Arch Microbiol (2005) 183: 121–129
DOI 10.1007/s00203-004-0754-5

ORIGINAL PAPER

**Pius Dahinden · Yolanda Auchli · Thierry Granjon
Malgorzata Taralczak · Markus Wild · Peter Dimroth**

Oxaloacetate decarboxylase of *Vibrio cholerae*: purification, characterization, and expression of the genes in *Escherichia coli*

Received: 8 October 2004 / Revised: 2 December 2004 / Accepted: 6 December 2004 / Published online: 13 January 2005

Abstract The oxaloacetate decarboxylase (OAD) Na^+ pump consists of subunits α, β, and γ, which are expressed from an *oadGAB* gene cluster present in various anaerobic bacteria. *Vibrio cholerae* has two copies of *oad* genes, which are termed *oad-1* and *oad-2*. The *oad-2* genes are part of the citrate fermentation operon, while the *oad-1* genes are flanked by genes encoding products not involved in a catabolic pathway. The gene sequences of *oad-1* and *oad-2* of *V. cholerae* strain O395-N1 were determined. The apparent frameshift in the published sequence of the *oadA-2* gene from *V. cholerae* El Tor N16961 was not present in strain O395-N1. Upon anaerobic growth of *V. cholerae* on citrate, exclusively the *oad-2* genes are expressed. OAD was isolated from these cells by monomeric avidin–Sepharose affinity chromatography. The enzyme was of higher specific activity than that from *Klebsiella pneumoniae* and was significantly more stable. Decarboxylase activity was Na^+ dependent, and the activation profile showed strong cooperativity with a Hill coefficient $n_H = 1.8$. Oxalate and oxomalonate inhibited the enzyme with half-maximal concentrations of 10 μM and 200 μM, respectively. After reconstitution into proteoliposomes, the enzyme acted as a Na^+ pump. With size-exclusion chromatography, the enzyme eluted in a symmetrical peak at a retention volume corresponding to an apparent molecular mass of approximately 570 kDa, suggesting a tetrameric structure for OAD-2. The two *oad* gene clusters were heterologously expressed in *Escherichia coli*, and the decarboxylases were isolated from the host cells.

P. Dahinden · Y. Auchli · T. Granjon
M. Taralczak · M. Wild · P. Dimroth (✉)
Institut für Mikrobiologie der ETH Zürich,
ETH Hönggerberg, Wolfgang-Pauli-Strasse 10,
8093 Zurich, Switzerland
E-mail: dimroth@micro.biol.ethz.ch
Tel.: +41-1-6323321
Fax: +41-1-6321378

Keywords *Vibrio cholerae* · Oxaloacetate decarboxylase · Na^+ pump

Introduction

Oxaloacetate decarboxylase (OAD) is a member of the Na^+-transporting carboxylic acid decarboxylase (NaT-DC) family of enzymes (Dimroth and Schink 1998; Busch and Saier 2002, 2004). Each of these vectorial catalysts performs a key reaction in the fermentation of a certain growth substrate and thereby conserves the free energy of the decarboxylation event by conversion into an electrochemical gradient of Na^+ ions (Dimroth 1997, 2004; Dimroth and Schink 1998; Buckel 2001). OAD is required for the fermentation of citrate or tartrate (Dimroth 1980; Woehlke and Dimroth 1994), glutaconyl-CoA decarboxylase for the fermentation of glutamate (Buckel 1980), and methylmalonyl-CoA decarboxylase for the fermentation of succinate (Hilpert et al. 1984; Bott et al. 1997).

Enzymes of the NaT-DC family are composed of highly conserved, membrane-bound β subunits involved in Na^+ translocation, and a peripheral α subunit generally consisting of a carboxyltransferase domain and a biotin-containing CO_2-acceptor domain. These subunits are assembled into a complex with the aid of a small, membrane-anchored γ subunit. In some decarboxylases, these domains exist as separate subunits. Some have an additional small subunit to increase the stability of the complex (Dimroth 1997).

In the following, we focus on OAD, which has been particularly well characterized. In *Klebsiella pneumoniae*, the genes encoding the three subunits of this enzyme are clustered and inserted into the citrate fermentation operon (Bott and Dimroth 1994; Bott et al. 1995). In *Salmonella typhimurium*, two copies of the *oad* genes have been identified, one being inserted into the citrate fermentation operon and the other one being associated with genes required for tartrate fermentation (Woehlke

and Dimroth 1994; McClelland et al. 2001). *Vibrio cholerae* is another organism with two copies of the *oad* genes, termed *oad-1* and *oad-2* (Heidelberg et al. 2000). The *oad-2* genes are part of the citrate fermentation operon, but the *oad-1* genes are not associated with genes for a specific fermentation pathway.

The catalytic reaction cycle of OAD starts with the transfer of the carboxyl group from position 4 of oxaloacetate to the biotin prosthetic group on the C-terminal biotin domain of the α subunit. The resulting carboxybiotin switches from the carboxyltransferase site on the N-terminal domain of the α subunit to the decarboxylase site on the β subunit. This integral membrane protein catalyses the decarboxylation of carboxybiotin coupled to the transport of two Na^+ ions from the cytoplasm into the periplasm and consumption of a periplasmically derived proton (Dimroth and Thomer 1993; Di Berardino and Dimroth 1996; Dimroth et al. 2001). The three universally conserved amino acids D203, Y229, and S382 have been recognized to play a key role in the ion-translocation mechanism (Jockel et al. 2000a, 2000b; Schmid et al. 2002). S382 is located in the center of the membrane within the highly conserved helix VIII that aligns the ion channel (Wild et al. 2003).

