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SHORT COMMUNICATION

Pius Dahinden · Klaas M. Pos · Malgorzata Taralczak
Peter Dimroth

Oxaloacetate decarboxylase of *Archaeoglobus fulgidus*: cloning of genes and expression in *Escherichia coli*

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Abstract *Archaeoglobus fulgidus* harbors three consecutive and one distantly located gene with similarity to the oxaloacetate decarboxylase Na^+ pump of *Klebsiella pneumoniae* (KpOadGAB). The water-soluble carboxyltransferase (AfOadA) and the biotin protein (AfOadC) were readily synthesized in *Escherichia coli*, but the membrane-bound subunits AfOadB and AfOadG were not. AfOadA was affinity purified from inclusion bodies after refolding and AfOadC was affinity purified from the cytosol. Isolated AfOadA catalyzed the carboxyltransfer from [4-^{14}C]-oxaloacetate to the prosthetic biotin group of AfOadC or the corresponding biotin domain of KpOadA. Conversely, the carboxyltransferase domain of KpOadA exhibited catalytic activity not only with its pertinent biotin domain but also with AfOadC.

Keywords *Archaeoglobus fulgidus* · Oxaloacetate decarboxylase · Na^+ pump · Archaeal membrane protein · Protein targeting and translocation · Refolding of inclusion bodies

Abbreviations Af: *Archaeoglobus fulgidus* · AHT: Anhydrotetracycline · DTT: Dithiothreitol · IPTG: Isopropyl-β-D-thiogalactopyranoside · Kp: *Klebsiella pneumoniae* · LDAO: *N*,*N*-dimethyldodecylamine-N-oxide · MBP: Maltose binding protein · OAD: Oxaloacetate decarboxylase · OadA: Oxaloacetate decarboxylase α-subunit · OadB: Oxaloacetate decarboxylase β-subunit · OadC: Oxaloacetate decarboxylase δ-subunit · OadG: Oxaloacetate decarboxylase γ-subunit · PPS: 3-(1-pyridino)-1-propanesulfate

P. Dahinden · K. M. Pos · M. Taralczak · P. Dimroth (✉)
Institut für Mikrobiologie der ETH Zürich,
ETH-Zentrum, Schmelzbergstrasse 7, 8092 Zürich,
Switzerland
E-mail: dimroth@micro.biol.ethz.ch
Tel.: +41-1-6323321
Fax: +41-1-6321378

Introduction

Genome sequencing of *Archaeoglobus fulgidus* (Af) (Klenk et al. 1997) revealed a cluster of three open reading frames (ORFs) with high similarity to genes encoding subunits of the Na^+ translocating decarboxylase enzyme family (for reviews see Dimroth 1997; Buckel 2001). Accordingly, the gene products of the ORFs AF2084, AF2085 and AF2086 were identified as the membrane-integral carboxybiotin decarboxylase (β-subunit), the biotin-binding protein and the membrane-anchored γ-subunit, respectively. At separate locations, genes encoding carboxyltransferases with specificities for oxaloacetate (AF1252) or methylmalonyl-CoA (AF2216) are found. The latter gene is succeeded by AF2217, encoding a biotin carrier protein. Based on this information, one may conclude that *A. fulgidus* has the capacity to synthesize an oxaloacetate decarboxylase and a methylmalonyl-CoA decarboxylase Na^+ pump and that these enzyme complexes share part of their subunits with each other.

