & Zadworny, 1980), and the gene product has been solubilized from the membrane, purified, reconstituted into proteoliposomes, and shown to be solely responsible for Na^+/proline transport (Chen & Wilson, 1986; Hanada et al., 1988). Based on hydropathy profile analysis of the primary amino acid sequence, a secondary structure model was proposed in which the protein consists of a short hydrophilic N-terminal region, 12 transmembrane domains in an α-helical conformation that traverse the membrane in zigzag fashion connected by hydrophilic loops, and a hydrophilic C-terminal tail (Nakao et al., 1987) (Figure 1). Immunological analysis revealed that the C-terminus of the permease is located on the cytoplasmic side of the membrane (Komeiji et al., 1989).

Labeling experiments and random and site-directed mutagenesis have been employed to identify amino acid residues involved in substrate and/or ion binding and translocation. Thus, Hanada et al. (1985, 1992) showed that the Na^+/proline permease is irreversibly inactivated by *N*-ethylmaleimide (NEM) and that protection is afforded by proline and Na^+ (or Li^+) ions. Based on site-directed mutagenesis, Cys281 and Cys344 were identified as substrate-protectable residues, suggesting that these residues are close to the binding site for the coupling ion and/or proline (Hanada et al., 1992; Yamato & Anraku, 1988). However, replacement of these Cys residues neither affected proline uptake nor altered the sensitivity of *E. coli* cells to the toxic proline analog L-azetidine-2-carboxylate significantly, thereby indicating that the two Cys residues may not be directly involved in binding (Hanada et al., 1992; Yamato & Anraku, 1988). Myers & Maloy (1988) reported the isolation and genetic characterization of *putP* mutants of *Salmonella typhimurium* with altered cation specificity, but the sites of the mutations were not determined. Yamato et al. (1990) showed that replacement of Gly22 or Cys141 results in a reduced affinity of the Na^+/proline permease to Na^+ ions with no effect on proline binding. In contrast, Arg376 which had been proposed to be part of a Na^+ binding motif was found to be dispensable with respect to energy coupling (Deguchi et al., 1990; Yamato et al., 1994). Recently, Quick et al. (1996)

† This work was financially supported by the Deutsche Forschungsgemeinschaft (SFB 171-C19).

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⊗ Abstract published in *Advance ACS Abstracts,* April 1, 1997.