For further insights into the reaction mechanism of this enzyme, structure information is of essence. Progress in this direction is limited to the crystal structure of the carboxyltransferase subunit of glutaconyl-CoA decarboxylase (Wendt et al. 2003). So far, we were unable to obtain suitable crystals of OAD from *K. pneumoniae*. A disadvantage of this enzyme may be the extended proline/alanine linker between the two domains of the α subunit providing structural flexibility. As these linker peptides are clearly reduced in both OADs from *V. cholerae*, we have selected these enzymes to study for their suitability to obtain crystals. Here we report on the purification and characterization of OAD isolated from citrate-fermenting *V. cholerae*. We also report on the expression of the *oad-1* and *oad-2* genes in *Escherichia coli* and on the purification of the corresponding OAD-1 and OAD-2 complexes.

Materials and methods

Strains and growth conditions

For anaerobic growth *V. cholerae* O395-N1 (Mekalanos et al. 1983) was cultivated in citrate medium, which contained 30.6 mM Na-citrate, 14.7 mM KH_2PO_4, 7.6 mM $(NH_4)_2SO_4$, 1.6 mM $MgSO_4$, 50 mM Tris, 0.5% yeast extract, 10% 20× M9 salts (956 mM Na_2HPO_4, 440 mM KH_2PO_4, 374 mM NH_4Cl, and 172 mM NaCl), 1.3 μM vitamin B_1, 2.5 μg casaminoacids per milliliter, and 20 μg Streptomycin sulfate per milliliter. Sterile 20× M9 salts, vitamin B_1, and casaminoacids were added to the medium after sterilization. The resulting pH of the medium at 37°C was 8.1. Cells were grown overnight anaerobically at 37°C in polypropylene carboy flasks (10 l) to an OD_{600} of approximately 0.5. With 20 l of these precultures, a fermenter containing 180 l medium was inoculated, which was gassed initially with nitrogen. The fermentation was performed without pH control. At 37°C and 80 rpm the cells grew exponentially to an average OD_{600} of 0.7 and reached stationary phase approximately 6 h after inoculation. Then the medium was cooled to approximately 8°C, and the cells were collected with a Westfalia chamber bowl continuous clarifier at a speed of approximately 100 l/h, resulting in the collection of approximately 350 g wet cell mass.

For cloning, sequencing, and heterologous expression, *E. coli* DH5α (Bethesda Research Laboratories, Mass., USA), *E. coli* C43(DE3) (Miroux and Walker 1996), and *E. coli* BL21 Star (DE3) (Invitrogen, USA) were used. For preparation of DNA as well as for expression of proteins, Luria Bertani (LB) medium containing 10 g NaCl per liter was used. For heterologous expression of *oad-1* or *oad-2* in *E. coli*, baffled Erlenmeyer flasks containing LB (200 ml–2 l) were inoculated with 1% of an overnight culture and incubated at 37°C and 180 rpm. At an OD_{600} of approximately 0.7, the cultures were cooled on ice and induced by the addition of IPTG (100 μM). The cells were harvested after incubation for 3–4 h at 30°C and 180 rpm.

Recombinant DNA techniques and sequencing

Genomic DNA was prepared by the CTAB method according to Ausubel et al. (1999). Extraction of plasmid DNA, restriction enzyme digestions, DNA ligations, and transformation of *E. coli* with plasmids were carried out by standard methods (Sambrook et al. 1989; Ausubel et al. 1999). PCRs were performed with an Air Thermocycler (Idaho Technology, Utah, USA, model 1605), using *Pfu* polymerase. Oligonucleotides used for mutagenesis were custom-synthesized by Microsynth (Balgach, Switzerland). All inserts derived from PCR as well as ligation sites were checked by DNA sequencing according to the dideoxynucleotide chain-termination method (Sanger et al. 1977) by Microsynth. In case of site-directed mutagenesis by PCR, whole plasmids were amplified, but only the sequence of the genes to be overexpressed was verified.

Construction of expression plasmids

Genomic DNA prepared from strain *V. cholerae* O395-N1 (Mekalanos et al. 1983) served as template for the amplification of the *oadGAB-1* and *oadGAB-2* genes by PCR. The *oadGAB-1* genes were amplified with the oligonucleotide primers TCAATCGGTCATATGTAAGAATGTCAGGATGACACGCAAAGG (VcoadG-1) and ACTGTGTCAGAGCTCCGCCATTTTGCGCAAAGTGCAACC (VcoadB-1), containing an *Nde*I or *Sac*I

site (underlined), respectively. The *oadGAB-2* genes were amplified with the oligonucleotide primers TCAATCGGTCATATGTAACGCACAAATATCTTCACGTCTGGG (VcoadG-2) and ACTGTGTCACTCGAGATTAACGGGGCTTATTGATTGGGG (VcoadB-2) containing an *Nde*I or *Xho*I site (underlined), respectively. The oligonucleotide primers VcoadG-1 and VcoadG-2 both featured a stop codon just after the *Nde*I restriction site and annealed 31 bp or 170 bp upstream of the respective *oadG* gene, whereas the oligonucleotide primers VcoadB-1 and VcoadB-2 containing a *Sac*I or an *Xho*I restriction site, respectively, annealed 33 bp or 49 bp downstream of the respective *oadB* gene. The PCR products were ligated directly with a pKS vector restricted with *Eco*RV and were then transferred into *E. coli* DH5α cells, which were plated on agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and isopropyl-β-D-thiogalactopyranoside (IPTG) for blue-white selection of clones with incorporated PCR fragments. Positive clones were restricted with *Nde*I and *Sac*I (*oadGAB-1*) or *Nde*I and *Xho*I (*oadGAB-2*), and the obtained *oadGAB* fragments were ligated with accordingly restricted pET24b or pET16b vectors to give pET24-VcoadGAB-1, pET24-VcoadGAB-2, and pET16-VcoadGAB-2 for the expression of untagged protein complexes.

