Nucleotide Sequence and Functional Properties of a Sodium-dependent Citrate Transport System from *Klebsiella pneumoniae* 

(Received for publication, November 7, 1992)

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The gene of the sodium-dependent citrate transport system from *Klebsiella pneumoniae* (*citS*) is located on plasmid pES3 (Schwarz, E., and Oesterhelt, D. (1985) *EMBO J.* 4, 1599–1603) and encodes a 446-amino acid protein. Transport of citrate via this citrate transport protein (CitS) is dependent on the presence of sodium ions and is inhibited by magnesium ions. The ΔpH (pH gradient across the membrane) is the major driving force for uptake. It is postulated that, in analogy with the proton-dependent citrate carrier (CitH) of *K. pneumoniae* (van der Rest, M. E., Abee, T., Molenaar, D., and Konings, W. N. (1990) *Eur. J. Biochem.* 195, 71–77), only one of the protonated species of citrate is recognized by CitS and that citrate is translocated across the membrane in symport with protons and sodium ions. The hydropathy profile of CitS suggests that the protein is very hydrophobic and contains 12 membrane-spanning segments. These segments are not centered around a hydrophilic core as has been suggested for other transport proteins, but the protein is asymmetrical with seven transmembrane segments in front of a large hydrophilic loop and five after this loop. The amino acid sequence is highly similar to a citrate transport system of *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* (CitP) (David, S., van der Rest, M. E., Driessen, A. J. M., Simons, G., and de Vos, W. M. (1990) *J. Bacteriol.* 172, 5789–5794) and less similar to CitH of *K. pneumoniae*. We conclude that the *citS* gene of *K. pneumoniae* encodes a sodium-dependent citrate transport system that belongs to a novel subclass of transport proteins.

*Klebsiella pneumoniae* shares with a wide range of bacteria of the family Enterobacteriaceae the ability to utilize citrate as a carbon and energy source. This property is one of several criteria used to distinguish between the different genera of this family (1). *Escherichia coli* normally does not grow on citrate (2). This inability has been attributed to the lack of a citrate transport system (3). However, rare isolates carrying antibiotic resistance plasmids or transposons encoding specific citrate transport systems have been investigated (4–6). In *K. pneumoniae* three citrate transport systems have been identified. The plasmids pES1, pES2, and pES3, isolated from a cosmid bank of *K. pneumoniae* DNA, encode different citrate transport systems (7). pES1 encodes a constitutive system which has been studied extensively. The *citH* gene coding for this citrate transporter has been cloned and sequenced (8). The gene product belongs to a class of transport proteins which have 12 transmembrane segments and a central hydrophilic core (9). The mechanism of transport has been characterized, and it was indicated that citrate2− is transported in symport with three protons (10). pES2 encodes an inducible citrate transport system which has only been studied superficially. pES3 encodes a citrate transport system that is probably induced under anaerobic conditions on citrate as a carbon source. Dimroth and Thomer (11, 12) showed that this citrate transport is sodium-dependent. Plasmid pES3 is also the determinant for the genes of the oxaloacetate decarboxylase (13), which is the key enzyme for citrate fermentation. The decarboxylase was found by Dimroth (14) to be a biotin-containing membrane protein consisting of three subunits and acting as a sodium pump. The free energy of the decarboxylation is used to translocate Na+ out of the cell thus generating an electrochemical gradient of sodium ions under fermentative conditions. This sodium gradient could subsequently be involved in Na+–citrate symport. Since anaerobic degradation of citrate by *K. pneumoniae* yields only one ATP by substrate level phosphorylation, the creation of a sodium motive force by the decarboxylase contributes significantly to the energetic yield of fermentation.

The fact that in the same organism a proton-dependent citrate transport protein has been characterized previously makes the sodium-dependent citrate transport system a very interesting system to study. In this report we describe the energetics of CitS from *K. pneumoniae*. The *citS* gene was isolated and its nucleotide sequence was determined. Analysis of the primary structural data shows that the *citS* gene product is a typical transport protein with alternating hydrophilic and hydrophobic regions. CitS has 12 α-helices with a large hydrophilic loop which is asymmetrically placed with seven α-helices in front of the loop and five after the loop.

