Subunit γ of the Oxaloacetate Decarboxylase Na^+ Pump: Interaction with Other Subunits/Domains of the Complex and Binding Site for the Zn^{2+} Metal Ion†

Markus Schmid, Markus R. Wild, Pius Dahinden, and Peter Dimroth*

Mikrobiologisches Institut der Eidgenössischen Technischen Hochschule, ETH-Zentrum, CH-8092 Zürich, Switzerland

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ABSTRACT: The oxaloacetate decarboxylase Na^+ pump of *Klebsiella pneumoniae* is an enzyme complex composed of the peripheral α subunit and the two integral membrane-bound subunits β and γ. The α subunit consists of the N-terminal carboxyltransferase domain and the C-terminal biotin domain, which are connected by a flexible proline/alanine-rich linker peptide. To probe interactions between the two domains of the α subunit and between α-subunit domains and the γ subunit, the relevant polypeptides were synthesized in *Escherichia coli* and subjected to copurification studies. The two α-subunit domains had no distinct affinity toward each other and could, therefore, not be purified as a unit on avidin-sepharose. The two domains reacted together catalytically, however, performing the carboxyl transfer from oxaloacetate to protein-bound biotin. This reaction was enhanced up to 6-fold in the presence of the Zn^{2+}-containing γ subunit. On the basis of copurification with different tagged proteins, the C-terminal biotin domain but not the N-terminal carboxyltransferase domain of the α subunit formed a strong complex with the γ subunit. Upon the mutation of γH78 to alanine, the binding affinity to subunit α was lost, indicating that this amino acid may be essential for formation of the oxaloacetate decarboxylase enzyme complex. The binding residues for the Zn^{2+} metal ion were identified by site-directed and deletion mutagenesis. In the γD62A or γH77A mutant, the Zn^{2+} content of the decarboxylase decreased to 35% or 10% of the wild-type enzyme, respectively. Less than 5% of the Zn^{2+} present in the wild-type enzyme was found if the two C-terminal γ-subunit residues H82 and P83 were deleted. Corresponding with the reduced Zn^{2+} contents in these mutants, the oxaloacetate decarboxylase activities were diminished. These results indicate that aspartate 62, histidine 77, and histidine 82 of the γ subunit are ligands for the catalytically important Zn^{2+} metal ion.

Members of the sodium ion transport decarboxylase family of enzymes include oxaloacetate decarboxylase, methylmalonyl-CoA decarboxylase, malonate decarboxylase, and glutaconyl-CoA decarboxylase from various anaerobic bacteria. These enzymes use the free energy of decarboxylation to pump Na^+ ions across the membrane. The generated $\Delta\tilde{\mu}Na^+$ can be used to energize the accumulation of nutrients or the synthesis of ATP (for reviews, see refs *1*−*4*).

The oxaloacetate decarboxylase of *Klebsiella pneumoniae* has been studied particularly well. This enzyme is composed of three different subunits (α, β, and γ), with molecular masses of 63.5, 44.9, and 8.9 kDa, respectively (*1*). A cartoon summarizing the structure and function of this enzyme is shown in Figure 1. The peripheral α subunit consists of the N-terminal carboxyltransferase domain and the C-terminal biotin domain, which are connected by a flexible proline/alanine-rich linker peptide (*5*). The β subunit (OadB) is a highly hydrophobic integral membrane protein with nine transmembrane helices (*6*). It catalyses the decarboxylation of carboxybiotin, which has been formed by the carboxyltransfer reaction on the α subunit. The decarboxylation is coupled to the transport of two Na^+ ions into the periplasm.

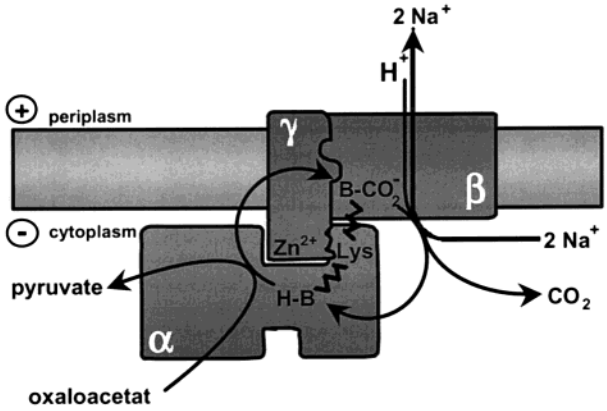

FIGURE 1: Cartoon showing the overall geometry of the oxaloacetate decarboxylase and features of the catalytic events: B−H, biotin; B−CO_2^-, carboxybiotin; Lys, biotin-binding lysine residue. The oxaloacetate decarboxylase complex is formed by an interaction of the γ subunit with the β subunit and with the C-terminal biotin-binding domain of the α subunit (αC). The N-terminal domain of the α subunit (αN) which harbors the carboxyltransferase catalytic site is kept in the assembly via a proline/alanine linker peptide bound to αC. A catalytic cycle includes the transfer of the carboxyl group of oxaloacetate to enzyme-bound biotin catalyzed by αN. As this step is accelerated by the Zn^{2+}-containing γ subunit, the Zn^{2+} must be near the catalytic site. The carboxybiotin residue switches to the decarboxylase site on the β subunit where it is decarboxylated, consuming a periplasmatically derived proton and pumping two Na^+ ions from the cytoplasm to the periplasm.