Purification of OAD from *V. cholerae* O395-N1 cells by monomeric avidin–Sepharose affinity chromatography

V. cholerae O395-N1 cells resuspended in buffer A [50 mM Tris-HCl (pH 8.0), 250 mM NaCl; 7 ml/g of cells] containing 1 mM MgK_2EDTA were disrupted after addition of 50 μg DNase I and 0.2 mM diisopropylfluorophosphate (DFP) by sonication (three cycles with a 9.5-mm probe at 16 micron and 23 kHz for 1 min). After centrifugation of the cell lysate (26,000 *g* for 10 min), membranes were collected by centrifugation of the supernatant at 200,000 *g* for 1 h. Membranes were resuspended in 1 ml buffer A per gram of cells, solubilized for 10 min at 4°C with 2% Triton X-100, and then centrifuged at 220,000 *g* for 35 min. The Triton X-100 extract was applied to a monomeric avidin–Sepharose column (Promega, 5 ml bed volume) (Dimroth 1986) and washed with eight bed volumes of buffer B [50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.5% Tween 20, and 0.05% Brij58]. Biotinylated protein was eluted with the same buffer containing 5 mM (+)-D-biotin.

Purification of heterologously expressed OAD-1 and OAD-2

OAD-1 and OAD-2 were synthesized in *E. coli* C43(DE3) harboring plasmids pET24-VcoadGAB-1 or pET24-VcoadGAB-2, respectively. The cells (1 g) were resuspended in 7 ml buffer A [50 mM Tris-HCl (pH 8.0), 250 mM NaCl] containing 1 mM MgK_2 EDTA. After addition of 0.2 mM DFP (final concentration) and approximately 50 μg DNase I, the cells were disrupted by three passages through a French pressure cell at 110 MPa. Intact cells and cell debris were removed by centrifugation (30 min at 8,000 *g*). Membranes were collected by ultracentrifuging (1 h at 200,000 *g*) the cell-free supernatant. The OAD-1 and OAD-2 isoforms were purified from the solubilized membranes, as described above for the purification of OAD from *V. cholerae* cells.

Protein concentrating method and size-exclusion chromatography

The elution fraction of the monomeric avidin–Sepharose column was applied to a Fractogel TMAE column (1-ml bed volume) and washed with 4 ml buffer B [50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.5% Tween 20 and, 0.05% Brij58]. The enzyme eluted with buffer B containing 250 mM NaCl in the fractions from 0.8 ml to 1.6 ml.

To determine the molecular mass of the OAD complex, the enzyme was eluted from monomeric avidin–Sepharose column with buffer C [50 mM Tris-HCl (pH 8.0), 250 mM NaCl, and 0.05% Brij58], loaded on a Superdex 200 HR 10/30 gel filtration column and eluted with the same buffer at a flow rate of 0.05 ml/min. The gel filtration HMW calibration kit from Amersham Biosciences was used to calibrate the column for buffer C.

Protein detection methods

Protein concentration was determined by the BCA method (Pierce, Ill., USA), using bovine serum albumin as standard. SDS-PAGE was performed as described (Schägger and von Jagow 1987). Gels were stained with Coomassie Brilliant Blue R-250 or with silver (Wray et al. 1981).

N-terminal sequencing

OAD purified from *V. cholerae* cells (10 μg) was subjected to SDS-PAGE (12% polyacrylamide) and transferred to a PVDF membrane by semidry blotting in a discontinuous buffer system (Kyhse-Andersen 1984) at a constant current of 0.8 mA/cm^2 for 1 h. The membrane was washed for 5 min in water, stained for 5 min with 0.1% Coomassie Brilliant Blue R-250 in 50% methanol, and destained for 5–10 min with 50% methanol. The bands were cut out separately, and the N-terminal amino acid sequence was determined by Edman degradation, using an Applied Biosystems Sequencer (model 470/A) with on-line phenylthiohydantoin derivative detection by HPLC (model 120A).

Enzyme assay

The decarboxylation activity was determined with the simple spectrophotometric assay at 265 nm as described (Dimroth 1981). To determine the effect of inhibitors on OAD activity the inhibitor substances were added to the assay mixture at the onset of measurement.

Dependence of OAD activity on Na^+ concentration or pH value

The activation of OAD activity by Na^+ was determined with enzyme purified in the absence of added Na^+ (15–20 μM Na^+, as determined by atomic absorption with a Shimadzu AA-646 spectrometer). Activity was measured at room temperature at fixed pH values in a Mes/Mops/Tris buffer system (20 mM) by the coupled spectrophotometric assay (Dimroth 1986). The Na^+ concentration was varied by the addition of NaCl (0–50 mM), essentially as described in (Schmid et al. 2002). Measured data were fitted to the Michaelis–Menten equation or the Hill equation. To determine the inhibition of OAD by high Na^+ concentrations, the activity tests were performed at NaCl concentrations from 100 mM to 1 M. The pH dependence of the enzyme was determined at a fixed Na^+ concentration of 40 mM in a pH range of 5.5 to 9.0.

Reconstitution of OAD from *V. cholerae* into phospholipid vesicles

Lipids (30 mg soybean L-α-phosphatidylcholine Type II-S, Sigma, St. Louis, Mo., USA) were dissolved in $CHCl_3$/MeOH (2:1, v/v). The solvent was removed under reduced pressure in a rotary evaporator at room temperature. The dry lipids were resuspended in $CHCl_3$, which was removed again at reduced pressure. This procedure was repeated three times. Residual solvent was removed by applying a high vacuum for 8 h.

The dry lipids were hydrated in 50 mM potassium phosphate (pH 7.5), 1 mM Na_2SO_4, and 20 μg of the Na^+-specific fluorophore Sodium Green tetra(tetramethylammonium) salt, S-6900 (Molecular Probes, Ore., USA) at a lipid concentration of 20 mg/ml. The lipid suspension was shaken to produce multilamellar vesicles, which were subjected to six freeze/thaw cycles, and then extruded 19 times through a polycarbonate membrane (Nucleopore) with 0.4-μm diameter pores, using a mini-extruder to yield homogeneous large unilamellar vesicles.