S0006-2960(96)03063-2 CCC: $14.00 © 1997 American Chemical Society

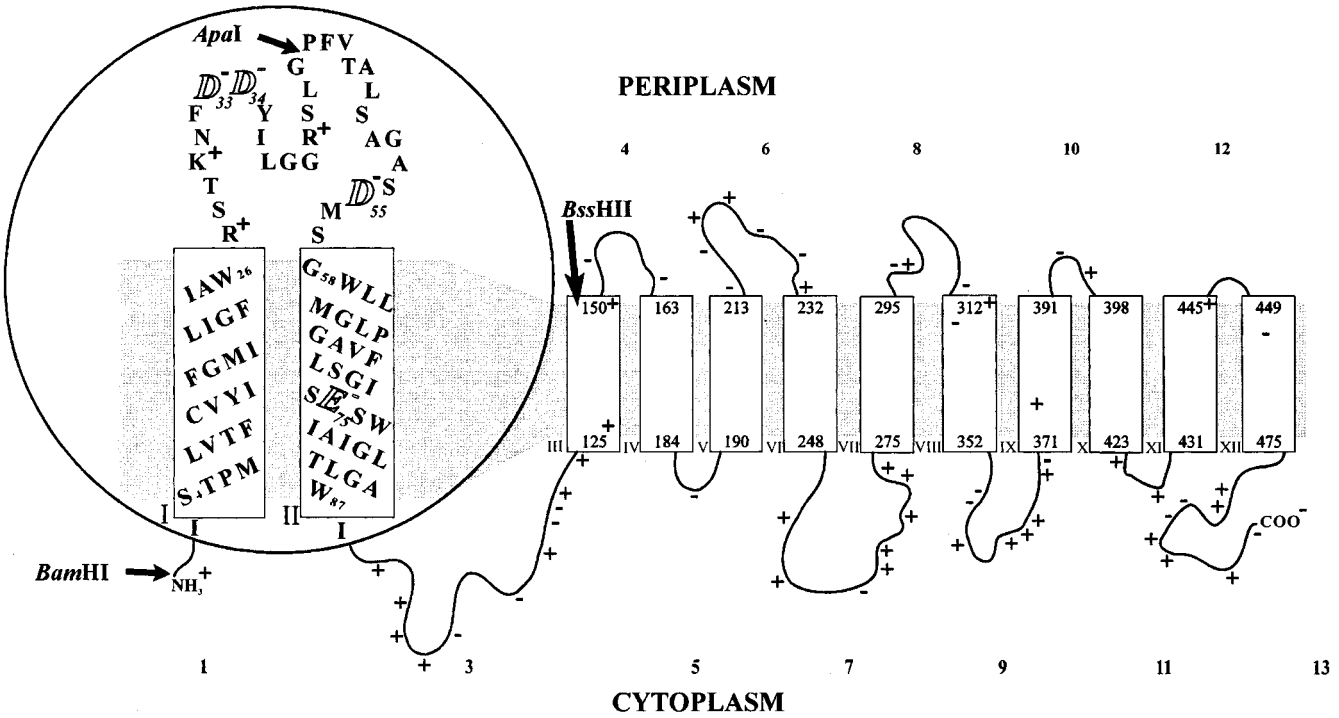

FIGURE 1: Secondary structure model of Na^+/proline permease based on the hydropathy analysis of the amino acid sequence (Nakao et al., 1987). Transmembrane domains are represented as rectangles and numbered with Roman numerals. Arabic numerals correspond to hydrophilic cytoplasmic and periplasmic loops. The first two putative N-terminal domains and the connecting periplasmic loop are shown in detail. Residues subjected to site-directed mutagenesis in the present study are highlighted. The positions of relevant restriction endonucleases sites in the corresponding DNA sequence are also indicated.

demonstrated that Ser57 is critical for high-affinity proline uptake. Thus, replacement with Ala, Cys, Gly, or Thr decreased the K_m for proline by up to 2 orders of magnitude with little influence on V_{max} values and reduced the sensitivity of *E. coli* to the toxic proline analogs L-azetidine-2-carboxylate and 3,4-dehydro-D,L-proline dramatically.

Although the functional importance of acidic amino acid residues was reported for melibiose permease (Franco & Wilson, 1996; Pourcher et al., 1993; Zani et al., 1993), only little is known about the role of acidic residues in other Na^+-coupled transport systems. In order to gain more information on the role of acidic residues in members of the Na^+/solute symporter family (Reizer et al., 1994), we have focused on the functional consequences of replacing acidic residues in the N-terminal portion of the Na^+/proline permease of *E. coli*. It is demonstrated that Glu75, the only negatively charged residue thought to be located in the middle of a transmembrane domain (helix II), Asp33, and Asp34 in the adjacent putative hydrophilic loop 2 are dispensable for active transport. In contrast, the carboxylate at position 55 (putative loop 2) is essential for Na^+-coupled proline uptake. Based on a kinetic analysis, it is suggested that Asp55 is part of the Na^+ binding domain of Na^+/proline permease.

EXPERIMENTAL PROCEDURES

Materials. Horseradish peroxidase-conjugated sheep anti-(mouse-IgG) antibody and the enhanced chemiluminescence kit were obtained from Amersham Corp. L-[U-^{14}C]Proline (279 μCi/μmol) was purchased from ICN Radiochemicals. Mouse anti-FLAGM2 IgG was from Integra Biosciences. Restriction endonucleases, alkaline phosphatase, *Taq* DNA polymerase, T4 DNA ligase, and T4 polynucleotide kinase were obtained from New England Biolabs or from Gibco/BRL, and small-DNA agarose was from Biozym. The Altered sites II *in vitro* mutagenesis system was purchased from Promega, and synthetic oligonucleotide primers were obtained from Eurogentec. Nitrocellulose membranes (0.45 μm pore size) were purchased from Schleicher & Schüll, and GF/F filters (0.7 μm pore size) were from Whatman. All other chemicals used were of analytical grade and purchased from commercial sources.