Most of the fundamental biochemistry of the Na^+ translocating decarboxylases has been studied with the oxaloacetate decarboxylase from *Klebsiella pneumoniae* (KpOAD). The catalytic cycle starts with the carboxyltransfer from oxaloacetate to the biotin group on KpOadA-C. Subsequently, KpOadB catalyzes the decarboxylation of carboxybiotin coupled to the transport of two Na^+ ions into the periplasmic reservoir and consumption of a periplasmically derived proton (Di Berardino and Dimroth 1996). By mutational studies, three amino acids (D203, Y229, S382) were identified to have a key function in the ion-transport-coupled decarboxylation event (Jockel et al. 2000a,b; Schmid et al. 2002a). The most highly conserved portion of the protein is helix VIII, which provides part of the ion channel (Wild et al. 2003). KpOadG harbors a Zn^{2+} ion that is part of the carboxyltransferase catalytic site and it is essential for the formation of a stable enzyme complex (Schmid et al. 2002b).

For detailed insights into the catalytic mechanism of the Na^+ pump, structural information is of essence. Progress in this direction was obtained recently with the crystal structure of the carboxyltransferase subunit of glutaconyl-CoA decarboxylase (Wendt et al. 2003). With the same aim we have investigated oxaloacetate decarboxylase from the thermophile *A. fulgidus*. Here, we report on the expression of the genes *AfoadA* and *AfoadC* in *Escherichia coli* and on the purification and characterization of the gene products.

Materials and methods

Strains and growth conditions *Archaeoglobus fulgidus* VC-16 (DSM 4304) was grown anaerobically at 83°C in sulfate–thiosulfate–lactate (STL) medium [medium 3, (Balch et al. 1979)] gassed with N_2/CO_2 (80%:20%, 200 kPa).

Recombinant DNA techniques and sequencing Standard microbiological methods were used as described (Ausubel et al. 1989; Sambrook et al. 1989). Polymerase chain reaction (PCR) was done with *Pfu polymerase and A. fulgidus chromosomal DNA as template. All inserts derived from PCR as well as the ligation sites were checked by DNA sequencing (Sanger et al. 1977).*

Cloning of the Afoad genes The oligonucleotide primers 5′-TCTGCATCTGGCTCATATGGCAAAAG-3′ (AfOA-*Nde*I) and 5′-ATATTTAGTCGCTCGAGCTTTTCTGGC-3′ (AfOA-*Xho*I-Hi), 5′-CGATCTAGCTCATATGCTCGGAAGCCTCG-3′ (AfOB-*Nde*I) and 5′-CCTATTCGATCTCGAGCCCAAGAATCTG-3′ (AfOB-*Xho*I-Hi), 5′-CGATCTAGCTCATATGAGGTTTTACAG-3′ (AfOC-*Nde*I) and 5′-GCTATTCGATCTCGAGGCTCACCGGCTGG-3′ (AfOC-*Xho*I-Hi), all including an *Nde*I or *Xho*I site (underlined), were used to amplify AF1252, AF2084 and AF2085, respectively. PCR products were digested with *Nde*I and *Xho*I and ligated to the adequately digested, dephosphorylated vector pET24b (Novagen). The resulting plasmids were designated pET24b-AfoadA, pET24b-AfoadB or pET24b-AfoadC, respectively, and all encoded the respective subunit including a C-terminal His-tag.

With the oligonucleotide primers AfOA-*Nde*I (see above) and 5′-AATGTCTCGCTCGAGCCACCTTGTCG-3′ (AfOA-*Xho*I) *AfoadA* was amplified, whereas *AfoadB* was amplified with AfOB-*Nde*I and 5′-CCTATTCGATCTCGAGCTACCCAAGAATCTG-3′ (AfOB-*Xho*I). The resulting PCR products included *Nde*I and *Xho*I restriction sites which were used for the unidirectional cloning into pET16b vector to obtain pET16b-AfoadA or pET16b-AfoadB, encoding oxaloacetate decarboxylase subunits α and β including an N-terminal His-tag.