To the vesicle suspension, β-octylglucoside was added to a final concentration of 3%, and 1.8 mg OAD-2 purified over a monomeric avidin–Sepharose column. This mixture was incubated 30 min at 4°C, with occasional shaking, and then dialyzed for 16 h at 4°C against 50 mM potassium phosphate (pH 7.5) and 1 mM Na_2SO_4. The proteoliposomes were collected by ultracentrifugation (200,000 *g* for 50 min) and the pellet resuspended in buffer [50 mM potassium phosphate (pH 7.5), 0.5 mM DTT, and 1 mM Na_2SO_4]. The same process was followed without addition of fluorophore or protein as negative control.

Real-time determination of Na^+ transport into proteoliposomes

The uptake of Na^+ into proteoliposomes was essentially performed as described (von Ballmoos and Dimroth 2004). Fluorescence was recorded with a RF-5001PC spectrofluorometer (Shimadzu, USA). The excitation and emission band-pass values were 1.5 nm and 3 nm, respectively. Spectra were recorded at 9°C, using a thermostated quartz cuvette and an excitation wavelength of 515 nm. Fluorescence intensity was recorded at an emission wavelength of 530 nm as a time function.

Measurement of $^{22}Na^+$ uptake into proteoliposomes

Proteoliposomes were prepared without fluorophore, as described above. The incubation mixture (25°C) of these assays contained in 0.5 ml: 50 mM potassium phosphate (pH 7.5), 0.5 mM DTT, 1 mM $^{22}Na_2SO_4$ (spec. activity: 580 cpm/nmol Na^+), and the proteoliposomes (50 μg protein). The $^{22}Na^+$-transport experiments were performed at 25°C, and $^{22}Na^+$ uptake was initiated by the addition of 1 mM oxaloacetate. At different time points, samples (100 μl) were passed over K^+-Dowex 50, equilibrated with 50 mM potassium phosphate (pH 7.5), and the columns were washed immediately with 0.9 ml deionized water. The radioactivity of $^{22}Na^+$ eluted from the columns, which reflects the $^{22}Na^+$ entrapped in the proteoliposomes, was determined by γ-scintillation counting. To inhibit Na^+ uptake, proteoliposomes were preincubated for 30 min, with an equimolar (with respect to OAD-2) amount of avidin.

MALDI-TOF mass spectrometry and protein identification

OAD purified from *V. cholerae* cells (25 μg) was subjected to SDS-PAGE (12% polyacrylamide). The gel was stained with Gelcode Blue Stain Reagent (Pierce), and the bands corresponding to subunits α, β, and γ were excised and digested in gel, using trypsin (Trypsin Gold, MS grade, Promega, Wallisellen, Switzerland). The tryptic peptides were extracted from the gel slices as previously described (Shevchenko et al. 1996; Becamel et al. 2002) and concentrated using Zip $Tips_{C18}$ (Millipore, Volketswil, Switzerland), from which they were eluted with 2.5 μl 70% acetonitrile, 0.1% trifluoroacetic acid, and 10 mg/ml α-cyano-4-hydroxycinnamic acid and spotted directly onto a MALDI target. Molecular masses were determined with the aid of a 4700

Proteomics Analyzer from Applied Biosystems. Analysis was performed in reflectron mode with external calibration. Identification of proteins was performed using both PeptIdent and Aldente software (available online at http://www.expasy.org/tools/peptident.html and http://www.expasy.org/tools/aldente/).

Results

Identification of two *oad* gene clusters in *V. cholerae*

All members of the NaT-DC family contain a membrane-bound β subunit that is highly conserved. A database search for proteins related to OadB from *K. pneumoniae* led to the identification of 27 similar sequences from eubacteria and four from archaea. In an effort to solve the structure of OAD, we selected the enzyme from *Archaeoglobus fulgidus*, for which we expected favorable stability properties. However, only the water-soluble subunits α and δ could be purified from this source after heterologous expression in *E. coli* (Dahinden and Dimroth 2004). Previously, we had intensively tried to obtain crystals of OAD from *K. pneumoniae*, which can be easily purified in large amounts and with which most of the biochemical investigations were performed. These efforts have not been successful, however, possibly due to the following problems: (1) the decarboxylase was unstable on storage under a great variety of conditions, typically leading to the loss of $>70\%$ of the activity within 15–20 h at 15°C; (2) the enzyme did not adopt a monodisperse form but existed in various aggregation states, as shown by gel filtration chromatography; and/or (3) the enzyme has an extended proline/alanine linker peptide in the α subunit. Such linkers are known to act as flexible hinges between different domains. The enzyme may therefore switch between different conformations, which would be unfavorable for the formation of crystals.

From sequence comparison, we found significantly shorter linker peptides in the α subunits of the two OADs of *V. cholerae*. We have therefore chosen to study whether these enzymes have more appropriate properties for structural investigations. *V. cholerae* has two *oadGAB* gene clusters, which are both located on chromosome 1 (Heidelberg et al. 2000). The *oad-1* gene cluster is flanked by genes encoding products not related to enzymes with a function in a fermentation pathway (tRNA genes downstream, quinone oxidoreductase genes upstream). The *oad-2* gene cluster, however, is inserted into the operon for citrate fermentation, comprising in addition genes for a citrate transporter, for citrate lyase, and for a two-component regulatory system. Similar operons are present in *S. typhimurium* and *K. pneumoniae*, where they provide the cells with the necessary enzymes for anaerobic growth on citrate (Dimroth 2004).

As expected from sequence information, *V. cholerae* could be grown anaerobically on citrate as carbon source. The growth conditions were optimized for the production of large amounts in a 250-l fermenter, as described in the "Materials and methods" section. A growth curve indicated that the cells grew with a doubling rate of 100 min and reached stationary phase after approximately 6 h. For enzyme activity measurements, the cells were disrupted by sonication and membranes isolated by ultracentrifugation. As expected, these membranes contained large amounts of OAD activity (1.2 U/mg protein). The enzyme was solubilized from the membranes with 2% Triton X-100 and purified by a monomeric avidin–Sepharose affinity column via its biotin prosthetic group. Overall, this procedure resulted in a 71-fold purification, with a yield of 85% (Table 1). The specific activity of the purified decarboxylase was approximately 50–80 U/mg protein, which is about twofold higher than that of the *K. pneumoniae* enzyme (20–47 U/mg) (Dimroth and Thomer 1986).