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* To whom correspondence should be addressed. Phone: 0041 1 632 33 21. Fax: 0041 1 632 13 78/11 48. E-mail: dimroth@micro.biol.ethz.ch.

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Table 1: Primers Used for Mutagenesis

primer	sequence (5′−3′)	restriction sites/comments
D62A*for*	CCGTCGCGCCCGCCGCGGATTTCGCCCGCC	mutation underlined
D62A*rev*	GGCGGGCGAAATCCGCGGCGGGCGCGACGG	mutation underlined
D63A*for*	CGCGCCCGCCGACGCGTTCGCCCGCCTGAAGCC	mutation underlined
D63A*rev*	GGCTTCAGGCGGGCGAACGCGTCGGCGGGCGCG	mutation underlined
H76A*for*	GTCGCCGCCATTGCGCACCATCGCCGTCTTCACC	mutation underlined
H76A*rev*	CGGCGATGGTGCGCAATGGCGGCGACAATCG	mutation underlined
H77A*for*	CGCCGCCATTCACGCGCATCGCCGTCTTCACCC	mutation underlined
H77A*rev*	GGGTGAAGACGGCGATGCGCGTGAATGGCGGCG	mutation underlined
H78A*for*	CGCCGCCATTCACCACGCGCGCCGTCTTCACCC	mutation underlined
H78A*rev*	GGGTGAAGACGGCGCGCGTGGTGAATGGCGGCG	mutation underlined
oadAC*for*	CG**GAATTCCATATG**GCAGAAAAACCTGCCGCC	*Eco*RI site in bold *Nde*I site in bold and underlined
oadAC*rev*	CG**GGATCCAAGCTT**ACGCCAGGGTCATCAG	*Bam*HI site in bold *Hind*III site in bold and underlined
oadAN*for*	CG**GAATTCCATATG**ACCGTTGCCATTACCG	*Eco*RI site in bold *Nde*I site in bold andunderlined
oadAN*rev*	CG**GGATCCAAGCTT**AGGCGGCAGGTTTTTCTGC	*Bam*HI site in bold *Hind*III site in bold and underlined
oadGC*for*	GC**GATATC**CGGGGAATGTCCCTC	*Eco*RV site in bold
oadGC*rev*	CGGG**AAGCTT**AAGGGTGAAGACGGCGA	*Hind*III site in bold
prgMut+	GGAAACAGCTATGACCATGATTAC	
prgMut-	CGTACCCTGGGCGTGGGCGC	
gUENde	GTCGCGG**CATATG**ACTGACAATGCCGTCCTCTGTTA	*Nde*I site in bold
T7	TAATACGACTCACTATAGGG	
oadBamHI-	CG**GGATCC**ATTAAGGGTGAAGACGGC	*Bam*HI site in bold

One periplasmic proton which crosses the membrane in the opposite direction is consumed at the cytoplasmic side in the decarboxylation event (*7*). On the basis of extensive mutational studies, a coupling mechanism of this pump was envisaged in which the membrane integral residues D203, Y229, and S382 perform a prominent role in the vectorial processes of Na^+ and H^+ translocation (*3*, *8*, *9*).

The role of the γ subunit has been less well-defined in the past. It is anchored in the membrane with a single N-terminal α helix. This is followed by a proline/alanine linker at the cytoplasmic surface and a short hydrophilic domain with a prominent motif of four histidines near the C terminus (*10*). Evidence indicates that the β and γ subunits form a subcomplex to which the α subunit is attached (*11*−*13*), but which proteins/domains are interacting to form the three subunit complex was unknown. We show here that the γ subunit is of essence for the formation of a stable oxaloacetate decarboxylase complex interacting specifically with the biotin-binding C-terminal domain of the α subunit.

