Site-Directed Mutants of *Escherichia coli* α-Ketoglutarate Permease (KgtP)

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ABSTRACT: To investigate an active site(s) in the *Escherichia coli* α-ketoglutarate premease, 11 point mutants were made in the corresponding structural gene, *kgtP*, by oligonucleotide-directed mutagenesis and the polymerase chain reaction. On the basis of sequences conserved in KgtP and related members of a transporter superfamily [Henderson P. J. F., & Maiden, M. C. (1990) *Philos. Trans. R. Soc. London B 326*, 391], Arg76 was replaced with Ala, Asp, or Lys; Asp88 with Asn or Glu; His90 with Ala; Arg92 with Ala or Lys; and Arg198 with Ala, Asp, or Lys. Mutant proteins expressed using the T7 polymerase system were in each case shown to be membrane-associated. However, they differed in transport activity. Mutants H90A and R198K had activities similar to that of wild type, and R76K and R198A retained 10–60% of the wild-type activity. In all other mutants, α-ketoglutarate transport was abolished. The results suggest that Arg92, which is highly conserved among other members of the transporter superfamily, is necessary for activity and also that Asp88 is critical for function, as observed for the tetracycline transporter. These data show further that a positive charge is essential at position 76 and is also important, but not absolutely required, at position 198 for α-ketoglutarate transport. Unlike *lacY* permease which was inactivated by deleting the last helix [McKenna, E., Hardy, D., Pastore, J. C., & Kaback, H. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2969], a KgtP truncation mutant missing the last putative membrane-spanning region was relatively stable and also retained 10–50% of the wild-type level of α-ketoglutarate transport activity.

α-Ketoglutarate permease (KgtP) is a hydrophobic, transmembrane protein that cotransports α-ketoglutarate and protons into *Escherichia coli* (Seol & Shatkin, 1990, 1991, 1992). The amino acid sequence of KgtP indicates that it belongs to subgroup V in the transporter superfamily (Henderson, 1991). This subgroup includes *E. coli* citrate and proline transporters whose sequences are also homologous to *E. coli* arabinose, xylose, and galactose transporters and several glucose carriers found in hepatoma cells, yeast, and *Arabidopsis* (all subgroup I) as well as to *E. coli* tetracycline transporter (subgroup VI). In addition to sequence homology, all of these transporters characteristically have 12 putative transmembrane domains connected by hydrophilic loops (Henderson, 1991), as in the prototypic carrier polypeptide *lacY* permease (Kaback et al., 1990). KgtP has the same type of organization, and a membrane topology model based on computer calculations of hydropathy values is shown in Figure 1.

To try to localize residues involved in the active site(s) in KgtP, five residues in the 432 amino acid protein (Arg76, Asp88, His90, Arg92, and Arg198) were selected for oligonucleotide site-directed mutagenesis on the basis of sequence homologies among transporter superfamily members (Seol & Shatkin, 1991). Mutant proteins were tested for the ability to associate with the cell membrane and to transport ^{14}C-labeled α-ketoglutarate. Arg76 and Arg198 were targeted for mutagenesis because they are the only positively charged amino acids in the two sequences GFLMRPIG (amino acids 72–79) and EWGWRIPF (amino acids 194–201) which are identical in the *E. coli* citrate transporter (Sasatsu et al., 1985), the transporter with the closest homology to KgtP (Seol & Shatkin, 1991). Asp88 was chosen since it has been shown that a negative charge in this position is critical for tetracycline transporter activity by TetA (Yamaguchi et al., 1990). Adjacent to Asp88 in KgtP is a motif R/KXGRR/K which in all members of the transporter superfamily are duplicated between hydrophobic segments II and III and segments VIII and IX (Henderson & Maiden, 1990; Seol & Shatkin, 1991). Therefore, Arg92 in the first copy of this motif was selected to test for an essential role in transport. Finally, His90 was used as a negative control because it lies between Asp88 and Arg92 but is not conserved in the motif. KgtP contains seven cysteines, but none were mutated because transport activity was not inhibited by thiol-reacting compounds including *N*-ethylmaleimide (NEM), iodoacetamide, and *p*-(chloromercuri)benzoic acid (Seol & Shatkin, 1992). By contrast, NEM decreased the activity of *E. coli* arabinose, xylose, and lactose transporters, presumably by alkylating important cysteine residues (Kaback, 1987; Henderson & Macpherson, 1986).