Properties of OAD from *V. cholerae*

Figure 1 shows an SDS-PAGE of OAD purified from citrate-grown *V. cholerae* (lane 2). The preparation consisted of the three decarboxylase subunits α, β, and γ,

Table 1 Purification tables of oxaloacetate decarboxylase OAD-1 purified from *Escherichia coli* and OAD-2 purified from *Vibrio cholerae* and *E. coli*

Purification step	Total activity (U)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification factor
OAD-1 heterologously expressed in *E. coli* (7 g wet mass)					
Membranes	319	134	1.4	(100)	(1)
Triton X-100 extract	31	66	0.5	10	0.4
Elution fraction	19	1.2	8	6	5.7
OAD-2 purified from *V. cholerae* cells (12 g wet mass)					
Membranes	1307	1103	1.2	(100)	(1)
Triton X-100 extract	1271	1029	1.2	97	1.0
Elution fraction	1114	13.7	81.3	85	67.8
OAD-2 heterologously expressed in *E. coli* (9 g wet mass)					
Membranes	471	194	2.4	(100)	(1)
Triton X-100 extract	198	159	1.2	42	0.5
Elution fraction	175	4.8	36.5	37	15.2

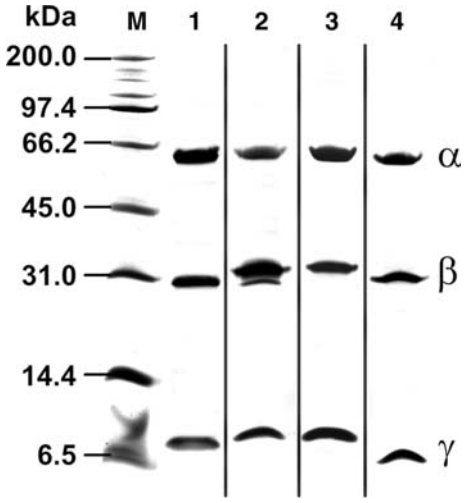

Fig. 1 SDS-PAGE of various preparations of oxaloacetate decarboxylase (OAD). Each lane was loaded with 2 μg purified protein, as described in the "Materials and methods" section. The gel was stained by silver. *M* Bio-Rad broad molecular mass standard, *1* OAD-1 purified from *Escherichia coli*, *2* OAD preparation from *Vibrio cholerae*, *3* OAD-2 purified from *E. coli*, *4* OAD preparation from *Klebsiella pneumoniae*. The predicted masses for the subunits α, β, and γ are 64.8, 39.1, and 9.2 for OAD-1; 65.0, 45.6, and 9.8 for OAD-2; and 63.4, 44.9, and 8.9 for OAD from *K. pneumoniae*

with only low amounts of other contaminating proteins. Upon N-terminal sequencing of the α subunit, the sequence TQAIKRVGV was obtained, which matches that predicted for OadA-2. This result indicates that, as expected, the *oad-2* genes are (preferentially) expressed during citrate fermentation. No sequences were obtained for the membrane-bound subunits β and γ. The gel shows a minor band moving just ahead of that assigned to OadB-2, which could indicate low amounts of OadB-1 or of another contaminating protein. The predicted molecular masses for OadB-1 (39.1 kDa) and OadB-2 (45.6 kDa) deviate sufficiently (see Fig. 1, lanes 1, 2, and 3) that separation of these proteins by SDS-PAGE can be expected. To distinguish between these possibilities, the four protein bands visible in Fig. 1, lane 2, were separately excised from the gel, digested with trypsin, and analyzed by MALDI-TOF mass spectrometry. The peptide fragments of the three main protein bands could be clearly assigned to the subunits α, β, and γ of OAD-2 from *V. cholerae*. No peptide fragments were identified, however, which matched the masses of OAD-1-derived fragments. The same applies to the protein moving just ahead of the OadB-2 subunit. The fragments resulting from this band corresponded to the masses of peptides derived from transaldolase (34.6 kDa), which is therefore probably present in our preparation as a (minor) contaminant. In summary, we conclude from these results that the *oad-2* genes are exclusively expressed by *V. cholerae* growing anaerobically on citrate.

The purified decarboxylase moved as a symmetrical peak on a size-exclusion chromatography column (Fig. 2), with an apparent molecular mass of 570 kDa. As the calculated molecular mass of a single OAD molecule is 121 kDa, we conclude that the native enzyme is present as a tetramer. The difference in the determined and calculated mass for the tetramer is probably due to the adhering Brij58 detergent micelles. In contrast, preparations of OAD from *K. pneumoniae* were always populated with various aggregation states (see Fig. 2), which seriously interfere with its crystallization.