With the oligonucleotide primers 5′-TCAATCGGTCTCGGCGCCATGCTCGGAAGCCTCGTG-3′ (AfOB-Str_fo) and 5′-ACCCTCGGTCTCTTATCACTACCCAAGAATCTGAATC-3′ (AfOB-Str_re), both including a *Bsa*I site (underlined), *AfoadB* was amplified from chromosomal *A. fulgidus* DNA. The PCR product and the vector pASK-IBA4 (IBA GmbH) were digested with *Bsa*I. The digested and ligated fragments resulted in plasmid pASK-AfoadB, encoding an *A. fulgidus* oxaloacetate decarboxylase β-subunit including an N-terminal Strep-tag.

To obtain a plasmid in which all putative *Afoad* genes are under the control of one promoter, the ORFs AF2086 (γ-subunit), AF2085 and AF2084 were amplified with the oligonucleotide primers 5′-ATCTCGGTATGACATATGATAGATTTGGCCATAATGCTCACTGTAGAGG-3′ (AfOadGCB-*Nde*I) and 5′-TC ATAGTATGAGCTCTCAGCTAGCCAAAATGCGATGATTAACCTACCC-3′ (AfOadGCB-*Sac*I), including *Nde*I and *Sac*I sites (underlined). ORF AF1252 was amplified with the oligonucleotide primers 5′-TGTAGTATTGAGCTCTGAGGATCCATTTCCCATCAGGCTTGAGACATGGC-3′ (AfOadA-*Sac*I) and 5′-TGATATATCCTCGAGTATGCGGCCGCAAGAGGTATATCACTTTTCTGGC-3′ (AfOadA-*Xho*I), including *Sac*I and *Xho*I sites (underlined). Plasmid pET24b-AfoadGCBA was obtained by ligation of the accordingly digested PCR products and vector pET24b.

Growth of recombinant E. coli and expression of genes For expression of the *Afoad* genes, baffled Erlenmeyer flasks containing LB (200 ml to 2 l) were inoculated 1:100 with an overnight culture and incubated at 37°C at 180 rpm until the culture reached an OD_{600} of approximately 0.6. After cooling to 4°C, the expression of the genes was induced by the addition of IPTG (100 μM) and the culture was incubated for another 4 h at 37°C and shaking at 180 rpm before harvesting the cells. In the case of $AfOadA_{His}$ synthesis, the cultures were grown at 30°C before induction and at 25°C after induction.

Refolding of AfOadA from inclusion bodies Cells containing inclusion bodies were resuspended in 5 ml per gram of cells 50 mM Tris–HCl, pH 7.0, 1 mM EDTA and 3 mM $MgCl_2$. After the addition of 7.5 mg lysozyme and 50 μg DNase I, the suspension was incubated for 25 min at 25°C and then passed three times through a French pressure cell at 110 MPa. Subsequently, 20 mM EDTA, pH 8.0, 0.5 M NaCl and 2% Triton X-100 (final concentration) were added and the mixture incubated for 30 min at 4°C. Inclusion bodies were harvested by centrifugation at 20,000×*g* and washed twice with 100 mM Tris–HCl, pH 7.5 and 20 mM EDTA (10 ml), before they were solubilized with 100 mM Tris–HCl, pH 8.0, 1 mM EDTA, 6 M guanidinium chloride and 100 mM dithiothreitol (at 10 mg protein per ml). After 2–3 h at 25°C, solubilization was complete. After adjusting the pH to 3–4 with 10 mM HCl and centrifugation, the supernatant (0.5 ml) was slowly diluted into

50 ml 100 mM Tris–HCl, 1 mM EDTA, 1 mM DTT containing 500 mM L-arginine or 1 M 3-(1-pyridino)-1-propanesulfate (PPS) and/or 0.1% LDAO (adjusted to a final pH of 8.5) at 15°C. After 10 min, another 0.5 ml of solubilized protein solution was slowly added. This cycle was repeated 19 times to reach a final guanidinium chloride concentration of just below 1 M. Precipitated protein was removed by passage through a 0.22-μm filter. Soluble, but higher aggregates of AfOadA were removed by precipitation with 40% ammonium sulfate. Soluble protein was then precipitated with 60% ammonium sulfate and the precipitate collected by centrifugation. The protein was dissolved in 20 mM Tris–HCl, pH 8.0, 100 mM NaCl and 0.1% LDAO to a concentration of about 1 mg/ml, dialyzed overnight against 2 l of 20 mM Tris–HCl, pH 8.0, containing 100 mM NaCl and subsequently passed through a 0.22-μm filter unit.