Bacterial Strains and Plasmids. *E. coli* JM109 [*endA1 recA1 gyrA96 thi hsdR17 supE44 relA1* Δ*(lac-proAB) (*F' *traΔ36 proAB*$^+$ *lacI*^{q} *ZΔM15)*] (Yanish-Perron et al., 1985) was used as carrier for the plasmids described. Plasmid pT7-5/*putP(Apa*I) (Quick et al., 1996), a derivative of pT7-5 (Tabor & Richardson, 1985) containing the *lac* promoter/operator for expression of the *putP* gene after induction with IPTG, was used as a source of the wild-type gene. *E. coli* WG170 [F' *trp lacZ rpsL thi* Δ*(putPA)101 proP219*] (Stalmach et al., 1983) harboring given plasmids was used for *in vitro* proline transport assays, proline-driven Na^+ uptake measurements, and immunoblots. *E. coli* WG175 [F′ *trp lacZ rpsL thi proP219 putP3*::Tn5] (Stalmach et al., 1983) was used for proline fermentation studies on different indicator/minimal media plates after transformation with given plasmids. Together with plasmid pAlter-1, *E. coli* ES1301*mutS* [*lacZ53 mutS201*::Tn5 *thyA36 rha-5 metB1 deoC* IN*(rrnD-rrnE)*] (Promega) was used for mutagenesis experiments with the Altered sites II *in vitro* mutagenesis system.

Site-Directed Mutagenesis and DNA Sequencing. Site directed mutagenesis was performed using synthetic mutagenic oligonucleotides. Substitutions of Asp33 or Asp34 by Cys were created using the polymerase chain reaction (PCR) with plasmid pT7-5/*putP*(*Apa*I) as template and mutagenic antisense primers (D33C: 5′-C GAA GGG CCC AAG ACT ACG ACC GCC CAG AAT ATA GTC **ACA** AAA GTT-3′; D34C: 5′-C GAA GGG CCC AAG ACT ACG ACC GCC CAG AAT ATA **ACA** GTC AAA GTT-

3′; altered codons are in boldface) and a sense primer binding upstream of the *Bam*HI recognition site. PCR fragments were digested with *Bam*HI and *Apa*I, and the resulting 136 bp DNA fragments were ligated to similarly treated plasmid pT7-5/*putP*(*Apa*I) incubated with alkaline phosphatase. For substitution of Glu75 by Cys or Asp, and Asp55 by Asn, Cys, or Glu, wild-type *putP* was cloned into plasmid pAlter-1 using the *Bam*HI/*Hin*dIII restriction endonuclease sites. Mutagenesis was performed according to the manufacturer's protocol for the Altered sites II *in vitro* mutagenesis system with the exception that 10 pmol of mutagenic sense primers (D55C: 5′-GGT GCG TCG **TGT** ATG AGC GGC-3′; D55E: 5′-GGT GCG TCG **GAA** ATG AGC GGC-3′; D55N: 5′-GGT GCG TCG **AAT** ATG AGC GGC-3′; E75C: 5′-TCC GGG ATT TCC **TGT** AGC TGG ATC GCC-3′; E75D: 5′-TCC GGG ATT TCC **GAT** AGC TGG ATC GCC-3′) was used. The resulting constructs were digested with *Apa*I and *Bss*HII, and the 312 bp DNA fragments were ligated into pT7-5/*putP*(*Apa*I) treated with the same restriction endonucleases and alkaline phosphatase. All constructs were verified by sequencing double-stranded plasmid DNA using dideoxynucleotide chain-termination (Sanger et al., 1977) after alkaline denaturation (Hattori & Sakaki, 1986).

Phenotype Testing. Transport by *E. coli* WG175 ($PutP^-A^+$) harboring plasmids encoding proline permease with given amino acid replacements was analyzed qualitatively by the following tests, each of which used an overnight culture in Luria−Bertani medium (LB medium) (Miller, 1992) as the inoculum. (i) TTC-proline indicator plates. Cells were spread on 2,3,5-triphenyltetrazolium chloride (TTC[1]) indicator plates (Bochner & Savageau, 1977) containing 0.2 mM IPTG, 0.1% proline, and 100 μg/mL ampicillin. (ii) Growth on proline plates. Cells were spread on agar plates containing low-Na^+ minimal medium (Shiota et al., 1984) supplemented with 0.1% proline, 20 mM NaCl, 0.2 mM IPTG, and 100 μg/mL ampicillin.

