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Purification of two active fusion proteins of the Na^+-dependent citrate carrier of *Klebsiella pneumoniae*

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Abstract

The sodium-ion-dependent citrate carrier of *Klebsiella pneumoniae* (CitS) was purified by means of bioengineerical methods. By fusing the biotin acceptor domain of the α-subunit of the oxaloacetate decarboxylase of *K. pneumoniae* to the C-terminus of CitS, purification of the carrier was achieved by use of a monomeric avidin-Sepharose column. Additionally, we were able to purify a CitS-protein with an N-terminal histidine-tag by immobilized metal chelate affinity chromatography (with Ni^{2+}-nitrilotriacetic acid-(NTA-) resin). Both purified fusion proteins showed citrate transport activity after reconstitution into liposomes by the freeze/thaw/sonication procedure.

Key words: citS gene; Secondary active transport; Citrate carrier; Sodium ion/citrate symport; Fusion proteins, Affinity chromatography; *Klebsiella pneumoniae*

1. Introduction

In *Klebsiella pneumoniae* a unique Na^+-dependent citrate carrier (CitS) is induced upon anaerobic growth on citrate. The properties of CitS have been investigated in a reconstituted proteoliposomal system with the carrier-protein derived from a Triton X-100 extract of *K. pneumoniae* membranes [1] and with vesicles from *citS* expressing *Escherichia coli* cells [2]. The DNA sequence of *citS* has recently been determined [2]; it encodes a highly hydrophobic protein of 446 amino acids with a predicted M_r of 47,531 and 12 putative membrane spanning α-helices. For more detailed analyses of the citrate carrier, we decided to purify CitS and reconstitute it into liposomes. In such a defined system, the ionic composition at both sides of the membrane is exclusively determined by the experimental design and not disturbed by other activities such as those of the sodium/proton antiporters present in *E. coli* membranes and *K. pneumoniae* Triton X-100 extracts. In order to purify CitS, we constructed fusions with the biotinylation domain of the oxaloacetate decarboxylase from *K. pneumoniae* or with an N-terminal tag of six histidine residues and purified these fusion proteins by affinity chromatography.

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Abbreviations: CitS, sodium-dependent citrate carrier; CitSα, CitS with a C-terminally attached biotinylation domain derived from the α-subunit of oxaloacetate decarboxylase; $CitS_{His}$, CitS with an N-terminally attached polyhistidine-tail; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; IPTG, isopropyl-β-D-thiogalactopyranoside; *oadGAB*, genes encoding the γ-, α-, and β-subunit of oxaloacetate decarboxylase from *Klebsiella pneumoniae*, respectively.

2. Experimental

2.1. Bacterial strains and plasmids

For plasmids pQE-8 (Qiagen Inc.) and $pCitS_{His}$ the *Escherichia coli* K12 strain M15[pREP4] (Qiagen) was used as host. All other plasmids were propagated in *E. coli* DH5α (Bethesda Research Laboratories).

2.2. Media and antibiotics

Luria broth (LB) and LB-agar were used for routine bacterial growth [3]. Ampicilline was used at 100 μg/ml (Amp^{100}) and kanamycin at 25 μg/ml (Km^{25}). Simmons' citrate agar [4], supplemented with 4 μg/ml thiamine and Amp^{100} was used to select for citrate utilizing clones.