Preparation of cytosolic fraction and membranes Cells from expression cultures (1 g wet weight) were suspended in 5 ml of a suitable buffer containing 0.2 mM DFP and 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*), and the cell-free supernatant was subjected to ultracentrifugation (1 h at 200,000×*g*) to separate the cytosolic fraction and the membrane fraction.

Purification of $AfOadA_{His}$ by Ni^{2+}-NTA chromatography The α-subunit was synthesized in *E. coli* C43(DE3) (Miroux and Walker 1996) harboring pET24b-AfoadA or pET16b-AfoadA. The cells were resuspended in HisBind buffer (20 mM Tris–HCl, pH 8.0, and 500 mM NaCl). The cytosolic fraction was prepared as described above and applied to a Ni^{2+}-NTA-agarose column (2 ml bed volume, Qiagen), pre-equilibrated with 10 bed volumes HisBind buffer containing 20 mM imidazole. The column was washed three times with 10 bed volumes of the same buffer containing 60, 70 and 80 mM imidazole, respectively. $AfOadA_{His}$ was then eluted in 2 bed volumes of HisBind buffer containing 250 mM imidazole.

Purification of AfOadC by affinity chromatography using monomeric avidin-Sepharose The δ-subunit was synthesized in *E. coli* C43(DE3) harboring pET24b-AfoadC in the presence of 10 mM (+)-D-biotin. The cells were resuspended in buffer A (20 mM Tris–HCl, pH 8.0, 500 mM NaCl, and 1 mM MgK_2EDTA). The cytosolic fraction was prepared as described above and applied to a SoftLink Soft Release avidin resin column (Promega) (Dimroth 1986). The column was washed with 7 bed volumes of buffer B (20 mM Tris–HCl, pH 8.0, 500 mM NaCl), and AfOadC was eluted with 2 bed volumes of buffer B containing 5 mM (+)-D-biotin.

Carboxyltransfer from oxaloacetate to the biotin-containing subunit The carboxyltransferase activity was measured with [4-^{14}C]-oxaloacetate and AfOadA and AfOadC or KpOadA-C, essentially as described before (Di Berardino and Dimroth 1995; Jockel et al. 2000).

Results and discussion

Identification, cloning and expression of genes similar to *oadGAB* of *K. pneumoniae*

Oxaloacetate decarboxylase of *K. pneumoniae* (KpOadGAB) is the prototype for the Na^+ transporting carboxylic acid decarboxylase (NaT-DC) family (Busch and Saier 2003). All members of this family contain a membrane-bound β-subunit involved in Na^+ translocation and a peripheral α-subunit. In oxaloacetate decarboxylase of *K. pneumoniae*, the α-subunit consists of the carboxyltransferase domain (OadA-N) and the biotin-containing CO_2 acceptor domain (OadA-C). Most members of the NaT-DC family have an additional small membrane-anchored subunit (designated γ or δ) which is required for the formation of a stable enzyme complex (Huder and Dimroth 1993; Bott et al. 1997; Schmid et al. 2002). A database search for proteins related to KpOadB resulted in the identification of 27 similar sequences from eubacteria and four from archaea. Because of our interest in the structure of the decarboxylase and the consideration that proteins from thermophilic sources might be more stable under ambient crystallization conditions, we decided to select and amplify the genes encoding the subunits of the putative oxaloacetate decarboxylase from *A. fulgidus* (Table 1). Based on homology searches with KpOAD, *A. fulgidus* encodes a putative oxaloacetate decarboxylase consisting of subunits α, β, δ and γ (ORFs AF1252, AF2084, AF2085, AF2086). See Table 1 and Fig. 1 for details. In *K. pneumoniae*, the *oadGAB* genes are clustered in an operon that is part of the citrate fermentation regulon (Bott et al. 1995). In contrast, *A. fulgidus* harbors the *AfoadGCB* genes and the *AfoadA* gene at separate locations on its genome (Fig. 1). In addition to the genes for a putative oxaloacetate decarboxylase, two ORFs were found with similarity to the methylmalonyl-CoA decarboxylase subunits α (PmMmdA, carboxyltransferase) and γ (PmMmdC, biotin-binding protein) of *Propionigenium modestum*. These genes were again not clustered with the genes for the membrane-bound subunits. The subunits encoded by the separate *AfoadGCB* gene cluster may therefore be used for both enzymes, the oxaloacetate and the methylmalonyl-CoA decarboxylase Na^+ pumps.