**MATERIALS AND METHODS**

**Bacterial Strains and Plasmids**—The *citS* gene was derived from genomic DNA of *K. pneumoniae* strain ATCC 19882. *E. coli* DH1 was
used as a host for the plasmid pES3 (7), which contains a 23-kb DNA fragment of _K. pneumoniae_ DNA. A subclone, pSCT, was obtained in the expression vector pNIIIA (15, 34). This plasmid conferred aerobic growth on citrate and was used for further studies. pH63 and pBSSG, the derivatives of pSCT. E. coli JM103 was used as a host for phage M13 mp18 and M13 mp19 (16).

*Media and Antibiotics—* Luria broth (LB) and LB agar (17) were used for routine bacterial growth. The selective medium for citrate utilization was Simmons citrate agar (Difco), supplemented with 4 \(\mu\)g/ml thiamine and 100 \(\mu\)M isopropyl-D-thiogalactopyranoside. Ampicillin was used at 100 \(\mu\)g/ml.

Preparation of Membrane Vesicles—E. coli DH1/pRS63-2 cells were grown to an \(A_{600nm}\) of 0.7 on LB medium containing 100 \(\mu\)g/ml ampicillin and 1 mM isopropyl-D-thiogalactopyranoside. Cells were harvested, and membrane vesicles were isolated as described by Kaback (18). Membrane vesicles were finally resuspended in 50 mM potassium phosphate buffer, pH 7.0 (8 mg of protein/ml), and stored in portions of 0.5 ml in liquid nitrogen until use. Protein concentration was measured by the method of Lowry et al. (19).

Solute Transport—Solute transport by membrane vesicles was measured at 30°C as described previously (10). Preincubation with 2 \(\mu\)M 2,7,9-tricarboxy-1H-pyrrolo-(2,3-f)-quinoline-4,5-dione (POQ) was carried out for 5 min after which 20 mM glucose was added to start electron transport in the vesicles. The uptake experiment was started by the addition of 4.5 \(\mu\)M [1,5-\(^{14}\)C]citrate. Uptake driven by artificial gradients and the magnitude of the \(\Delta\Psi\) (pH gradient across the membrane) and \(\Delta\Psi\) (electrical potential across the membrane) were determined as described previously (10).

DNA Manipulations—General procedures for cloning and DNA manipulations were essentially performed as described by Maniatis et al. (20).

Restriction enzymes and T4 DNA ligase were used as recommended by the manufacturer (Pharmacia-LKB, Sweden).

DNA Sequence Analysis—DNA sequencing was performed by the dideoxy-chain terminator procedure of Sanger et al. (21). Most of the M13 mp18/19 templates were generated as deletion clones by the method described in the manual of the pBluescript II Eko/Mung DNA sequencing system, Stratagene (La Jolla, CA).

Missing parts of the sequence were sequenced with the use of sitespecific oligonucleotide primers. The DNA sequence was analyzed both with an automatic DNA sequencer (Applied Biosystems model 370A) as described by Smith et al. (22) and with \(^{35}\)S-dATP. Oligonucleotides were synthesized with an automatic DNA synthesizer (Applied Biosystems model 381A).

Chemicals—Radioactively labeled solutes were obtained from the Radiochemical Center (Amersham, Buckinghamshire, United Kingdom) with the following specific activities: [1,5-\(^{14}\)C]citrate, 111 mCi/mmole; [\(^{35}\)S]-dATP, 3700 Ci/mmol. The Sequenase kit was obtained from the United States Biochemical Corp. All other chemicals were reagent grade and obtained from commercial sources.