2.3. Recombinant DNA work, DNA sequence analysis and PCR

For routine work with recombinant DNA, established protocols were used [3]. Sequence analysis was performed according to the dideoxynucleotide chain termination method [5] using a Taq DyeDeoxy terminator cycle sequencing kit and the model 370A DNA sequencer from Applied Biosystems. The following mixture (100 μl) was used for PCR: 20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 500 μM of each deoxynucleoside triphosphate, 1 μM forward primer, 1 μM reverse primer and 1 μg pGW234 (carrying the *K. pneumoniae* genes for *citS* and *oadGAB*) [6] as template. Two units of Vent DNA polymerase were added after an initial denaturation step at 94°C for 10 min. Thirty cycles were carried out for 90 s at 94°C, 60 s at 50°C and 60 s at 70°C. PCR fragment A: the forward primer (5′ GTC AGC GAC GGC GGT GAC GTC 3′) is identical to bases 1502–1522 of the KPOXD (*K. pneumoniae* oxaloacetate decarboxylase) sequence (GenBank/EMBL Data Bank, accession number J03885). The reverse primer (5′ GTC GAC TCT AGA TTA CGC CAG GGT CAT CAG GG 3′) is complementary to the bases 1800–1819 of the same sequence entry. The primer extends at the 5′ end by additional 12 bp comprising a *Xba*I restriction-site. PCR fragment B: the forward primer (5′ CGG CGA TCG GTG GCT GGC TGA 3′) is identical to bases 1450–1471 of the KPCITS (*K. pneumoniae* Na^+-dependent citrate carrier) sequence (GenBank/EMBL Data Bank, accession number M83146). The reverse primer (5′ CTC GAT GGA TCC AAT CAT CAT GCC GAA CAC GA 3′) is complementary to bases 1639 to 1658 of the same sequence entry. It has a 12 bp extension at the 5′ end containing a *Bam*HI restriction site. PCR fragment C: the forward primer (5′ GCT CGG TAC CCG GCC GGG GAT CCA TCG AGG GTA GGA TGA CTA ACA TGA GTC AG 3′) is identical to bases 321–338 of the KPCITS sequence mentioned above and contains at the 5′ end 35 bp matching the sequence of the multiple cloning site and the factor Xa recognition site encoding region of pMal-c

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(New England Biolabs). The reverse primer (5' CAC CGG AGC GCC ACC G 3') is complementary to bases 557–572 of the KPCITS sequence.

2.4. Construction of the CitSα fusion and the 6xHis-tagged CitS-fusion

PCR fragment B was restricted with *StuI/BamHI* and ligated into *StuI/BamHI* restricted pKP-1 (a pGW234 derivate carrying only *citS*) to obtain pKP-1Δstop. PCR fragment A was restricted with *PvuII* and *XbaI* and cloned into a *StuI/XbaI* restricted pMAL-c vector to obtain pMA-9. pMA-9 was restricted with *BamHI/XbaI* and the resulting 319 bp fragment (including a factor Xa recognition site) was ligated into *BamHI/XbaI* restricted pKP-1Δstop to obtain pFXa (see Fig. 1). PCR fragment C was restricted with *KpnI/SphI* and ligated into a *KpnI/SphI* restricted pFXa to obtain pFXa-2 (Fig. 1). pFXa-2 was restricted with *BamHI* and the resulting 1355 bp fragment was isolated and ligated into a *BamHI* restricted, dephosphorylated pQE-8 vector to obtain $pCitS_{His}$. The DNA sequence of the fusion sites and the PCR derived sequences were controlled by sequencing.

2.5. N-terminal sequencing and protein determination

For N-terminal sequencing, 18 μg of purified CitSα were blotted onto a polyvinylidene difluoride-(PVDF-)membrane and treated as described [7]. N-terminal sequence analysis was performed using a protein sequencer with on-line PTH-(phenylthiohydantoin) amino acid detection by HPLC. Protein was determined according to [8] using bovine serum albumin dissolved in the same buffer as the samples as standard.

2.6. Determination of expression of the fusion protein and growth of E. coli *DH5α/pFXa and* E. coli *M15 [pREP4]/pCitS$_{His}$*

E. coli DH5α/pFXa cells were grown on LB Amp^{100} to an OD_{600} of 0.8 to 1.2. After addition of 50 μM IPTG, the culture was grown for another 2 h and then harvested. *E. coli* DH5α harbouring pGW234 or pMA-9 was grown overnight in LB Amp^{100} and then harvested. Cells from 1 ml culture with an $OD_{600} = 1$ were resuspended in 100 μl of SDS-sample buffer and boiled for 5 min. Proteins from a 10 μl sample were separated by SDS-PAGE (10% acrylamide) [9], blotted onto a nitrocellulose filter [10] and stained with avidin-peroxidase [11]. Four liters of LB Amp^{100} were inoculated with 2 ml overnight culture of *E. coli* DH5α/pFXa grown in the same medium at 37°C and 180 rpm. After the culture reached an OD_{600} of 0.7 to 0.8 , 50 μM IPTG was added. The cells were allowed to grow for another 2 h before harvest. Two liters of LB Amp^{100} Km^{25} were inoculated with 1 ml overnight culture of *E. coli* M15 [pREP4]/$pCitS_{His}$ grown in the same medium at 37°C and 180 rpm. The cells were grown until they reached the stationary phase ($OD_{600} = 2.2$) and then harvested. Induction of *E. coli* M15 [pREP4]/$pCitS_{His}$ with IPTG (even with 50 μM) was lethal for the cells and therefore avoided.