Oxaloacetate decarboxylase is specifically required for the citrate or tartrate fermentation pathways. Consequently, the *oadGAB* genes are clustered either with the genes for citrate lyase and for a citrate transporter (*K. pneumoniae* or *S. typhimurium*) or with the genes for tartrate dehydratase (*S. typhimurium*) (Bott 1997). In *A. fulgidus*, however, the *oadGCB* and *oadA* genes are

Table 1 Comparison of oxaloacetate decarboxylase subunits of *Archaeoglobus fulgidus* and *Klebsiella pneumoniae*. The molecular mass is the theoretical molecular mass, deduced from amino acid sequence. The Blosum62 similarity matrix was used to compute identities/similarities by BioEdit (Hall 1999). Values are given in % identity/similarity

Subunit	*A. fulgidus*			*K. pneumoniae*	
	Molecular mass (kDa)	Amino acids	Similarity to KpOAD (identities/similarities)	Molecular mass (kDa)	Amino acids
OadA (α)	53.9	480	50/67[a]	63.4	596
OadB (β)	36.9	355	48/62	44.9	433
OadC (δ)	15.8	143	24/34[b]	–	–
OadG (γ)	7.6	69	19/32	8.9	83
MmdA (α)	56.8	516	16/32	–	–
MmdC (δ)	15.7	140	28/37	–	–

[a]AfOadA or AfMmdA was compared with the transferase domain of KpOadA (N-terminal 497 amino acids)
[b]AfOadC or AfMmdC was compared with the biotin carrier domain of KpOadA (C-terminal 99 amino acids)

neither clustered with the genes for citrate lyase or tartrate dehydratase nor could these genes be identified elsewhere on the genome. We conclude therefore that *A. fulgidus* is unable to perform citrate or tartrate fermentation and, hence, no specific role could be assigned to the oxaloacetate-decarboxylase-encoding genes in a catabolic pathway. This conclusion is consistent with the inability of *A. fulgidus* to grow on citrate as sole carbon and energy source. *A. fulgidus* cells grown on lactate did not contain any significant amount of oxaloacetate decarboxylase activity; consequently, no decarboxylase complex could be isolated by the standard procedure with avidin-Sepharose affinity chromatography.

As oxaloacetate decarboxylase could not be obtained from wild-type *A. fulgidus* cells, we tried to synthesize the enzyme in *E. coli*. For this purpose, the appropriate *A. fulgidus* genes were cloned into pET vectors and transferred into *E. coli* BL21(DE3). Synthesis of the desired proteins was analyzed by SDS-PAGE and Western blotting following purification by affinity chromatography via specific tags. The results of Table 2 and Fig. 2 show that pure AfOadA and AfOadC were isolated from recombinant *E. coli* BL21(DE3) harboring pET24-*AfoadA* and pET24-*AfoadC*, respectively. Expression of *AfoadA* resulted mainly in the formation of inclusion bodies, even when the cells were grown at 25°C. From 1 g of cells (wet weight), 0.3 mg of AfOadA was affinity purified from the cytoplasm while 100 mg were present in inclusion bodies.