RESULTS

Subcloning of the citS Determinant—Three _E. coli_ clones (DH1/CIT1, DH1/CIT2, and DH1/CIT3) capable of utilizing citrate as a sole carbon and energy source had been isolated from a cosmid bank of _K. pneumoniae_ DNA (7). One of these clones, _E. coli_ DH1/CIT3, was found to express a citrate transport system (CitS) which could be induced under anaerobic conditions (7). The 23-kb DNA fragment determining the Cit* phenotype of _E. coli_ DH1/CIT3 is located on plasmid pES3 (7). Plasmid pES3 is very unstable and breaks down spontaneously (23). Subsequent ligation of the fragments in the expression vector pNIIIA digested with _BamHl_ yielded plasmid pSCT (34). _E. coli_ DH1 transformants with pSCT are both chloramphenicol- and ampicillin-resistant, indicating that this plasmid also contains, besides pNIIIA (15), the Cm resistance gene of the original cosmid vector pSUP205 (23). pSCT was found to be unstable, and the correct size of the insert in this plasmid could not be precisely determined. After partial digestion of pSCT with _Sau_III, DNA fragments were ligated into _BamHl_-digested plasmid pNIIIA. This way plasmid pRS63 was constructed with a DNA insert size of about 4 kb. An EcoRI site was found 2.1 kb downstream of the lac promoter of the vector pNIIIA. This site, together with the HindIII site of the vector, was used to subclone a 2.1-kb fragment containing the citrate transport gene. This plasmid was called pRS63-2. Transformants containing pRS63-2 grow on Simmons minimal citrate agar supplemented with isopropyl-D-thiogalactopyranoside without a lag phase. The colour selection on this medium allowed the identification of citrate utilizing transformants. These cells which expressed the citrate transport system under the control of the lac promoter were further used for citrate transport assays. The plasmid pRS63-2 isolated from these cells served for nucleotide sequence determination of the citS gene.

Citrate Transport Assays—In this study the role of the proton motive force (\(\Delta\Psi\)) and the nature of the symported ions have been analyzed in right-side-out membrane vesicles prepared from _E. coli_ DH1/pRS63-2. In these membrane vesicles a PQQ-dependent glucose dehydrogenase is present which is functionally linked to the respiratory chain. In the presence of PQQ, glucose is oxidized and a \(\Delta\Psi\), inside negative and alkaline, is generated (24). Membrane vesicles prepared from _E. coli_ DH1 lack a citrate transport system and in these membrane vesicles no uptake of citrate could be observed (8).

Membrane vesicles from _E. coli_ DH1/pRS63-2, energized aerobically with glucose/PQQ, accumulated citrate at a low rate, and low steady state levels of accumulation were reached (Fig. 1). Sodium ions were found to have a strong stimulating effect on uptake of citrate (Fig. 1). Maximal stimulation of transport was achieved at 10 mM NaCl. Increasing the sodium ion concentration to 100 mM did not further stimulate transport activity (results not shown). Lithium ions at concentrations of 10 and 100 mM could not replace sodium ions. To investigate whether the observed effects of Na were directly related to the transport process, the influence of different sodium ion concentrations on the components of the \(\Delta\Psi\) and \(\Delta\Psi\) were studied. Under the conditions employed the \(\Delta\Psi\) was found to be 70 mV and the \(\Delta\Psi\) was -60 mV. These values were not affected by the presence of Na. It is therefore concluded that citrate transport is directly dependent on the presence of sodium ions. In _Bacillus subtilis_ citrate is transported as a magnesium-citrate complex (25). The role of Mg in transport mediated by CitS was investigated in membrane vesicles of _E. coli_ DH1/pRS63-2. Transport of citrate was studied in the presence of 10 mM Na and different concentrations of magnesium ions. Citrate uptake was already significantly inhibited by 100 \(\mu\)M Mg and this inhibition increased with the Mg concentration (Fig. 2). As shown for Na also, the addition of magnesium ions at concentrations up to 10 mM did not affect the composition nor the magnitude of the \(\Delta\Psi\). These results indicate that citrate is transported as an uncomplexed species. The formation of a Mg-citrate complex results in a decrease of the rate and extent of citrate uptake.