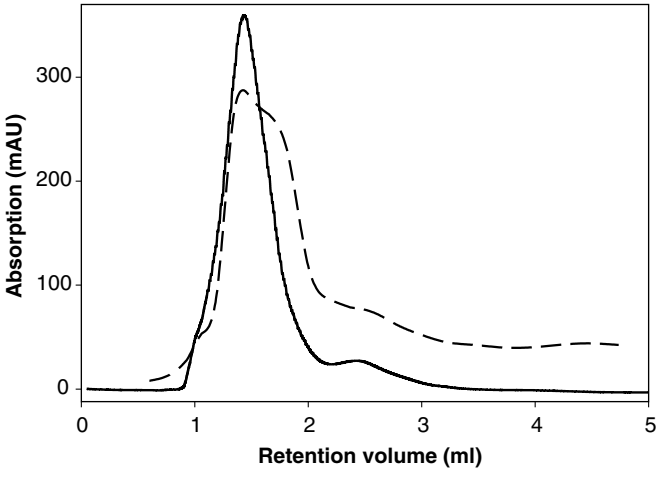

Fig. 2 Size-exclusion chromatogram of OAD-2 from *V. cholerae* and OAD from *K. pneumoniae*. The affinity-purified enzymes were concentrated and subjected to size-exclusion chromatography by loading them onto a Superdex 200 HR 10/30 gel filtration column. The protein was eluted with 50 mM Tris-HCl (pH 8.0), 250 mM NaCl, and 0.05% Brij58, at a flow rate of 0.05 ml/min. OAD-2 from *V. cholerae* eluted in a symmetrical peak (*solid line*), whereas OAD from *K. pneumoniae* was present in different aggregation states (*dashed line*)

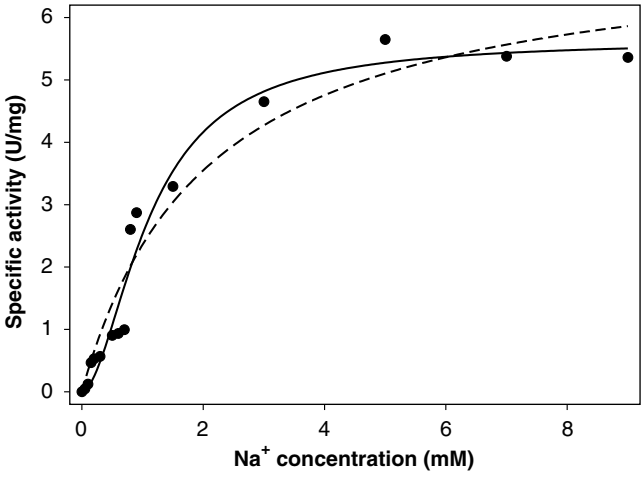

Fig. 3 Dependence of OAD activity on Na^+ concentration. Experiments were carried out as described in the "Materials and methods" section. The measured data points were fitted according to the Michaelis–Menten equation (*dashed line*) or to the Hill equation (*solid line*). The fittings were performed with SigmaPlot, version 8.02

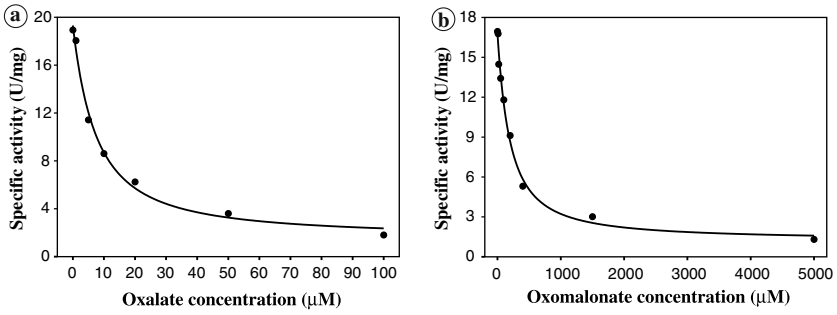

Fig. 4 Inhibition of OAD-2 by oxalate and oxomalonate. Inhibition of OAD-2 was measured in the presence of the competitive inhibitors oxalate (**a**) or oxomalonate (**b**). Half-maximal inhibition was achieved at 10 μM oxalate (**a**) or at 200 μM oxomalonate (**b**)

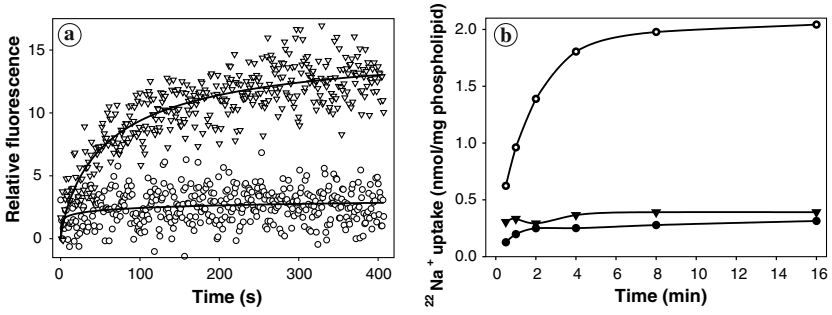

Fig. 5 Na^+ uptake into proteoliposomes by OAD was determined in a continuous (**a**) and a discontinuous (**b**) assay. **a** OAD-driven Na^+ uptake into proteoliposomes was followed continuously by recording the change of the emission intensity of the fluorophore Sodium Green contained in the proteoliposomes interior, as described in "Materials and methods" (*open inverted triangles*). To inhibit Na^+ uptake, proteoliposomes were preincubated for 30 min, with an equimolar (with respect to OAD-2) amount of avidin (*open circles*). **b** In a discontinuous assay the uptake of $^{22}Na^+$ into proteoliposomes was determined. $^{22}Na^+$ uptake was initiated by the addition of oxaloacetate (*open diamonds*). To inhibit $^{22}Na^+$ uptake proteoliposomes were preincubated for 30 min, with an equimolar (with respect to OAD-2) amount of avidin (*filled inverted triangles*). Without addition of oxaloacetate no $^{22}Na^+$ was accumulated in the proteoliposomes (*filled diamonds*)

A peculiar property of OAD is its specific activation by Na^+ ions. We therefore investigated the effect of Na^+ ions on OAD from *V. cholerae*. The results of Fig. 3 show that the enzyme was inactive in the absence of added Na^+ ions, and that the activity increased with increasing Na^+ concentrations to reach half-maximal activation at 1 mM and saturation at about 10 mM. The activation curve followed the Hill equation, with a Hill coefficient of 1.8, indicating that at least two Na^+ ions bind to the enzyme in a cooperative manner. This is consistent with the transport of two Na^+ ions per decarboxylation event and a recent model with two Na^+ binding sites operating together during transport (Dimroth and Thomer 1993; Schmid et al. 2002). At very high Na^+ concentrations (>200 mM), the enzyme was inhibited to reach less than 10% of the maximal activity at 1 M NaCl (data not shown). This observation is also in accord with data obtained with the *K. pneumoniae* decarboxylase (Schmid et al. 2002).