Proline Transport Assays. Active transport was measured in *E. coli* WG170 ($PutP^-A^-$) harboring plasmids encoding proline permease with given amino acid replacements. Cells were grown aerobically in LB medium supplemented with ampicillin (100 μg/mL) at 37 °C, and expression of the *putP* gene was achieved by addition of 0.5 mM IPTG at the middle of the exponential growth phase. After harvesting by centrifugation, cells were washed with 250 mM Tris/Mes buffer, pH 6.0, and resuspended in the same buffer to give a final protein concentration of 0.35 mg/mL (Quick et al., 1996). Under standard test conditions, aliquots of the cell suspension (200 μL) were assayed for L-[U-^{14}C]proline transport (final proline concentration 5 μM; 25 μCi/μmol) in the presence of 20 mM D-lactate (Na^+ salt) and 50 mM NaCl at 25 °C using rapid filtration (Chen et al., 1985). Kinetics of transport were calculated from the initial linear portion of the time courses between 0 and 10 s, except for D55E[2] permease, where the initial linear portion between 0 and 3 min was used. Analysis of the dependence of proline uptake on the Na^+ concentration was carried out in plastic vials at 25 °C using cells resuspended in 250 mM Tris/Mes buffer, pH 6.0, as described above. The Na^+ content of the buffer prior to addition of NaCl (Na^+ free buffer) was determined to be 6 μM by atomic absorption spectroscopy (Eppendorf, Elex6361). To initiate uptake, different amounts of NaCl and 5 μM L-[U-^{14}C]proline (25 μCi/μmol) were added simultaneously. For downhill proline transport, cells were completely deenergized by incubation with 5 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 5 μM monensin at 25 °C for 30 min in Na^+-free buffer or in the presence of 100 mM NaCl before adding 20 μM L-[U-^{14}C]proline (15 μCi/μmol). All experiments were performed in triplicate at least.

Proline-Induced Na^+ Transport. Proline-induced Na^+ uptake was assayed essentially as described (Chen et al., 1985). *E. coli* WG170 harboring plasmids encoding proline permease with given amino acid replacements were grown as described for proline transport. After harvesting, cells were washed 3 times with 100 mM Tris/Mops, pH 7.0, and resuspended in the same buffer to yield a protein concentration of 25 mg/mL. For the Na^+ uptake assay, 40 μL of cells was diluted in 100 mM Tricine/tetramethylammonium hydroxide (TMA-OH), pH 8.0, containing 25 μM NaCl and 20 μM 5-(*N*,*N*-hexamethylene)amiloride to yield a protein concentration of 0.1 mg/mL (final volume 10 mL) and placed in a closed plastic vessel. Holes were made in the cap of the vessel to accommodate a Na^+-selective electrode (ISEC221Na, Radiometer) and to allow additions. The cell suspension was incubated anaerobically under a constant stream of N_2 with continuous stirring at 22 °C for at least 45 min. Na^+ uptake was induced by the addition of 100 μL of 200 mM L-proline. Changes in the Na^+ concentration of the extracellular medium were monitored with a PHM95 ion/pH meter (Radiometer) and were processed via an analog/digital modul with the Origin (MicroCal Software, Inc.) calculation program.

Immunological Analysis. The amount of permease in the membrane of *E. coli* WG170, harboring each plasmid described, was estimated by immunoblotting with mouse anti-FLAG-IgG against the FLAG epitope at the carboxyl terminus of the protein followed by incubation with horseradish peroxidase-linked sheep anti-mouse-IgG antibody (Quick et al., 1996).

Protein Determination. Protein determination was performed using a modification of the method of Lowry (Peterson, 1977) with bovine serum albumin as standard.

RESULTS

Generation and Verification of Amino Acid Replacements in Proline Permease. Four acidic residues (Asp33, -34, -55, and Glu75) located in the N-terminal portion of the Na^+/proline permease were independently replaced with Cys. In addition, Asp55 was replaced with Asn or Glu and Glu75 with Asp. Mutagenic DNA fragments were cloned into pT7-5/*putP*(*Apa*I) using appropriate restriction endonucleases sites (Figure 1). All mutations were verified by sequencing of double-stranded plasmid DNA, and, except for the desired base changes, the sequences were identical to those of the wild-type *putP* gene.