The relation between the \(\Delta\Psi\) and citrate transport was investigated with artificially imposed ion gradients. A \(\Delta\Psi\) can be generated by equilibrating membrane vesicles with relatively high concentrations of acetate followed by a 100-
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FIG. 1. Effect of Na⁺ on citrate uptake in membrane vesicles of E. coli DH1/pRS63-2. Assays were conducted aerobically at 30 °C in 50 mM Pipes (potassium salt), pH 6.0. Membrane vesicles (0.4 mg/ml) were preincubated in the presence of PQQ (2 μM) before the addition of the electron donor glucose (20 mM). Uptake experiments were initiated by addition of 4.5 pM [1,5-¹⁴C]citrate. 0, no Na⁺ added; 0, 100 μM NaCl; V, 1 mM NaCl; O, 4 mM NaCl; □, 7 mM NaCl; Δ, 10 mM NaCl.

FIG. 2. Effect of Mg⁺ on citrate uptake in membrane vesicles of E. coli DH1/pRS63-2. Assays were conducted as described in the legend to Fig. 1 in the presence of 10 mM NaCl. Uptake experiments were started by addition of 4.5 μM [1,5-¹⁴C]citrate. 0, no Mg⁺ added; V, 100 μM MgCl₂; O, 1 mM MgCl₂; C, 5 mM MgCl₂; □, 10 mM MgCl₂.

high rate in the presence of sodium ions (Fig. 3, □—□). In the absence of sodium ions a lower level of citrate uptake was observed (Fig. 3, O—O). An inwardly directed sodium gradient (ΔΔνNa⁺: chemical gradient of sodium ions across the membrane) was created by a 100-fold dilution of potassium-loaded membrane vesicles of E. coli DH1/pRS63-2 into a potassium-free medium with a high concentration of sodium ions. This ΔΔνNa⁺ could drive uptake of citrate only to a low level (Fig. 3, △—△). Addition of valinomycin to the reaction mixture results in the creation of a ΔΔνNa⁺ (electrochemical gradient of sodium ions across the membrane). In this case an increase in the citrate accumulation is observed, indicating that citrate uptake is electrogenic (Fig. 3, Δ—Δ). The imposition of an artificially generated Δp + ΔΔνNa⁺ (membrane vesicles were also 100-fold diluted) resulted in the highest citrate uptake rates (Fig. 3, □—□). It is clear from these results that plasmid pRS63-2 codes for a secondary sodium-dependent citrate transport protein. The ΔpH, Δψ, and ΔΔνNa⁺ are driving forces for citrate uptake.

DNA Sequence Determination—The nucleotide sequence of the fragment in pRS63-2 determining the Cit⁺ phenotype was determined with the dideoxynucleotide method of Sanger et al. (21). The sequence of 2083 base pairs is shown in Fig. 4. Several open reading frames (ORF) could be detected but only one is of sufficient size, from start codon ATG at position 321 to the stop codon TAA at position 1659, to code for a transport protein (26). The transcription of this reading frame is controlled by the promoters of the vector pINIIIA. The ORF consists of 1338 base pairs. The corresponding amino acid sequence is also given in Fig. 4. The molecular mass of the protein calculated from the 446 amino acids is 47,531 Da. The amino acid sequence shows a composition which is typical for membrane proteins. It contains 29% polar residues and 71% non-polar residues. It contains 61 charged residues, of which 35 are basic and 26 acidic, giving an excess of nine positive charges at neutral pH. The citrate carrier is thus a...
FIG. 4. Nucleotide sequence of the fragment in pRS63-2 determining the Cit+ phenotype. The sequence is shown in the 5' to 3' direction. The amino acid sequence predicted from the DNA sequence of the open reading frame between position 321 and 1661, coding for CitS, is given in the three-letter code below the DNA sequence. The shaded boxes indicate the 12 membrane-spanning regions predicted by the method of Eisenberg et al. (33). The RBS sequence and the -35 and -10 regions of the putative promoter are underlined. Also underlined is the ORF coding for the γ-subunit of the oxaloacetate decarboxylase (13), from position 1817 to 2065.

basic protein with a theoretical isoelectric point of 8.6.

A second ORF is found in the DNA sequence of pRS63-2 which starts with an ATG codon at position 1817 and ends with a TAA stop codon at position 2066. This gene codes for the γ-subunit of the oxaloacetate decarboxylase from K. pneumoniae described by Laufermair et al. (13).