The substrate analogue oxalate was a potent inhibitor of OAD from *V. cholerae* (see Fig. 4a). Half-maximal inactivation by oxalate was reached at 10 μM. Another substrate analogue, oxomalonate, also inhibited OAD, but half-maximal inactivation was accomplished at 20 times higher concentrations than with oxalate (Fig. 4b).

Na^+ transport was demonstrated in a continuous fluorescence assay (von Ballmoos and Dimroth 2004) performed with proteoliposomes reconstituted with OAD purified form *V. cholerae*. The emission response of the fluorophore Sodium Green contained in the inner compartment of the proteoliposomes depends on Na^+ concentration, and therefore, Na^+ uptake can be observed in real time by recording the change in fluorescence emission intensity at 530 nm. Figure 5a shows that no Na^+ uptake was observed if the proteoliposomes were preincubated with avidin, which binds to the prosthetic biotin group and inhibits OAD. In a discontinuous assay, $^{22}Na^+$ uptake was shown with the same proteoliposomes without fluorophore addition (Fig. 5b).

Sequencing, cloning, and expression of the *oad-1* and *oad-2* gene clusters from *V. cholerae* in *E. coli*

For practical reasons, we have chosen to work with the *V. cholerae* strain O395-N1 (Mekalanos et al. 1983), which has a deletion of the cholera toxin genes, rather than with the pathogenic strain *V. cholerae* El Tor N16961, for which the genome sequence is available (Heidelberg et al. 2000). As the sequences could deviate between both strains, we sequenced the *oad-1* and *oad-2* genes from the toxin deletion strain O395-N1. The results are deposited in the EMBL nucleotide sequence database, under accession nos. AJ784855 (*oad-1*) and AJ784856 (*oad-2*). The only difference in the *oad-1* sequences is an exchange of a G to a T in the intergenic region between *oadA-1* and *oadB-1*. The differences between the *oad-2* genes from the two different strains are

more significant. The two sequences of 3,408 base pairs in length differ at 5% of positions. The changes are preferentially in the third base of codons and are synonymous substitutions. The most remarkable difference is found at the position of the triplet CGT coding for R408 of the α subunit in strain *O395-N1*. In the published sequence for strain El Tor N16961, the T of this triplet is missing. The resulting frame shift would lead to an α subunit with a deletion of 191 amino acids at the C terminus. However, with the T present in the El Tor strain, identical α subunits would be formed, with the exception of the mutations H330R and Y426H in the O395-N1 strain. The only other notable difference is the mutation S369T in the β subunit of *V. cholerae* O395-N1.

The two gene clusters *oad-1* and *oad-2* were cloned into pET vectors to express them separately in *E. coli* under the control of the T7 promoter. Per gram of cells, up to 0.4 mg of OAD-1 was purified with a specific activity of maximally 8 U/mg (Table 1; see also Fig. 1, lane 1). On the other hand, up to 1.1 mg OAD-2 was isolated per gram of cells, and the specific activity of the heterologously purified OAD-2 was up to ten times higher than that of OAD-1 and corresponds to the specific activity of the enzyme purified from *V. cholerae* cells (data not shown).

Discussion

V. cholerae grown anaerobically on citrate is a suitable source for the large-scale preparation of OAD. The OAD-2 isoform is exclusively synthesized by the wild-type cells. This is consistent with the localization of the *oad-2* genes within the gene cluster for citrate fermentation. The *oad-1* genes, on the other hand, are not connected with genes of a specific fermentation pathway and may therefore be remnants from a gene duplication event. It was shown by heterologous expression in *E. coli* that not only the *oad-2* genes but also the *oad-1* genes encode a functional OAD. Both isoforms could be isolated in pure form from the expression hosts by avidin–Sepharose affinity chromatography, but the specific activity of the isolated OAD-1 was about 6–13 times lower than that of OAD-2. Furthermore, the yield of the purified OAD-1 was only 6% compared to 37% for purified OAD-2. This lower yield probably reflects a low stability of OAD-1 complex. Taken together, these data indicate superior properties of the OAD-2 over the OAD-1 isoform, which would be consistent with the presence of *oad-1* as silent genes that are no longer expressed by these cells.

OAD-2 from *V. cholerae* is similar to the decarboxylase from *K. pneumoniae* with respect to kinetic properties, but its overall stability seems to be much better, which makes it a superior subject for structural investigations. The pure OAD-2 has about twice the specific activity of the pure OAD from *K. pneumoniae* and is significantly more stable during storage at 4°C. Another difference is the aggregation behavior between both enzymes. Whereas OAD-2 was found to run as a monodisperse species with the molecular mass of the tetramer on size-exclusion chromatography, the decarboxylase of *K. pneumoniae* existed in multiple aggregation states. Furthermore, the absence of an extended proline/alanine linker in OAD-2 from *V. cholerae* probably provides less flexibility to this protein, which may be another advantage for crystallization studies. Taken together, these results show that investigation of a particular enzyme from different organisms is a suitable approach to increase the chances of success in crystallization studies, especially those with membrane proteins, which are notoriously difficult to crystallize.

Acknowledgements This work was supported by Swiss National Science Foundation. We thank Christoph von Ballmoos for performing the MALDI-TOF analysis.