Phenotype Testing. The ability of *E. coli* WG175 ($PutP^-A^+$) cells to use L-proline as source of carbon and

[1] Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; IPTG, isopropyl 1-thio-β-D-galactopyranoside; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; TMA-OH, tetramethylammonium hydroxide; TTC, 2,3,5-triphenyltetrazolium chloride.

[2] Amino acid replacements are designated as follows: The one-letter amino acid code is used followed by a number indicating the position of the native residue in wild-type Na^+/proline permease. The sequence is followed by a second letter denoting the substitution at this position.

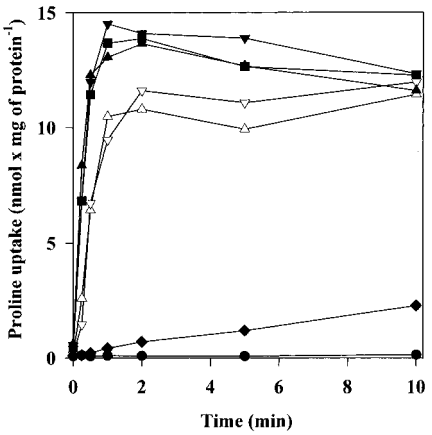

FIGURE 2: Time course of proline uptake by *E. coli* WG170 containing wild-type permease or permease with given amino acid replacements. Cells were grown and treated as described under Experimental Procedures. Transport of L-[U-^{14}C]proline (5 μM final concentration) was assayed in the presence of 50 mM NaCl and 20 mM D-lactate (Na^+ salt) as the electron donor at 25 °C under aerobic conditions. Wild-type PutP (■); PutP-D33C (△); PutP-D34C (▽); PutP-D55E (◆); PutP-E75C (▲); PutP-E75D (▼); PutP-D55C, PutP-D55N, pT7-5 (●).

nitrogen was tested on TTC indicator plates. Cells transformed with plasmid encoding functional Na^+/proline permease are expected to utilize proline and form red colonies on TTC indicator plates while those lacking active permease should be white. *E. coli* WG175 transformed with plasmid pT7-5/*putP*(*Apa*I) encoding wild-type permease or D33C, D34C, E75C, or E75D permease appeared as red colonies. In contrast, cells producing D55C, D55N, or D55E permease grew as white colonies, as observed for cells containing plasmid pT7-5 without *putP*. In addition, strain WG175 producing wild-type permease, D33C, D34C, E75C, or E75D permease was able to grow on minimal medium supplemented with proline as the sole source of carbon. In contrast, cells containing D55C, D55N, or D55E permease or plasmid pT7-5 without *putP* did not grow under these conditions.

Active Proline Uptake. Active transport of proline was assayed by using *E. coli* WG170 which lacks proline permease and proline dehydrogenase, and therefore, cannot metabolize proline. Cells transformed with plasmid encoding D33C, D34C, E75C, or E75D permease accumulated proline in a manner essentially indistinguishable from the wild-type (Figure 2). On the other hand, replacement of Asp55 caused a severe defect in proline accumulation. Thus, replacement of this residue with Asn or Cys resulted in complete loss of proline transport activity, whereas D55E permease retained low but significant activity (about 1% of the initial rate of proline uptake and about 30% of the steady-state level of proline accumulation compared to wild-type permease).

Kinetics of Active Transport. Initial rates of uptake were determined at proline concentrations varying between 0.2 and 200 μM, and data were analyzed according to Eadie−Hofstee. In agreement with the uptake measurements, replacement of Asp33, Asp34, or Glu75 resulted in only little or no change in the apparent K_m or V_{max} (Table 1). With D55E permease, the apparent K_m for proline was increased by a factor 5. The V_{max} for proline uptake by D55E permease was altered more dramatically, reduced to only 3% of wild-type. No transport was observed with D55C or D55N permease.