During the course of confirming the sequences of the *kgtP* mutants, R198D was found to contain a spontaneous termination mutation at position 400, in addition to the directed replacement of Arg by Asp at position 198. Using this spontaneous mutation, a KgtP mutant missing the last putative membrane-spanning region was generated and tested for transport activity and stability.

Experimental Procedures

Materials

α-[U-^{14}C]ketoglutarate was purchased from NEN, Vent DNA polymerase was from New England Biolabs, and pET11C was from Novagen. Bacteriophage DE3 lysogen was kindly provided by Dr. A. Rosenberg (Brookhaven National Laboratory, Upton, NY).

Methods

Bacterial Strains and Plasmids. The *E. coli* MC1061 *kgtP* negative strain (Seol & Shatkin, 1991) was used throughout this study. Wild-type KgtP was expressed from plasmid pES16 which includes the intact KgtP coding region (but is missing part of the putative RNA polymerase binding site) under T7 RNA polymerase promoter regulation (Seol & Shatkin, 1992). pET11C, the starting vector without the *kgtP* insert, was used to construct expression plasmids containing mutated *kgtP*. MC1061 *kgtP*$^-$ cells were infected with phage DE3 containing

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Table I: Primers Used for Mutagenesis[a]

amino acid change	primer	DNA sequence WT	DNA sequence mutant
R76A and R76D	5′ GA TTC CTG ATG GA/CC CCA ATA GGC G 3′	CG	GA/C
R76K	5′ GA TTC CTG ATG AAA CCA ATA GGC G 3′	CGC	AAA
D88N	5′ CGC ATA GCC AAC AAA CAT GGT CGC 3′	GAT	AAC
D88E	5′ GC ATA GCC GAA AAA CAT GGT CG 3′	T	A
H90A	5′ GCC GAT AAA GCT GGT CGC AAA AAA TC 3′	CA	GC
R92A	5′ GAT AAA CAT GGT GCC AAA AAA TCG ATG 3′	CG	GC
R92K	5′ GAT AAA CAT GGT AAA AAA AAA TCG ATG 3′	CGC	AAA
R198A and R198D	5′ G TGG GGA TGG GC/AT ATT CCT TTC GC 3′	CG	GC/A
R198K	5′ G TGG GGA TGG AAA ATT CCT TTC GC 3′	CGT	AAA

[a] Each primer was synthesized as a complementary pair for use in the overlap extension method. Some primers (R76A, R76D, R198A, and R198D) were synthesized as mixed oligonucleotides, and the correct plasmids were selected by sequencing of the corresponding regions. Underlined residues indicate sequence changes.

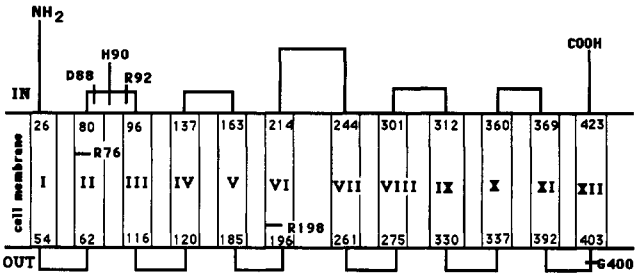

FIGURE 1: Proposed model of KgtP in the cell membrane. The model is based on hydropathy calculations using the PEPTIDESTRUCTURE program in the GCG sequence analysis software package (Devereux et al., 1984). The 12 membrane-spanning segments are depicted as rectangles with their end residues numbered. The five point mutation sites and the truncated mutation position in 400Ter are indicated.

the T7 RNA polymerase gene, and the resulting lysogens were selected as described (Studier & Moffatt, 1986) for the expression of wild-type and mutant KgtP protein.