References

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1999) Short protocols in molecular biology, 4th edn. Wiley, New York

Ballmoos C von, Dimroth P (2004) A continuous fluorescent method to measure Na^+ transport. Anal Biochem (in press)

Becamel C, Galeotti N, Poncet J, Jouin P, Dumuis A, Bockaert J, Marin P (2002) A proteomic approach based on peptide affinity chromatography, 2-dimensional electrophoresis and mass spectrometry to identify multiprotein complexes interacting with membrane-bound receptors. Biol Proced Online 4:94–104

Bott M, Dimroth P (1994) *Klebsiella pneumoniae* genes for citrate lyase and citrate lyase ligase: localization, sequencing, and expression. Mol Microbiol 14:347–356

Bott M, Meyer M, Dimroth P (1995) Regulation of anaerobic citrate metabolism in *Klebsiella pneumoniae*. Mol Microbiol 18:533–546

Bott M, Pfister K, Burda P, Kalbermatter O, Woehlke G, Dimroth P (1997) Methylmalonyl-CoA decarboxylase from *Propionigenium modestum:* cloning and sequencing of the structural genes and purification of the enzyme complex. Eur J Biochem 250:590–599

Buckel W (1980) The reversible dehydration of (R)-2-hydroxyglutarate to (E)-glutaconate. Eur J Biochem 106:439–447

Buckel W (2001) Sodium ion-translocating decarboxylases. Biochim Biophys Acta 1505:15–27

Busch W, Saier MH Jr (2002) The transporter classification (TC) system, 2002. Crit Rev Biochem Mol Biol 37:287–337

Busch W, Saier MH (2004) The IUBMB-endorsed transporter classification system. Mol Biotechnol 27:253–262

Dahinden P, Dimroth P (2004) Oxaloacetate decarboxylase of *Archaeoglobus fulgidus*: cloning of genes and expression in *Escherichia coli*. Arch Microbiol 182:414–420

Di Berardino M, Dimroth P (1996) Aspartate 203 of the oxaloacetate decarboxylase β-subunit catalyses both the chemical and vectorial reaction of the Na^+ pump. EMBO J 15:1842–1849

Dimroth P (1980) A new sodium-transport system energized by the decarboxylation of oxaloacetate. FEBS Lett 122:234–236

Dimroth P (1981) Characterization of a membrane-bound biotin-containing enzyme: oxaloacetate decarboxylase from *Klebsiella aerogenes*. Eur J Biochem 115:353–358

Dimroth P (1986) Preparation, characterization, and reconstitution of oxaloacetate decarboxylase from *Klebsiella aerogenes*, a sodium pump. Methods Enzymol 125:530–540

Dimroth P (1997) Primary sodium ion translocating enzymes. Biochim Biophys Acta 1318:11–51

Dimroth P (2004) Molecular basis for bacterial growth on citrate or malonate. In: Curtiss III R (ed) *EcoSal -Escherichia coli* and *Salmonella*: cellular and molecular biology, 3.4.6 edn. ASM, Washington, DC

Dimroth P, Schink B (1998) Energy conservation in the decarboxylation of dicarboxylic acids by fermenting bacteria. Arch Microbiol 170:69–77

Dimroth P, Thomer A (1986) Kinetic analysis of the reaction mechanism of oxaloacetate decarboxylase from *Klebsiella aerogenes*. Eur J Biochem 156:157–162

Dimroth P, Thomer A (1993) On the mechanism of sodium ion translocation by oxaloacetate decarboxylase of *Klebsiella pneumoniae*. Biochemistry 32:1734–1739

Dimroth P, Jockel P, Schmid M (2001) Coupling mechanism of the oxaloacetate decarboxylase Na^+ pump. Biochim Biophys Acta 1505:1–14

Heidelberg JF, Eisen JA, Nelson WC, Clayton RA, Gwinn ML, Dodson RJ, et al (2000) DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature 406:477–483

Hilpert W, Schink B, Dimroth P (1984) Life by a new decarboxylation-dependent energy-conservation mechanism with Na^+ as coupling ion. EMBO J 3:1665–1670

Jockel P, Schmid M, Choinowski T, Dimroth P (2000a) Essential role of tyrosine 229 of the oxaloacetate decarboxylase β-subunit in the energy coupling mechanism of the Na^+ pump. Biochemistry 39:4320–4326

Jockel P, Schmid M, Steuber J, Dimroth P (2000b) A molecular coupling mechanism for the oxaloacetate decarboxylase Na^+ pump as inferred from mutational analysis. Biochemistry 39:2307–2315

Kyhse-Andersen J (1984) Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J Biochem Biophys Methods 10:203–209

McClelland M, Sanderson KE, Spieth J, Clifton SW, Latreille P, Courtney L et al (2001) Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature 413:852–856

Mekalanos JJ, Swartz DJ, Pearson GD, Harford N, Groyne F, de Wilde M (1983) Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development. Nature 306:551–557

Miroux B, Walker JE (1996) Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. J Mol Biol 260:289–298

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Schägger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166:368–379

Schmid M, Vorburger T, Pos KM, Dimroth P (2002) Role of conserved residues within helices IV and VIII of the oxaloacetate decarboxylase β subunit in the energy coupling mechanism of the Na^+ pump. Eur J Biochem 269:2997–3004

Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 68:850–858

Wendt KS, Schall I, Huber R, Buckel W, Jacob U (2003) Crystal structure of the carboxyltransferase subunit of the bacterial sodium ion pump glutaconyl-coenzyme A decarboxylase. EMBO J 22:3493–3502

Wild MR, Pos KM, Dimroth P (2003) Site-directed sulfhydryl labeling of the oxaloacetate decarboxylase Na^+ pump of *Klebsiella pneumoniae*: helix VIII comprises a portion of the sodium ion channel. Biochemistry 42:11615–11624

Woehlke G, Dimroth P (1994) Anaerobic growth of Salmonella typhimurium on L(+)- and D(−)-tartrate involves an oxaloacetate decarboxylase Na^+ pump. Arch Microbiol 162:233–237

Wray W, Boulikas T, Wray VP, Hancock R (1981) Silver staining of proteins in polyacrylamide gels. Anal Biochem 118:197–203