Table 1: Kinetic Analysis of Na^+-Coupled Proline Uptake by Na^+/Proline Permease Bearing Replacements of Given Acidic Residues[a]

	$K_{m(proline)}$ (μM)	$V_{max(proline)}$ [nmol min^{-1} (mg of protein)$^{-1}$]	$K_{m(Na^+)}$ (μM)
PutP-wild-type	2.1 ± 0.2	27 ± 1	32 ± 4
PutP-D33C	4.8 ± 0.3	58 ± 2	29 ± 2
PutP-D34C	2.1 ± 0.1	26 ± 1	27 ± 3
PutP-D55C	−[b]	−	−
PutP-D55E	8.9 ± 2.0	1 ± 0.4	1500 ± 200
PutP-D55N	−	−	−
PutP-E75C	1.6 ± 0.1	28 ± 2	41 ± 1
PutP-E75D	1.6 ± 0.4	30 ± 3	29 ± 1

[a] Initial rates of proline uptake by *E. coli* WG170 producing either wild-type or permease with given replacements were measured at proline concentrations from 0.2 to 200 μM in the presence of 50 mM NaCl. Apparent Na^+ affinity constants [$K_{m(Na^+)}$] were determined at NaCl concentrations varying from 0.006 to 100 mM at a proline concentration of 5 μM. The data were plotted according to Eadie−Hofstee. Standard deviations were determined from at least three independent experiments. [b] no detectable transport.

Facilitated Diffusion. To study facilitated influx of proline, *E. coli* WG170 containing wild-type, D55C, D55N, or D55E permease was completely deenergized by preincubation with CCCP and monensin. Deenergized cells bearing D55E permease exhibited downhill proline transport in the presence of 100 mM NaCl with an initial rate of 0.36 nmol min^{-1} (mg of protein)$^{-1}$ (18% of the wild-type rate) and a steady-state level of proline within the cells that was essentially indistinguishable from the wild-type value (2.2 nmol/mg of protein). Significant activity was not observed with cells containing permease with Cys or Asn in place of Asp55. However, at very low Na^+ concentrations (6 μM), cells containing D55E permease did not show significant influx of proline down its concentration gradient within 1 h of incubation, while downhill transport catalyzed by wild-type permease was only slightly altered (data not shown). Furthermore, the Na^+ concentration had no effect on downhill transport by D55C or D55N permease.

Proline-Induced Na^+ Uptake. Addition of 2 mM L-proline to a suspension of *E. coli* WG170 cells containing wild-type PutP resulted in an immediate fall of the external Na^+ concentration as indicated by the downward deflection of the signal from a Na^+ selective electrode (Figure 3). A smaller but significant decrease in Na^+ concentration was observed with cells containing D55E permease. Alternatively, cells bearing D55N permease exhibited only a small decrease in external Na^+ concentration that was at the border of the sensitivity of the electrode. Finally, no decrease in Na^+ concentration was detected with cells containing D55C permease or cells transformed with plasmid pT7-5 devoid of *putP*.

Effect of Na^+ Concentrations on Uphill Proline Transport. The rate of proline transport by cells with wild-type permease increased markedly as the Na^+ concentration was increased from 6 to about 200 μM and is essentially constant above 400 μM (Figure 4A). In marked contrast, the rate of proline transport by cells with D55E permease increased dramatically from 0.2 mM to about 20 mM and remained constant at higher concentrations (Figure 4B). Analysis of the data according to Eadie−Hofstee yielded an apparent K_m for Na^+ [$K_{m(Na^+)}$] of 32 ± 4 μM for the wild-type and about 1500 ± 200 μM for D55E permease. Thus, the affinity of D55E permease for Na^+ appears to be markedly decreased. As shown in Table 1, D33C, D34C, E75C, or E75D exhibited