Construction of Mutant Plasmids. Site-directed mutagenesis was done by the overlap extension method using the polymerase chain reaction (PCR; Saiki et al., 1988) as described (Ho et al., 1989). Deoxynucleotide primers with one of each pair containing the desired mismatches (Table I) were annealed to *kgtP* sequences in denatured pES16 DNA. PCR was then carried out at 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 2 min for 30 cycles with Vent DNA polymerase. Purified products from these reactions were used as templates for a second round of PCR under the same conditions but with the external primers 5′ GGCTCTAGAAATATTTCCTTTACAAAAAAAATAAAC 3′ and 5′ CGCGGATCCAATATTCACAGCATCAGGGGATG 3′. The products were purified, filled by incubation with Klenow fragment to make blunt ends, digested with *Xba*I and *Bam*HI at the corresponding sites in the external primers underlined above, and ligated into pET11C digested with the same restriction enzymes. The resulting plasmids, which contained the mutated *kgtP* downstream of the T7 promoter with T7 transcription initiation also under *lac* regulation (Studier et al., 1990), were used to transform MC1061 *kgtP*⁻(DE3) cells. In each case, the entire *kgtP* coding regions were confirmed by dideoxynucleotide sequencing (Sanger et al., 1977).

Construction of a Nonsense Mutant. During sequencing of the *kgtP* mutant plasmids, spontaneous nonsense mutations were found in pESR198A* and pESR198D*. The latter, which contained one termination mutation in addition to the designed single amino acid change, was then used to construct pESR198D and pES400Ter, a truncated form of *kgtP* containing a TGA instead of a GGA (Gly) codon at position 400. To accomplish this, pESR198D* and pES16 were digested with *Nsi*I and *Hin*dIII which resulted in two fragments from each plasmid. The smaller fragment, which includes the region in KgtP corresponding to amino acid 275 to the C-terminus, was ligated to the larger fragment from the other plasmid. pESR198A containing only the desired single amino acid replacement was constructed by the same method starting with pESR198A*. The plasmids were transformed into MC1061 *kgtP*⁻(DE3) cells, and mutated regions were sequenced to confirm the changes.

Transport Assay. Uptake of α-[U-^{14}C]ketoglutarate by MC1061 *kgtP*⁻(DE3) cells transformed with various plasmids was measured in cultures grown in M9 minimal medium containing glycerol as a sole carbon source, i.e., without α-ketoglutarate as inducer (Seol & Shatkin, 1991). After the cells were washed four times with 10 mM $MgSO_4$/100 mM potassium phosphate (pH 7.5), appropriate volumes of cell suspensions adjusted to OD_{680nm} 2.0 were incubated with 40 μM α-[U-^{14}C]ketoglutarate (final specific activity, 45 Ci/mol; 1 Ci = 37 GBq) at room temperature. At the indicated times, 50-μL samples were withdrawn, diluted with 1 mL of 100 mM LiCl/20 mM $HgCl_2$/100 mM potassium phosphate (pH 5.5), filtered, and washed with the same solution (Menezes et al., 1990). Filters were dried immediately, and radioactivity was determined by liquid scintillation counting.

Expression of Mutated KgtP. E. coli MC1061 *kgtP*⁻(DE3) cells containing different mutated plasmids were grown, radiolabeled, fractionated, and analyzed as described (Seol & Shatkin, 1992).

Pulse–chase experiments were also done to determine the stability of the 400Ter KgtP mutant. After being labeled with [^{35}S]methionine, cells were incubated with nonradioactive methionine (0.5% final concentration), and samples were taken at the indicated times for fractionation and analysis as described (Seol & Shatkin, 1992).

RESULTS

Arg76 and Arg198 Replacements. It was previously shown that most of the wild-type KgtP in *E. coli* is present in the cell membrane (Seol & Shatkin, 1992). To begin to identify sites in the protein involved in membrane association and α-ketoglutarate transport function, arginine residues in the two regions of KgtP (72-GFLMRPIG-79 and 194-EWGWRIPF-201) were changed to an uncharged (pESR76A and pESR198A), negatively charged (pESR76D and pESR198D), or positively charged amino acid (pESR76K and pESR198K).