2.7. Preparation of membranes and purification of the fusion proteins

All phosphate buffers used in this study were prepared from K_2HPO_4 or Na_2HPO_4 by adjusting the pH with HCl. Bacterial membrane vesicles were prepared by suspending 1 g cells (wet weight) of *E. coli* DH5α/pFXa or *E. coli* M15[pREP4]/$pCitS_{His}$ in 4 ml 20 mM K^+-phosphate buffer pH 7.5 containing 0.5 M NaCl, 0.2 mM diisopropylfluorophosphate (DFP), 1 mM Mg-EDTA and a trace amount of deoxyribonuclease and passing the suspension twice through a French pressure chamber at 82.7 MPa (12,000 psi). The suspension was centrifuged at 4°C for 10 min at 10,000 × *g* and the supernatant was centrifuged at 4°C for 45 min at 180,000 × *g*. The membrane pellet was washed twice with 5 ml 20 mM potassium-phosphate buffer pH 7.5 and resuspended in 0.5 ml 20 mM potassium-phosphate buffer pH 7.5 containing 0.5 M NaCl. The membrane vesicles were thoroughly mixed with 3% Triton X-100 and allowed to stand for 15 min on ice with occasional shaking before the mixture was centrifuged for 45 min at 180,000 × *g*. The supernatant (Triton X-100 extract) could be stored at 4°C for a week without significant loss of citrate transport activity. For the purification of CitSα, a Triton X-100 extract of *E. coli* DH5α/pFXa (8 ml, 80 mg protein) was applied to a monomeric avidin-Sepharose column (15 ml bed volume, prepared as described [12]) preequilibrated with 6 bedvolumes buffer A1 (20 mM K^+-phosphate pH 6.0, 0.5 M NaCl, 1 mM K^+-citrate, 0.1% dodecylmaltoside, 0.2 mM DFP) or buffer A2 (20 mM K^+-phosphate pH 6.0, 20 mM NaCl, 20 mM K^+-citrate, 0.1% Triton X-100, 0.25 mg/ml *E. coli* phospholipids (Avanti Polar Lipids), 0.2 mM DFP) . The column was washed with 7 bed volumes of buffer A1 (or A2) to remove all unbound proteins. The biotinylated fusion protein was eluted with buffer A1 (or A2) containing 1 mM (+)-biotin . The flow rate was 0.5 ml/min and 2 ml fractions were collected. Fractions containing CitSα were pooled and concentrated 6- to 7-fold in an Amicon ultrafiltration chamber using a Diaflo PM-10 membrane. This concentrated protein solution could be stored for 3 weeks at 4°C or for several months in liquid nitrogen without significant loss of activity.

For the purification of $CitS_{His}$, a Triton extract of *E. coli* M15[pREP4]/$pCitS_{His}$ (0.7 ml, 3.8 mg protein) was applied to a Ni^{2+}-NTA-column (Qiagen) with 1 ml bedvolume preequilibrated with buffer B (20 mM K^+-phosphate buffer, pH 7.8 containing 20 mM NaCl, 1 mM K^+-citrate, 0.1% dodecylmaltoside and 0.2 mM DFP). The column was washed with 6 bedvolumes of buffer B and afterwards with 6 bedvolumes of buffer B adjusted to pH 6.0. The His-tagged protein was eluted with buffer B, pH 6.0, containing 100 mM imidazole. The flow rate was determined by gravitation and 1 ml fractions were collected. The purified protein could be stored at 4°C for at least a week without significant loss of activity.