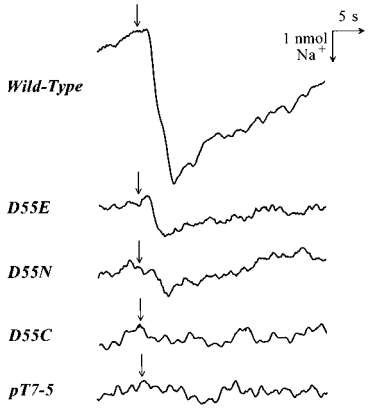

FIGURE 3: Proline-induced Na^+ uptake by *E. coli* WG170 containing wild-type Na^+/proline permease or permease with given Asp55 replacements. Cells were grown in Luria−Bertani medium supplemented with ampicillin (100 μg/mL), harvested, washed 3 times, and resuspended in 100 mM Tris/Mops, pH 7.0, to a final protein concentration of 25 mg/mL. For uptake measurements, cells were diluted to a final protein concentration of 0.1 mg/mL in 10 mL of Tricine/TMA-OH, pH 8.0, and were incubated in the presence of 20 μM 5-(*N*,*N*-hexamethylene)amiloride and 25 μM NaCl under anaerobic conditions as described under Experimental Procedures. L-Proline was added to a final concentration of 2 mM as indicated by the arrow. Changes in Na^+ concentration of the medium were monitored with a Na^+-selective electrode.

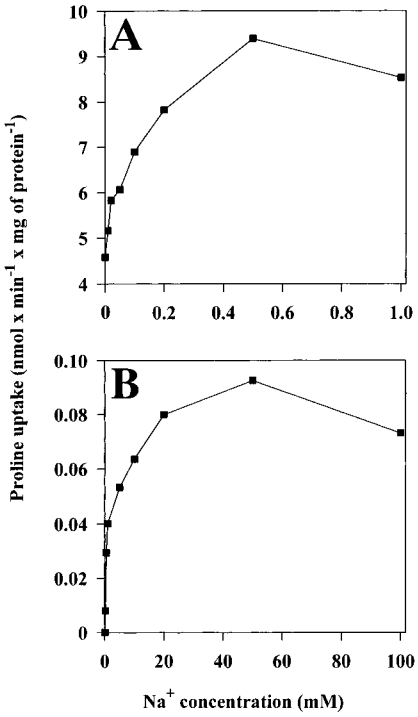

FIGURE 4: Effect of Na^+ concentration on initial rates of proline transport by *E. coli* WG170 containing wild-type (A) or D55E permease (B). Cells were prepared as described in Figure 2. Transport of L-[U-^{14}C]proline (5 μM final concentration) was assayed in plastic vials. The NaCl concentrations varied from 0.006 mM (Na^+-free medium) to 1 mM for wild-type permease, and from 0.1 mM to 100 mM for D55E permease. Initial rates of proline uptake were calculated as described under Experimental Procedures.

apparent $K_{m(Na^+)}$ values that are similar to that of wild-type proline permease. Cells containing D55C or D55N permease exhibited no activity whatsoever, up to 100 mM Na^+.

Immunological Analysis. The relative concentration of the permease molecules bearing replacements of Asp55 in membranes of *E. coli* WG170 was approximated by Western blot analysis using an anti-FLAG antibody against an engineered FLAG epitope at the C-terminus of the constructs

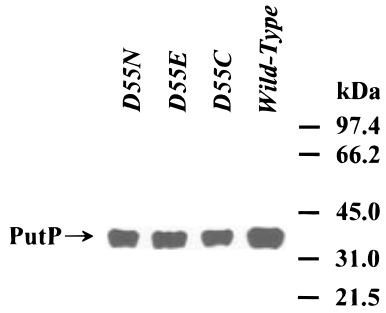

FIGURE 5: Western blot of membranes from *E. coli* WG170 cells containing permease with given Asp55 replacements. Membranes prepared from IPTG-induced cultures were subjected to 10% SDS/PAGE (40 μg of protein/lane) and electroblotted onto nitrocellulose membranes. The blot was incubated with mouse monoclonal antibodies against the FLAG epitope at the C-terminus of the permease, followed by incubation with sheep anti-(mouse-IgG) horseradish peroxidase conjugate. Proline permease was detected by the enhanced chemiluminescence method. Positions of the protein standards are indicated (kDa).

(Quick et al., 1996). Wild-type permease and also permease with Asn, Cys, or Glu in place of Asp55 were present in comparable amounts (Figure 5). Therefore, the differences described between wild-type permease and permease molecules with Asp55 replacements cannot be attributed to defective insertion of the permease into the membrane or to enhanced proteolytic degradation of the modified proteins subsequent to insertion.