The mutated plasmids were expressed in MC1061 *kgtP*⁻(DE3) cells using the T7 polymerase system, and the cells were fractionated into soluble and membrane fractions. In each case, the KgtP mutant proteins, like the wild-type KgtP expressed from pES16, fractionated with cell membranes (Figure

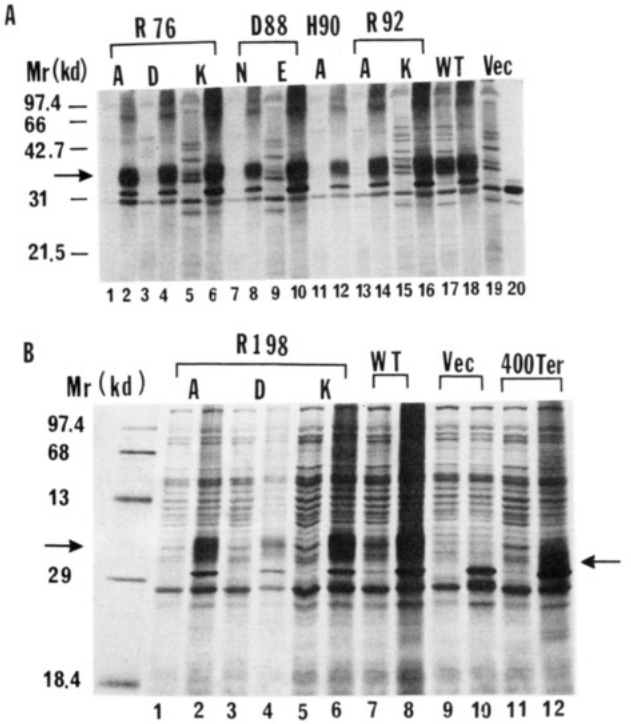

FIGURE 2: Membrane association of the wild-type and mutant KgtP proteins expressed using the T7 polymerase system. (A) KgtP replacement mutants R76, D88, H90, and R92 with the corresponding amino acid substitution shown above each lane of the paired samples. (B) R198 replacement and the 400Ter mutant. Cell membrane fractions from IPTG-induced cells (even-numbered lanes) and uninduced cells (odd-numbered lanes) were heated at 50 °C for 15 min and analyzed by 12% SDS–polyacrylamide gel electrophoresis followed by autoradiography. Arrows indicate the position of wild-type (WT) or mutated KgtP. Proteins expressed from MC1061 *kgtP*⁻(DE3)/pET11C (labeled Vec) are also shown.

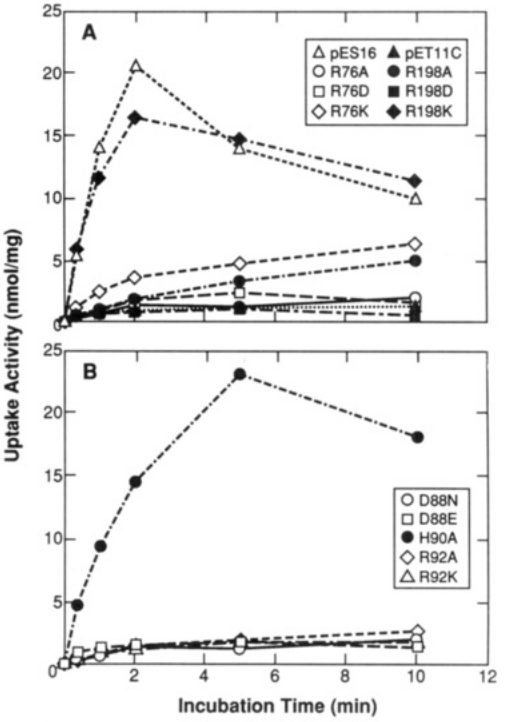

FIGURE 3: α-Ketoglutarate transport activity of mutant proteins. MC1061 *kgtP*⁻(DE3)cells transformed with different pES plasmids were grown in minimal medium containing glycerol, induced with IPTG, and assayed for α-[^{14}C]ketoglutarate transport. Wild-type KgtP activity expressed from pES16 and background activity using pET11C are shown in comparison to 11 point mutants.