Another interesting feature of the γ subunit is the presence of a tightly bound Zn^{2+} metal ion (*13*, *14*) which was proposed to play an essential role as a Lewis acid in polarizing the carbonyl oxygen bond of oxaloacetate, thereby accelerating the carboxyl-transfer reaction. In accord with this notion is the observation that the velocity of the carboxyl-transfer reaction as catalyzed by the α subunit is increased by at least 3 orders of magnitude in the additional presence of the Zn^{2+}-containing γ subunit (*13*). Furthermore, decarboxylases acting on substrates such as methylmalonyl-CoA or glutaconyl-CoA which do not contain a carbonyl oxygen were not reported to contain Zn^{2+} (*15*). Interestingly, the γ subunits from several oxaloacetate decarboxylases contained a polyhistidine motif near the C terminus, which was missing in the methylmalonyl-CoA or glutaconyl-CoA decarboxylases. These histidine residues or part of them could, therefore, provide the ligands for Zn^{2+} binding to the oxaloacetate decarboxylase γ subunit.

To investigate this possibility, multiple mutants were generated within the C-terminal region of the γ subunit which contains the polyhistidine motif. The Zn^{2+} content of the oxaloacetate decarboxylase was drastically reduced in the mutants γD62A, γH77A, and the C-terminal deletion of γH82 and γP83, identifying γD62A, γH77A, and γH82 as Zn^{2+} binding ligands. The prominent role of the Zn^{2+} ion in catalysis was confirmed by the decrease of the catalytic rate of the enzyme in parallel to the decrease of its Zn^{2+} content.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids. The bacterial strains used in this study are *Escherichia coli* DH5α (Bethesda Research Laboratories, MD), *E. coli* BL21(DE3) (Novagen, Darmstadt, Germany), and *E. coli* C43(DE3) (*16*). All strains transformed with pBluescript derivatives were routinely grown at 37 °C in Luria Bertani (LB)[1] medium (*17*). For the synthesis of the C-terminal domain of OadA, LB medium was supplemented with 10 μM biotin (final concentration). Strains transformed with pET or pMal-c derivatives were inoculated with 1% of an overnight culture grown under aerobic conditions at 37 °C until OD600 of 0.5−0.7 before induction with 0.5 mM IPTG (final concentration). Cells were grown for another 4−7 h at 30 °C before harvest. Plasmid-containing strains were supplemented with the selective antibiotics ampicillin (100 μg/mL) or kanamycin (50 μg/mL).

Recombinant DNA Techniques. Standard recombinant DNA techniques were performed essentially as described by Sambrook et al. (*17*). Oligonucleotides used for mutagenesis were custom-synthesized by Microsynth (Balgach, Switzerland). All inserts derived from polymerase chain reaction (PCR) and ligation sites were checked by DNA sequencing according to the dideoxynucleotide chain-termination method (*18*) using a *Taq* dye-dideoxy terminator cycle sequencing kit and the ABI PRISM 310 genetic analyzer from Applied Biosystems (Foster City, CA).

[1] Abbrevations: IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria Bertani; LDAO, lauryldimethylamine oxide; MBP, maltose binding protein; NTA, nitrilotriacetic acid; OAD, oxaloacetate decarboxylase; OadA, oxaloacetate decarboxylase α subunit; OadB, oxaloacetate decarboxylase β subunit; OadG, oxaloacetate decarboxylase γ subunit; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)-aminomethane.

Construction of Deletion Mutants and Site-Directed Mutagenesis of the γ Subunit. Primers used for site-directed mutagenesis are listed in Table 1. For convenient cloning of the deletion mutants of the γ subunit, the *EcoRI* restriction site located in the multiple cloning site of pSKGAB (*13*) was removed as follows: pSKGAB was digested with *ClaI*, and the resulting 6822 bp vector-containing fragment was isolated and religated to obtain the desired plasmid pSKGABΔEcoRI. For the construction of deletion mutants γΔH76, γΔH78, or γΔH82, the respective coding region of OadG was amplified from plasmid pSKGAB using primers pskNot1+ and pskgΔH76, pskgΔH78, or pskgΔH82, respectively. PCR fragments were digested with *NotI* and *EcoRI* and cloned into pSKGABΔEcoRI treated with the same enzymes, yielding the plasmids pSKGABΔH76−P83, pSKGABΔH78−P83, and pSKGABΔH82P83.