DISCUSSION

This report demonstrates that the carboxylate at position 55 (putative loop 2) of Na^+/proline permease is essential for Na^+-coupled proline uptake. Glu in place of Asp55, which lengthens the side chain by only one methylene group, the most conservative change possible, yields permease with highly diminished transport activity, and replacement with other residues abolishes activity. Kinetic analysis of active transport catalyzed by D55E permease reveals a 50-fold decrease of the apparent affinity of the protein for Na^+ ions compared to the wild-type transporter. On the other hand, only a relatively small alteration of the apparent affinity for proline is observed, a result that may be related to the close proximity of Asp55 and Ser57. Ser57 has been suggested to be located at or near the proline binding domain of the permease (Quick et al., 1996). The fact that D55E permease catalyzes significant downhill transport of proline in de-energized cells in the presence of high Na^+ concentrations only is in agreement with a reduced affinity of the permease for Na^+ ions. In addition, the latter result indicates that transport of Na^+ and proline is still coupled. The marked effect of the Asp55→Glu replacement on V_{max} clearly shows that beside the charge also the size of the amino acid side chain at position 55 is critical for transport. Since the alteration in transport activities cannot be attributed to changes in production or insertion of the permease into the membrane, the results suggest strongly that Asp55 is involved in binding of the coupling ion. Interestingly, an Asp at position 55 is also essential for Na^+ (Li^+)-coupled galactoside uptake via the melibiose permease of *E. coli* (Pourcher et al., 1991). Based on transport kinetics and substrate binding studies of *melB* mutants, it was suggested that Asp55, as well as Asp31, Asp51, and Asp120, forms a cation binding site in melibiose permease (Pourcher et al., 1993; Zani et al., 1993). In the case of the Na^+/glucose transporter

(SGTL1), that belongs together with Na^+/proline permease to the family of Na^+/solute symporters (Reizer et al., 1994), replacement of Asp28 causes glucose/galactose malabsorption, and replacement of Asp176 alters the kinetics of charge transfer without changing the kinetics of Na^+/glucose transport (Turk et al., 1991; Panayotova-Heiermann et al., 1994).

As an alternative to direct involvement of Asp55 of proline permease in Na^+ binding, the residue could be involved in transmitting a functional important conformational alteration caused by Na^+ binding elsewhere. Thus, based on the analysis of binding properties of the Na^+/proline permease, it was proposed that binding of Na^+ causes a conformational change that results in increased affinity for substrate (Yamato & Anraku, 1993). If replacement of Asp55 effects a long-range conformational alteration, it would be expected that the substitution would alter proline binding affinity rather than cause a marked decrease in Na^+ affinity. However, replacement of Asp55 with Glu causes a relatively large effect on the apparent Na^+ affinity, and Cys or Asn replacement abolishes transport. Therefore, the notion that Asp55 is at or near a Na^+ binding site is favored.

Finally, it has been shown for transporters such as lactose permease, melibiose permease, and Na^+/H^+ antiporter that the acidic residues critical for function are located on transmembrane domains (Kaback et al., 1995; Pourcher et al., 1991, 1993; Inoue et al., 1995). In this context, it is rather surprising that the only acidic residue postulated to be in the middle of a transmembrane domain in the Na^+/proline permease (Glu75) is not important for transport. Taking into account that insertion of charged amino acid residues into a region of low dielectric is energetically unfavorable (Honig & Hubbell, 1984), a question arises as to whether the proposed topology of transmembrane domain II is correct. Indeed, preliminary studies in this laboratory on a series of *putP*−*phoA* fusions suggest that the middle of transmembrane domain II should be shifted toward the N-terminus, thereby placing Asp55 and Ser57 in putative transmembrane domain II (K. Leifker and H. Jung, unpublished information). In this respect, it is interesting that the hydrophilic segment between domains I and II was postulated to be buried within the membrane because antibody raised against a synthetic peptide corresponding to this region does not react with the permease (Yamato & Anraku, 1993).

ACKNOWLEDGMENT

We thank Dr. K. Altendorf (University of Osnabrück) for his generous support of the project, Dr. J. Wood (University of Guelph) for providing strains WG170 and WG175, and Dr. H. R. Kaback (University of California, Los Angeles) for critical reading of the manuscript.

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BI963063W