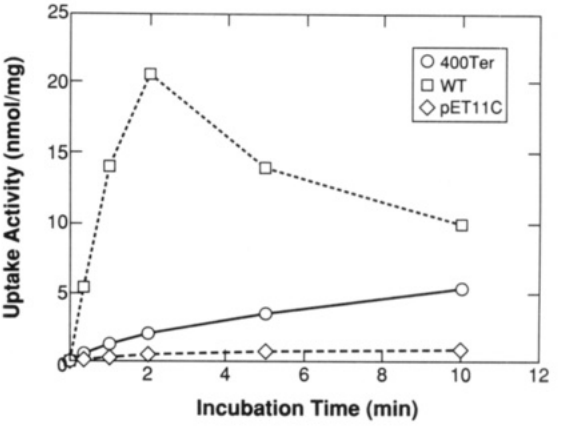

FIGURE 4: α-Ketoglutarate transport activity of the 400Ter mutant and wild-type KgtP. Also shown is the activity measured with MC1061 *kgtP*⁻(DE3)/pET11C.

2; lanes 1–6 in panel A and lanes 1–6 in panel B). However, α-ketoglutarate transport into cells expressing mutant proteins differed. Activity relative to that of the wild type was reduced with R76K and R198A, and only R198K retained full transport capacity (Figure 3A). All other mutants were inactive; i.e., α-ketoglutarate uptake values were similar to a negative control, MC1061 *kgtP*⁻(DE3) containing pET11C. Although the expression level of R198D was reduced in the experiment shown in Figure 2B, in other experiments the amount was similar to R198K, but transport activity was also lost, indicating that inactivity of the R198D mutant was not due to low expression (data not shown). The results suggest that the arginine at position 76 in the first conserved sequence of KgtP and CitA is important for transport activity. In contrast, a positive charge at position 198 in the second conserved sequence appears to be sufficient for KgtP function.

D88, H90, and R92 Replacements. To determine the importance of the highly positive conserved R/KXGRR/K motifs among members of the transporter superfamily, mutations were made in three residues within or adjacent to the first motif which is located between hydrophobic segments II and III in KgtP (amino acids 89–93, see Figure 1). The mutant polypeptides, D88N, D88E, H90A, R92A, and R92K, were present in cell membranes and by gel electrophoresis had the same mobility as that of the wild-type polypeptide (Figure 2A, lanes 7–16). However, when Asp88 was replaced by another negatively charged amino acid (Glu) or a neutral residue (Asn), the ability to transport α-ketoglutarate was abolished (Figure 3B). Conversely, His90 which is not conserved in the motif R/KXGRR/K apparently is not important for activity. Its replacement by Ala resulted in a KgtP transporter with wild type or even higher levels of activity (Figure 3B). The Arg at position 92 in KgtP is one of the most conserved amino acids among the members of the superfamily. Not surprisingly, it appears to be essential for transport activity. Mutant KgtP proteins R92K and R92A were both devoid of uptake activity (Figure 3B), suggesting that the Arg itself and not only a positive charge at this position is required for function.

Termination Mutation at Position 400. pES400Ter lacks the last putative transmembrane region XII and a part of the loop connecting hydrophobic segments XI and XII, on the basis of a model constructed from hydropathy calculations (see Figure 1). pES400Ter was expressed in the MC1061 *kgtP*⁻(DE3) cells induced with isopropyl thiogalactoside (IPTG), and the truncated KgtP appeared in the membrane fraction (Figure 2B, lane 12). In addition, the 400Ter KgtP maintained 10–50% of the wild-type activity (Figure 4). The stability and function of the truncated KgtP were also tested

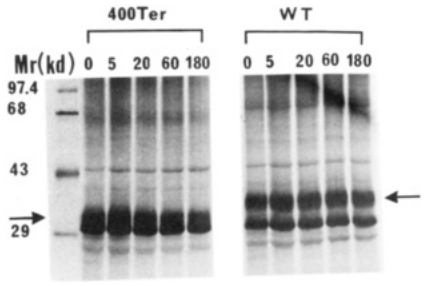

FIGURE 5: Stability of wild-type (WT) and truncated 400Ter KgtP. Samples of [^{35}S]methionine-labeled cultures were taken at 0, 5, 20, 60, and 180 min after the addition of excess unlabeled methionine as described in Experimental Procedures. Arrows on the left and right indicate positions of truncated and wild-type KgtP, respectively.

in a pulse–chase experiment and by assaying for α-ketoglutarate transport after storage of mutant-expressing cells in ice. Both experiments indicate that the 400Ter protein is relatively stable; i.e., degradation products were not evident after a 3-h chase at 37 °C (Figure 5), and transport was maintained for at least 30 h at 0 °C (data not shown).