2.8. Reconstitution of purified fusion protein and citrate transport assay

To form liposomes a suspension of 14 mg of phosphatidylcholine (Sigma, type II S) in 930 μl reconstitution-buffer (50 mM K^+-phosphate pH 6.0) was vigorously agitated with a vortex mixer for 3 min and afterwards sonicated for 3 × 1 min using a water bath-type sonicator. The purified fusion protein (70 μl, 6 μg of protein) was added to the preformed liposomes and the mixture was allowed to stand for 15 min on ice with occasional shaking. In order to remove the undesired salts (citrate, NaCl) added with the protein, the mixture (1 ml) was applied to a NAP-10 column (Pharmacia) preequilibrated in reconstitution buffer and the proteoliposomes were eluted in this buffer. The first three drops containing proteoliposomes (detected by turbidity) were discarded and the next 0.5 ml were collected, frozen in liquid nitrogen, thawed in a water/ice bath and sonicated for 2 × 4 s in a water bath-type sonicator. The proteoliposomes were collected by centrifugation at 180,000 × *g* for 50 min and resuspended in 14 μl of reconstitution buffer, usually resulting in a total volume of 80 μl. Proteoliposomes (10 μl) loaded with 50 mM K^+-phosphate pH 6.0 were diluted into 490 μl 50 mM Na^+-phosphate pH 6.0 containing 4.5 μM [1,5-^{14}C]citrate (140–160 cpm/pmol). 100 μl samples were taken at various times, diluted into 900 μl ice-cold 0.1 M LiCl, and rapidly filtered through 0.22 μm GSTF filters (Millipore). The filters were washed once with 1 ml ice-cold 0.1 M LiCl and placed into scintillation vials. After addition of 4 ml scintillation fluid (Ready-safe, Beckman), the entrapped [1,5-^{14}C]citrate was determined with a liquid scintillation counter. Experimental values were corrected for zero-time controls by diluting proteoliposomes (2 μl) into 98 μl 50 mM Na^+-phosphate pH 6.0 containing 4.5 μM [1,5-^{14}C]citrate that had already been diluted with 900 μl ice-cold 0.1 M LiCl, followed by rapid filtration and washing of the sample as described above.

3. Results and discussion

3.1. Construction of the fusion proteins

The construction of fusion proteins by genetic methods and their purification by affinity chromatography is a powerful technique of protein isolation that can be applied if the gene for the desired protein is available. As reported recently for the lactose permease (LacY) [13] and the $IIBC^{Glc}$-subunit of the glucose transporter from *E. coli* [14], purification by this method yields highly purified proteins. The gene for the Na^+-dependent citrate carrier from *K. pneumoniae* (*citS*) was subcloned from plasmid pGW234 which contains in addition three genes encoding the oxaloacetate decarboxylase (*oadGAB*) downstream of *citS* [6]. In the resulting plasmid, pKP-1, the stop codon of *citS* was deleted by PCR-methodology. A DNA fragment encoding the biotin acceptor domain

of the *K. pneumoniae* oxaloacetate decarboxylase α-subunit (residues 500–595) was amplified by PCR with pGW234 as template and cloned in-frame behind the DNA region of pMal-c encoding the recognition site of the endoproteinase factor Xa. Unfortunately, all clones obtained had a deletion of one 'G' at the fusion junction. The resulting frame-shift led to a TGA-stop codon directly behind the gene of the maltose binding protein (see also Fig. 1). We concluded therefore that the maltose binding protein α-fusion was toxic and created a highly selective pressure in favour of the frame-shift mutant. Consequently, induction of expression from the *tac* promoter of pMal-c by IPTG was avoided and the clones were screened by restriction analysis. Under these conditions pMA-9 was obtained. A DNA fragment comprising the factor Xa recognition site plus the biotin acceptor domain was then fused in-frame behind the last codon of *citS* yielding pFXa. The correct sequence of the fusion sites and of DNA fragments derived from PCR products was verified by sequencing. In pFXa, the gene encoding the CitS-α peptide fusion protein is expressed from the *lac* promoter present on the vector pBluescript KS+ (data not shown). pFXa-2 was obtained by cloning a