Biotinylation of AfOadC was shown by Western blot analysis and staining with avidin-conjugated alkaline phosphatase. The amount of AfOadC isolated by avidin-Sepharose chromatography was approximately half of that obtained by Ni^{2+}-NTA chromatography; therefore, approximately 50% of AfOadC was equipped with the biotin prosthetic group. Extensive attempts were made to express AfOadB either individually or in various combinations with AfOadA, AfOadC, and AfOadG using various expression hosts at different growth stages. Details of these experiments, compiled in Table 2, indicated no increase in the expression level under all examined conditions.

We therefore considered the possibility that the presence of rare codons prevented the synthesis of AfOadB in *E. coli*. However, the mean difference in codon usage for *AfoadB* (21.5%), calculated using the *E. coli* codon usage table with the gcua program (Fuhrmann M, Ferbitz L, Hausherr A, Schrodl T, and Hegemann P, in preparation), did not significantly deviate from the value for *AfoadA* (20.5%), which was synthesized in large amounts (100 mg/g cells). Moreover, no expression of *AfoadB* was detected with expression hosts containing extra copies of tRNAs recognizing rare codons.

Since specifically the two membrane-bound proteins AfOadB and AfOadG were not successfully synthesized in *E. coli*, their insertion and/or proper folding into the native structure could have been the main problem. For most membrane proteins of *E. coli*, targeting and insertion into the membrane requires the signal recognition particle and the Sec pathway (Fekkes and Driessen 1999). In addition, YidC has been recognized to be re-

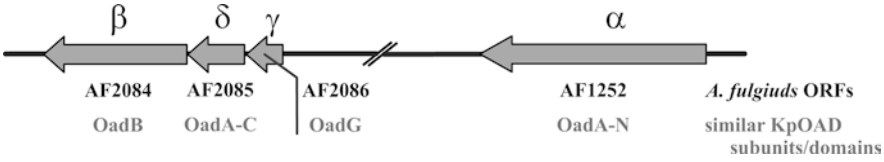

Fig. 1 ORFs of *Archaeoglobus fulgidus* encoding proteins with similarity to OAD subunits/domains from *Klebsiella pneumoniae*. The ORFs AF2084, AF2085, and AF2086 encode proteins similar to KpOadB, KpOadA-C, and KpOadG, respectively, of the OAD from *K. pneumoniae* (KpOadGAB). The ORF AF1252, located on a remote part of the *A. fulgidus* genome, is similar to KpOadA-N. The latter is the carboxyltransferase domain of KpOadA, comprising the N-terminal 497 amino acids, whereas KpOadA-C is the biotin-binding domain of KpOadA, comprising the C-terminal 99 amino acids

Table 2 Constructs for heterologous expression of oad genes of *A. fulgidus* in *Escherichia coli*

Construct		Yield (mg/g cells)
Name	Comment	
pET16b-AfoadA +	Encoding AfOadA including an N-terminal HisTag[a] and 20 additional vector-encoded amino acids at the C-terminus	0.02–0.3[b]
pET16b-AfoadA	Encoding AfOadA including an N-terminal HisTag[a]	0.02–0.3[b]
pET24b-AfoadA	Encoding AfOadA including a C-terminal HisTag[c]	0.02–0.1[b]
pET43.1b-AfoadA	Encoding AfOadA including an N-terminal NusTag[d] and a C-terminal HisTag[c]	0.1[b]
pET24b-AfoadC	Encoding AfOadC including a C-terminal HisTag[c]	0.15–0.45[e]
pASK-AfoadB[f]	Encoding AfOadB including an N-terminal OmpA signal sequence and a StrepTag	No expression
pET16b-AfoadB	Encoding AfOadB including an N-terminal HisTag[a]	No expression
pET24b-AfoadB	Encoding AfOadB including a C-terminal HisTag[c]	No expression
pET24b-AfoadCB	Encoding AfOadCB	No expression
pET24b-AfoadCBA	Encoding AfOadCBA	No expression
pET24b-AfoadGCB	Encoding AfOadGCB	No expression
pET24b-AfoadGCBA	Encoding AfOadGCBA	No expression