DISCUSSION

The nucleotide sequence of the citS gene coding for the sodium-dependent citrate carrier from K. pneumoniae has been determined. The DNA sequence in Fig. 4 shows the entire citS gene and its flanking regions. In front of the ATG start codon a potential ribosomal binding site is found at position 309. The region in front of the citS ORF is very AT-rich and contains an eubacterial promoter motif. The -35 and -10 promoter regions of the citS gene and the spacing between the two regions are similar to that of the consensus promoter sequences that are recognized by the RNA polymerase of E. coli (27). In addition to the citS gene, a second ORF was found starting at position 1817 and ending at position 2066. This ORF codes for the γ-subunit of the oxaloacetate decarboxylase. Between the ORF coding for the citS gene and the genes coding for the oxaloacetate decarboxylase are no regions found which could act as a promoter sequence. This suggests that the citS gene and the genes coding for the oxaloacetate decarboxylase are cotranscribed. Laufermair et al. (13) indicated the existence of a polycistronic messenger of about 5600 nucleotides in length coding for the oxaloacetate decarboxylase genes. The citS gene and the genes coding for the oxaloacetate decarboxylase require about 4.4 kb. This would leave room for a gene of 1.2 kb which could be located downstream.
Fig. 5. Alignment of citrate carriers with the method of Higgins and Sharp (31). CitA, citrate carrier from E. coli (6); CitH, sodium-dependent citrate carrier from K. pneumoniae (5); CitS, sodium-dependent citrate carrier from K. pneumoniae (this article); CitP, citrate carrier from L. lactis (30).

The gene of the oxaloacetate decarboxylase β-subunit (13). Currently these regions are analyzed at the nucleotide level. The nucleotide sequences of the genes coding for six citrate carriers from bacterial origin have now been determined. The citA genes from E. coli (5, 6) located on a naturally occurring plasmid or transposon, the citA genes from Citrobacter amalonaticus (28) and Salmonella typhimurium (29) located on the chromosome, the citH and citS genes from K. pneumoniae (Ref.8 and this paper) and also the citP gene of L. lactis subsp. lactis var. diacetylaclactis (30) which is plasmid-located. The genes from K. coli, C. amalonaticus, and S. typhimurium are virtually identical (28, 29). Analysis of the different citrate transport proteins by the method developed by Higgins and Sharp (31) for the alignment of multiple protein sequences shows that the CitH and CitS of K. pneumoniae are related proteins with an overall similarity of 57% (Fig. 5). CitS of K. pneumoniae and CitP of Lactococcus lactis subsp. lactis var. diacetylactis (30) have an overall similarity of 76% (Fig. 5). The similarity between the citrate carrier of E. coli and CitH of K. pneumoniae is 89%. It was therefore postulated that these transport proteins have evolved from one ancestral gene (32). Alignment of all citrate transport proteins shows 32% similar amino acids between the citrate transport proteins of the different bacteria throughout the sequence (Fig. 5). The ion dependence of CitP from L. lactis subsp. lactis var. diacetylactis has not yet been investigated. The similarity with CitS of K. pneumoniae would suggest that CitP has the same ionic requirement of citrate translocation. Recent experiments, however, argue against a role of Na⁺ in the translocation of citrate by the L. lactis carrier. The similarity between CitH and CitS from K. pneumoniae is less pronounced. The difference between the two citrate transport proteins from K. pneumoniae is confirmed by their hydropathy profiles (Fig. 6). The algorithm of Eisenberg et al. (33) predicts 12 hydrophobic transmembrane α-helices separated by hydrophilic domains for all citrate carriers. However, the hydropathy