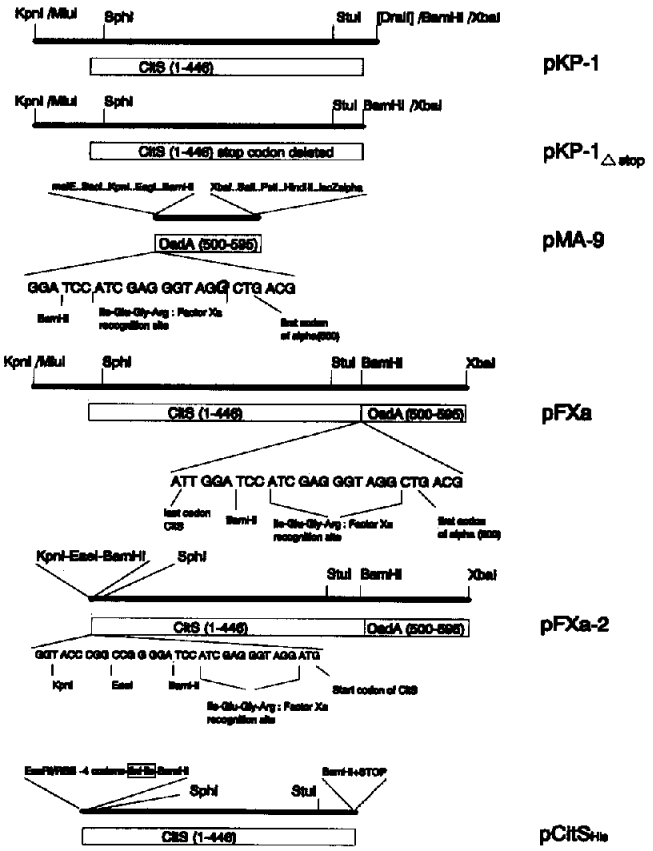

Fig. 1. Schematic representation of plasmids encoding CitS (pKP-1, pKP-$1_{\Delta stop}$), CitSα (pFXa, pFXa-2), $CitS_{His}$($pCitS_{His}$) and the MalE-α(500–595) fusion (pMA-9). Only the inserts and the vector parts used in the construction are shown. The deleted base ('G') in pMA-9 is shown in bolder letter-type (for details see text).

PCR-fragment encoding residues 1–25 of CitS and comprising 35 base-pairs of the pMal-c polylinker region at the 5′ end, into pFXa. By inserting the polylinker region of pMal-c upstream of *citS-oad A* (500–595), *citS* could easily be cloned into pQE-8, the source of the His-tag, yielding $pCitS_{His}$ (Fig. 1).

3.2. Expression of biotin-containing fusion proteins

The expression of biotinylated fusion proteins was verified by Western blotting of cell extracts and staining with avidin-peroxidase (data not shown). The biotin protein expressed by pMA-9 showed an apparent M_r of 52,000 on SDS-PAGE as expected for maltose binding protein (M_r 42,000) fused to the biotin acceptor domain ($M_r \approx 10{,}000$). The biotin protein expressed by *E. coli* DH5α[pFXa] exhibited an apparent M_r of 50,000 which is smaller than expected (theoretical value ≈57,500) probably due to abnormal binding of SDS to the membrane protein. Plasmid pFXa led to modest growth inhibition of *E. coli* DH5α in LB Amp^{100} liquid medium because the cells reached the stationary growth phase already at an OD_{600} of 1–2 whereas the vector control grew to an OD_{600} of 3–4. pFXa conferred the Cit^+-phenotype to *E. coli* under aerobic conditions proving that the CitSα fusion protein is a functional citrate carrier in vivo. This was confirmed by citrate transport experiments with proteoliposomes that were reconstituted with Triton X-100 extract from membranes of DH5α[pFXa] (data not shown).

3.3. Purification of the fusion proteins

Membranes prepared from 12 g cells (wet weight) of *E. coli* DH5α[pFXa] were extracted with 3% Triton X-100 and the Triton extract (8 ml, 80 mg protein) was applied to a monomeric avidin-Sepharose column equilibrated with a buffer containing 0.1% dodecylmaltoside. After washing off all unbound proteins, the biotin-containing fusion protein (about 0.5 mg) was specifically eluted with column buffer containing 1 mM (+)-biotin. Alternatively, column buffers containing 0.1% Triton X-100 could be used, but in this case phospholipids from *E. coli* or *K. pneumoniae* (0.25 mg/ml) had to be present in the buffer to retain transport activity. The presence of citrate in the column buffers was essential for the stability of the carrier. Citrate carrier with an N-terminal histidine-tag was extracted from membranes of *E. coli* M15[pREP4]/$pCitS_{His}$ with 3% Triton X-100 and 0.7 ml Triton extract containing 3.8 mg protein were applied to a Ni^{2+}-NTA-column equilibrated with buffer containing 0.1% dodecylmaltoside. The column was successively washed with column buffer of pH 7.8 and 6.0 to remove unbound proteins. $CitS_{His}$ was specifically eluted with column buffer pH 6.0 containing 100 mM imidazole. The yield was 72 μg of pure protein indicating that at least 2% of the protein present in the Triton-extract was $CitS_{His}$.