[a]Ten consecutive histidine residues followed by nine amino acids (SSGHIEGRH)
[b]Most of the overproduced protein (>99%) was present as inclusion bodies (approximately 100 mg g^{-1} cells)
[c]Six consecutive histidine residues
[d]Fusion with 491 amino acid NusA (NusTag) protein (Harrison 2000)
[e]The values indicated are the yield of biotinylated protein (purified via SoftLink-avidin column). Twice the amount can be purified via Ni^{2+}-NTA affinity chromatography (biotinylated and non-biotinylated species)
[f]Derivative of pASK-IBA4 (Skerra and Schmidt 2000)

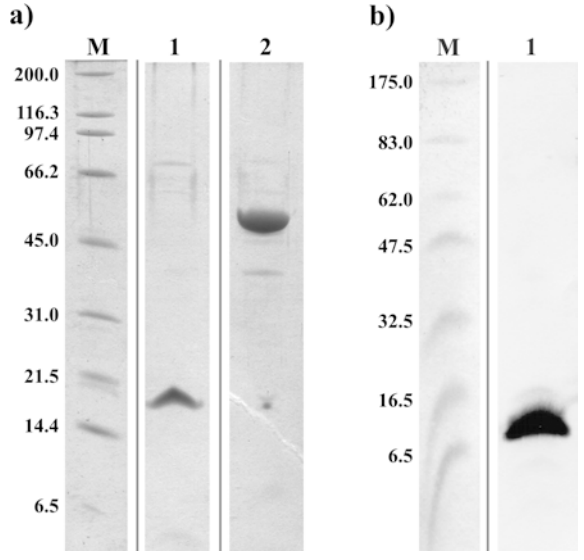

Fig. 2 SDS-PAGE of heterologously expressed AfOadC and AfOadA and Western blot of AfOadC. Each lane was loaded with 2 μg of protein purified as described in "Materials and methods." **a** Silver-stained SDS-PAGE gels; *M* BioRad Broad Range marker, *lane 1* AfOadC purified via SoftLink-avidin column, *lane 2* AfOadA purified via Ni-NTA-agarose column. **b** Western blot of AfOadC; *M* NEB Prestained Broad Range marker, *lane 1* AfOadC purified via Ni-NTA-agarose column. The blot was incubated with alkaline-phosphatase-conjugated avidin to show the biotinylation of AfOadC

quired for the insertion or proper folding of specific membrane proteins (Nagamori et al. 2004; Pohlschroder et al. 2004). If specific archeal targeting and folding proteins are required for the integration of archeal membrane proteins, this would prevent their successful synthesis in *E. coli* (Eichler 2000; Pohlschroder et al. 2004).

The *A. fulgidus* OadA subunit transfers CO_2 from oxaloacetate to OadC

Transfer of the labeled carboxyl group from [4-^{14}C]-oxaloacetate to the biotin group of AfOadC was measured in the purified AfOadA and AfOadC proteins. Figure 3 shows the kinetics of the carboxyl transfer with various combinations of carboxyltransferase and biotin carrier protein from *A. fulgidus* and *K. pneumoniae*. Incorporation of radioactivity into the biotin-containing CO_2 acceptor domain was dependent on the carboxyltransferase and was found for each of the four pairs tested, albeit at different rates. With both *K. pneumoniae* proteins, the transfer was approximately two times faster than with the similar *A. fulgidus* pair. Surprisingly, the fastest rates were observed with AfOadA/KpOadA-C pair. $^{14}CO_2$ labeling of the KpOadA-N/AfOadC pair was slow and the steady state level was reduced to about one third compared to the other combinations of transferase and biotin carrier protein. Taken together, these experiments identify AfOadA as carboxyltransferase and AfOadC as the appropriate CO_2 acceptor.