Fig. 6. Normalized hydrophobicity profiles of the amino acid sequences of CitS (A) and CitH (C) of K. pneumoniae and CitP of L. lactis subsp. lactis var. diacetylactis (B). The hydrophobicity was calculated with an averaging window of 21 amino acids. The black bars indicate the membrane-spanning regions predicted with the method of Eisenberg et al. (33)

profiles of CitS of K. pneumoniae and CitP of L. lactis subsp. lactis var. diacetylactis are somewhat different than expected for members of the family proposed by Baldwin and Henderson (9) which have six α-helices on either side of a central hydrophilic loop. Both carriers are asymmetrical proteins with seven α-helices in front and five after their hydrophilic loop (Fig. 6). The algorithm of Eisenberg et al. (33) predicts an additional hydrophobic α-helix in CitP of L. lactis subsp. lactis var. diacetylactis from amino acid 320 to 341, but this helix has a comparably low hydrophobicity. Therefore, it is not likely that this helix crosses the membrane. It is clear that CitS of K. pneumoniae and CitP of L. lactis subsp. lactis var. diacetylactis are similar proteins which differ from the class of transport proteins which have 12 membrane spanning α-helices and a central hydrophilic core.

Citrate transport in E. coli membrane vesicles, catalyzed by CitS, is clearly sodium-dependent. Low uptake rates of citrate can be explained by contamination of the reaction media with small amounts of sodium ions. Uptake of citrate is stimulated by an artificial ΔpH and a ΔΨ only in the presence of sodium ions. A ΔΨ+- can drive uptake of citrate but only at a low rate. Recently, Dimroth and Thomet (12) suggested that only the ΔΨ+- is a driving force for citrate uptake via this carrier. The results presented in Fig. 3, however, indicate that in addition to sodium ions also protons are cotransported with citrate by this transport system. This cotransport can occur by a symport of citrate with Na⁺ and H⁺ or by symport of a sodium-citrate complex with H⁺. Further studies are neces-

2 M. E. van der Rest, unpublished results.

The nucleotide sequences of the genes coding for six citrate carriers from bacterial origin have now been determined. The citA genes from E. coli (5, 6) located on a naturally occurring plasmid or transposon, the citA genes from Citrobacter amalonaticus (28) and Salmonella typhimurium (29) located on the chromosome, the citH and citS genes from K. pneumoniae (Ref.8 and this paper) and also the citP gene of L. lactis subsp. lactis var. diacetylactis (30) which is plasmid-located. The genes from K. coli, C. amalonaticus, and S. typhimurium are virtually identical (28, 29). Analysis of the different citrate transport proteins by the method developed by Higgins and Sharp (31) for the alignment of multiple protein sequences shows that the CitH and CitS of K. pneumoniae are related proteins with an overall similarity of 57% (Fig. 5). CitS of K. pneumoniae and CitP of Lactococcus lactis subsp. lactis var. diacetylactis (30) have an overall similarity of 76% (Fig. 5). The similarity between the citrate carrier of E. coli and CitH of K. pneumoniae is 89%. It was therefore postulated that these transport proteins have evolved from one ancestral gene (32). Alignment of all citrate transport proteins shows 32% similar amino acids between the citrate transport proteins of the different bacteria throughout the sequence (Fig. 5). The ion dependence of CitP from L. lactis subsp. lactis var. diacetylactis has not yet been investigated. The similarity with CitS of K. pneumoniae would suggest that CitP has the same ionic requirement of citrate translocation. Recent experiments, however, argue against a role of Na⁺ in the translocation of citrate by the L. lactis carrier. The similarity between CitH and CitS from K. pneumoniae is less pronounced. The difference between the two citrate transport proteins from K. pneumoniae is confirmed by their hydropathy profiles (Fig. 6). The algorithm of Eisenberg et al. (33) predicts 12 hydrophobic transmembrane α-helices separated by hydrophilic domains for all citrate carriers. However, the hydropathy

2 M. E. van der Rest, unpublished results.
sary to discriminate between these two options. In solution, citrate is composed of different protonated species. The citrate transport system CitH of *K. pneumoniae* was shown to translocate citrate\(^{3-}\) in symport with three protons. The experimental data of the CitS transport system also indicate that this system translocates citrate\(^{3-}\) in symport with three positive ions, which are protons or sodium ions. For a more detailed analysis of the mechanism of energy coupling, attempts will be made to isolate CitS and to reconstitute this protein functionally into liposomes. In this model system the ionic composition at both sides of the membrane can be controlled.

Acknowledgment—We thank Dr. K. Rodewald for her help with the DNA sequence determination.

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