3.4. SDS-PAGE, Western blot analysis, and N-terminal sequencing

The purified fusion proteins CitSα and $CitS_{His}$ were analysed by SDS-PAGE. The results shown in Fig. 2 indicate that both fusion proteins were highly purified by the procedure applied. Interestingly, purified CitSα consisted of two about equally strong stained proteins with apparent M_r's of 50,000 and 33,000. The N-terminal 14 amino acids of both polypeptides were identical and started with the threonine next to the methionine encoded by the start codon of *citS* [2]. The proteinchemically determined sequence matched the DNA-derived sequence with the exception of an alanine at position 6 that had been predicted to be a proline. Resequencing of the 5′ part of *citS* in our laboratory revealed that the first base of the 6th codon is a 'G' rather than a 'C'. The correct codon is therefore 'GCT' which codes for alanine in accord with our protein sequence analysis. The identical N-termini of the two proteins indicated that the polypeptide with an apparent M_r of 33,000 derived from that with an apparent M_r of 50,000 by C-terminal cleavage. However, besides these two polypeptides no other bands were visible, indicating that no proteolytic cleavage occurred during the purification. These observations were confirmed by Western-blot analysis showing that only the polypeptide with an apparent M_r of 50,000 was biotinylated (Fig. 2, lane c). The co-purification of a biotinylated and a non-biotinylated polypeptide by monomeric avidin-Sepharose chromatography indicated that CitS is an oligomeric protein. Moreover, since the ratio of the two forms after purification was about 1:1, whereas the non-biotinylated form was present in excessive amounts in the Triton X-100 extract (data not shown), native CitS is most probably a dimer. A similar approach was recently used to identify the dimeric structure of the $IIBC^{Glc}$ protein [14]: if a mixture of wild-type $IIBC^{Glc}$ and His-tagged $IIBC^{Glc}$ was loaded onto a Ni^{2+}-NTA-resin affinity column, both proteins were retained and eluted together, whereas wild-type

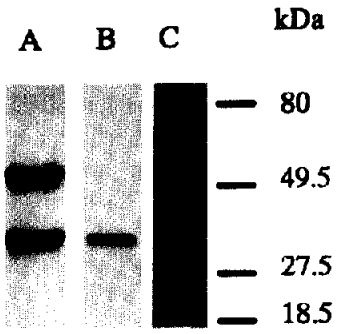

Fig. 2. SDS-PAGE and Western blot analysis of the purified fusion proteins. Lane A : CitSα purified by avidin-Sepharose affinity chromatography (6 μg protein); lane B: $CitS_{His}$ purified by a Ni^{2+}-NTA-affinity column (0.5 μg of protein); the gels were stained with silver [15]; lane C: Western blot analysis of purified CitSα (1 μg protein) stained with avidin-peroxidase.