Discussion

To investigate the functional importance of conserved amino acids among the members of the transporter superfamily, 11 separate point mutations in five different sites were introduced into *kgtP*, and one termination mutant was also constructed. All were expressed, and the mutated KgtP proteins, like the wild-type counterpart, fractionated with cell membranes. In addition, the mutant proteins migrated in polyacrylamide gels as broad bands with the same mobility as that of the wild-type KgtP (with the exception of the 400Ter truncation mutant). These properties, i.e., broad bands migrating faster than predicted from deduced molecular weights, are characteristic of hydrophobic proteins like KgtP (Seol & Shatkin, 1991) and have been observed for other transporters (Henderson & Macpherson, 1986).

The transport assays demonstrated that the positive charges carried by the arginines in positions 76 and 198 are important for α-ketoglutarate uptake. These residues are in the middle of two eight amino acid stretches that are conserved between KgtP and CitA, both transporters of negatively charged substrates. Therefore, these positive charges located in putative membrane-spanning domains II and VI may serve to neutralize the acidic substrates, thus facilitating transport in the hydrophobic environment of the cell membrane. However, it is also possible that they neutralize negatively charged amino acids in other transmembrane domain(s).

Three residues within or close to the first copy of the duplicated motif, R/KXGRR/K (positions 89–93), one of the most conserved sequences among the members of the transporter superfamily, were replaced to test the importance of this sequence for KgtP function. Aspartate at position 88 is also conserved in TetA, and its negative charge has been shown to be critical for the transport of tetracycline (Yamaguchi et al., 1990). When Asp88 was replaced in KgtP by either Asn or Glu, the resulting mutants lost all uptake activity, suggesting that the Asp itself, not a negative charge only, was critical to KgtP activity. Similarly, the Arg at position 92 is required because its replacement by Lys destroyed uptake activity, consistent with this amino acid being the most conserved residue among all the superfamily transporters.

In contrast, the His at position 90 in KgtP could be replaced by Ala without loss of activity. Although this result is expected because position 90 varies in the motif, there is another interesting point related to the H90A mutation. In *lacY* permease, the most extensively investigated membrane transporter, His325 and Glu322 have been reported to play an important role as components of a H^+ relay with Arg302 for cotransport of lactose substrate (Kaback et al., 1990). If α-ketoglutarate transport involves a similar H^+ relay, His90 and Asp88 (or His185 and Glu188) could comprise a similar pair since they are the only sites in KgtP where a histidine lies close to a negatively charged amino acid. However, the H90A mutant retained wild-type α-ketoglutarate transport activity, suggesting that the transport mechanisms of KgtP and *lacY* permease are different or that His90 and Asp88 do not comprise a pair for H^+ relay.

The activity of the truncated mutant, 400Ter, is striking in comparison to the lack of activity of the similarly truncated *lacY* permeases. For instance, *lacY* permease terminated at residue 396 or 397, and missing the last turn of putative helix XII is completely defective with respect to transport (McKenna et al., 1991). Conversely, the 400Ter KgtP mutant, which has lost part of the last connecting hydrophilic loop and the entire putative membrane-spanning domain XII, retained 10–50% of wild-type transport activity (Figure 4). The differences between *lacY* permease and KgtP are consistent with the absence of primary sequence homology between *lacY* permease and the transporter superfamily, despite overall structural similarities such as the 12 transmembrane regions.

Experimental data are not yet available either for or against the KgtP membrane topology model which we have constructed on the basis of hydropathy calculations (Figure 1). Consequently, it remains a possibility, although an unlikely one, that the last membrane-spanning domain is retained in the 400Ter mutant. To test the validity of the topology model, several *kgtP–phoA* fusion mutants (Calamia & Manoil, 1990) have been isolated, and analyses of phosphatase activities are being carried out. The resulting data should help to define more accurately the putative transmembrane segments in KgtP predicted from computer calculations.

Acknowledgments

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