Reconstitution of AfOadA from inclusion bodies

To make at least part of the AfOadA deposited in inclusion bodies available for structural investigations, refolding into the native structure was attempted. For this purpose, the inclusion bodies were dissolved in 6 M guanidinium chloride and then diluted into buffer containing appropriate additives to facilitate refolding (Lilie et al. 1998). Most of the solubilized protein precipitated during this procedure, but the residual protein was essentially pure AfOadA (Table 3). The recovery of

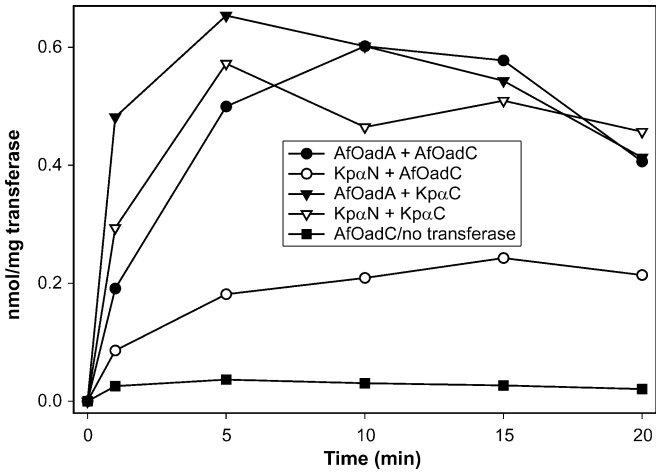

Fig. 3 $^{14}CO_2$ labeling of biotin carboxyl carrier proteins (BCC) by the carboxyl transferases from oxaloacetate decarboxylase of *K. pneumoniae* or *A. fulgidus*. The BCC subunits (AfOadC or KpOadA-C) were labeled as described in "Materials and methods." The experiments were carried out with the following combinations of BCC and transferase subunits: AfOadC and AfOadA (*filled circles*), AfOadC and KpOadA-N (*open circles*), KpOadA-C and AfOadA (*filled inverted triangles*), KpOadA-C and KpOadA-N (*open inverted triangles*). A control experiment was done with AfOadC only (*filled squares*)

Table 3 Refolding yields and recovered transferase activity of AfOadA. The recovered activity was calculated from the initial rates (nmol min^{-1} mg^{-1} OadA) as determined by the transferase activity assay. AfOadA inclusion bodies were prepared and solubilized with 6 M guanidinium chloride. The yield was calculated from the amount of soluble protein present after dilution of the solubilized protein into refolding buffer. See "Materials and methods" for details

AfOadA refolded in the presence of	Recovered specific activity (%)	Yield (%)
L-Arg	25.4	11
PPS	30.3	8
L-Arg + 0.1% LDAO	37.6	18
PPS + 0.1% LDAO	59.0	24
0.1% LDAO	3.7	4
No additive	6.8	4
Soluble AfOadA	100	–

transferase activity from inclusion bodies strongly depended on the additive. Without additives, only 4% of the inclusion bodies refolded into soluble protein. This refolded, soluble AfOadA showed only 7% of the specific transferase activity compared to soluble, cytoplasmically derived AfOadA. Using additives such as L-arginine and PPS in the presence of 0.1% LDAO, yields could be dramatically improved (up to 24%) and specific activity recovered to almost 60% compared to soluble expressed AfOadA (Table 3). These experiments impressively demonstrated the effectiveness of additives for refolding protein solubilized in guanidinium chloride and have provided us with the necessary amount of protein to initiate crystallization experiments.

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