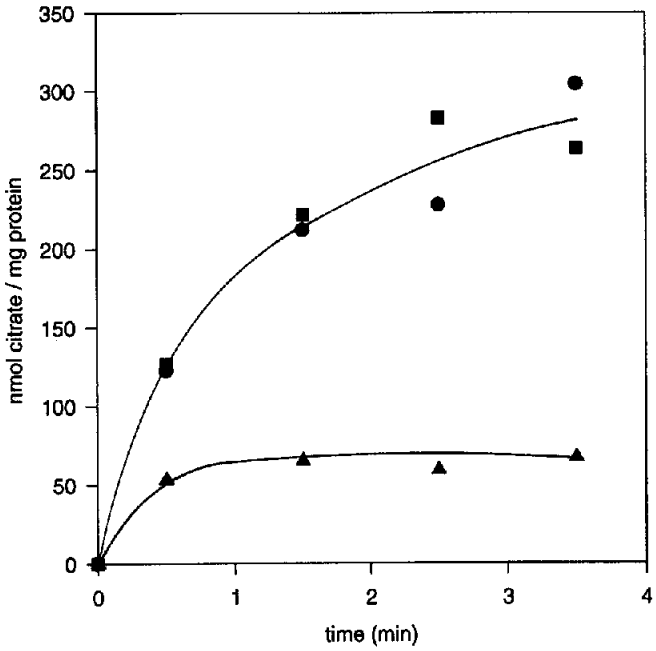

Fig. 3. Citrate uptake into proteoliposomes containing purified CitSα (●) or $CitS_{His}$ (■). The proteoliposomes were prepared in the presence of 50 mM K^+-phosphate buffer of pH 6.0. The uptake of [1,5-^{14}C]citrate was initiated by diluting 10 μl proteoliposomes into 490 μl 50 mM Na^+-phosphate buffer of the same pH containing 4.5 μM [1,5-^{14}C]citrate (ΔpNa^+). In the controls the proteoliposomes containing either CitSα or $CitS_{His}$ were diluted into K^+-phosphate buffer, pH 6.0 containing 4.5 μM [1,5-^{14}C]citrate (▲). The control experiments led to similar results for CitSα and $CitS_{His}$, therefore only one set of data is shown. Each time point represents the mean value of three assays.

$IIBC^{Glc}$ alone did not bind to the column. In Fig. 2 (lane b), the N-terminally His-tagged CitS purified by the Ni^{2+}-NTA-affinity column was applied. Obviously, only one polypeptide is apparent in this material which exhibits an apparent M_r almost identical to the non-biotinylated form of the CitSα fusion protein. This result indicated that the polypeptide with an apparent M_r of 33,000 detected in purified CitSα represents most probably CitS without the attached α-peptide. Attempts to cleave off the α-peptide in purified samples of CitSα with the endoproteinase factor Xa failed (data not shown). The question how the shortened CitSα derivative was formed cannot be answered definitively. Since a biotinylated degradation product of $M_r \leq 10{,}000$ could be detected neither in the cell extract nor in the Triton extract nor in the avidin-Sepharose column effluent, it appears unlikely that the 33K protein results from proteolytic cleavage. A more plausible explanation for the appearance of the 33K protein is by a mutation at the fusion junction leading to the generation of a stop codon. Exactly such an event had occurred during the construction of the maltose binding protein-α peptide fusion in pMA-9, indicating that there is a selective pressure in favour of it. Additional support for a mutation comes from the obser-

vation that the formation of biotinylated CitSα was considerably diminished after several transfers of *E. coli* DH5α/pFXa into fresh medium. Such a behaviour is to be expected if cells carrying the mutated plasmid grow faster than cells with the original plasmid.

3.5. *Transport of citrate into proteoliposomes containing purified CitS fusion proteins*

Purified CitSα or $CitS_{His}$ was reconstituted into proteoliposomes by the freeze/thaw/sonication procedure as outlined in section 2. When proteoliposomes were prepared in K^+-phosphate buffer pH 6.0 and diluted into Na^+-phosphate buffer of the same pH, citrate was rapidly accumulated in response to the imposed ΔpNa^+ (Fig. 3). Without imposition of a ΔpNa^+, by diluting the proteoliposomes into K^+-phosphate buffer pH 6.0, citrate accumulation was poor and ceased after the first 30 seconds. Accumulation of citrate in liposomes without incorporated CitSα or $CitS_{His}$ was 20-fold less than in the proteoliposomes containing CitSα or $CitS_{His}$ (data not shown). With the data obtained an 8-fold increase of specific activity in comparison with reconstituted Triton X-100 extracts of *E. coli* DH5α[pFXa] or *E. coli* M15[pREP4]/pCitS_{His} was estimated. The purified preparations of $CitS_{His}$ and CitSα exhibited the same specific citrate transport activity (≈250 nmol·min^{-1}·mg $protein^{-1}$), indicating that both proteins of the CitSα/CitS heterodimer are catalytically active.

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