Site-directed mutants of the γ subunit were obtained as follows: the PCR fragments containing the mutations were constructed in a two-step protocol. For the 5′ part of the PCR fragments of the γ subunit, primer prgMut+ and primers with the affix *rev* were used. For the corresponding 3′ part of the PCR, primer prgMut- and primers with the affix *for* were used. In both reactions, pSKGAB (*13*) served as a template. The purified mutagenic PCR fragments were used as a template for the subsequent PCR reaction with primers prgMut+ and prgMut-. The resulting PCR products were digested with *Spe*I and *Rsr*II and cloned into pSKGAB digested wih the same restriction enzymes. To construct the γH77A site-directed mutant, a new plasmid (pET derivative) encoding the whole oxaloacetate decarboxylase was created. For this, the coding region of OadG was amplified using the plasmid pSKGAB as a template and primers gUENde (introduces a *Nde*I restriction site at the initiation codon (CATATG)) and prgMut-. The resulting PCR product was digested with *Nde*I and *Eco*RI and cloned into the expression vector pET24b digested with the same enzymes, yielding the plasmid pEToadG. The plasmid pSKGAB was digested with *Bst*1107I, treated with T4 DNA polymerase, and digested with *Eco*RI. The 3290 base pair long insert was ligated into pEToadG (digested with *Eco*RI and *Bpu*1102I), to yield plasmid pETGAB. This plasmid was subsequently used as a template for the two-step PCR protocol as described previously. For the 5′ part of the PCR fragment, the T7 primer and primer H77A*rev* were used, and for the corresponding 3′ part of the PCR product, primer prgMut- and primer H77A*for* were used. The purified mutagenic PCR fragments were used as a template for the subsequent PCR reaction using primers T7 and prgMut-. PCR products were digested with *Nde*I and *Rsr*II and cloned into *Nde*I/*Rsr*II-digested pETGAB.

Construction of Single Domain Expression Plasmids. To obtain a plasmid coding for the γ subunit with an N-terminal His_{10} tag, the coding region of OadG was amplified from plasmid pETGAB using the T7 primer and primer poadBam- that introduced a new *Bam*HI restriction site downstream of the stop codon. The obtained PCR fragment was digested with *Nde*I/*Bam*HI and ligated into the cloning vector pET16b treated with the same enzymes, yielding plasmid pET16bγ. For the construction of a plasmid coding only for the cytoplasmic domain of OadG fused to maltose binding protein (MBP), the coding region of the cytoplasmic part of OadG (R34 to P83) was amplified from plasmid pETGAB using primers OadGC*for* introducing an *Eco*RV restriction site and OadGC*rev* introducing a *Hin*dIII site. After digestion of the PCR product with *Eco*RV and *Hin*dIII, this DNA fragment was introduced into *Stu*I/*Hin*dIII-digested vector pMal-c to yield plasmid pMalcγC.

Construction of Expression Plasmids Encoding the N-Terminal Transferase Domain or C-Terminal Biotin Carrier Domain of the α Subunit (OadA). To obtain expression plasmids encoding the N-terminal or the C-terminal domain of OadA, we amplified the respective regions using primers that introduced an *Eco*RI and a *Nde*I restriction site comprising the initiation codon and a *Hind*III and *Bam*HI site downstream of the stop codon. The region encoding the C-terminal domain was amplified using primers oadACfor and oadACrev. This PCR product was digested with *Nde*I/*Bam*HI and cloned into the expression vectors pET24b and pET16b to yield the plasmids pET24bαC and pET16bαC or digested with *Eco*RI/*Hind*III and cloned into the vector pMal-c yielding pMalcαC. The same strategy was used to clone the DNA fragment encoding the N-terminal domain of the α subunit, using primers oadANfor and oadANrev resulting in plasmids pET16bαN, pET24bαN, and pMalcαN.

Purification of Oxaloacetate Decarboxylase Mutants and Enzyme Assays. Oxaloacetate decarboxylase and mutant derivatives were purified by affinity chromatography of solubilized membrane extract on a SoftLink monomeric avidin-sepharose column (Promega, Madison, WI) (*19*). Large-scale purification was performed according to Di Berardino and Dimroth (*7*). The decarboxylation activity was determined with the simple spectrophotometric assay at 265 nm as described (*19*).

Purification of His_{10}-γ by Ni^{2+}-NTA Chromatography. His_{10}-γ was synthesized by expression of plasmid pET16bγ in *E. coli* C43(DE3). The cell extract was centrifuged at 200 000*g* for 1.5 h, and the membrane pellet was resuspended in 3 mL of a wash buffer (20 mM Tris HCl, 500 mM NaCl, and 0.05% LDAO (pH 7.9)). The recombinant protein was solubilized from the membrane with 2% LDAO (final concentration) and stirred for 15 min at 25 °C. After centrifugation (30 min at 70 000*g*), the supernatant was diluted 10-fold with a wash buffer and applied twice onto a column of Ni^{2+}-NTA agarose (2 mL) (Qiagen), preequilibrated with 10 mL of wash buffer. The column was washed with 10 volumes wash buffer each containing 5, 60, and 100 mM imidazole. His_{10}-γ was then eluted with 10 mL wash buffer containing 400 mM imidazole.

Purification of the N-Terminal Domain of OadA by Ni^{2+}-NTA Chromatography. The N-terminal domain of the α subunit was synthesized by expression of plasmid pET16bαN in *E. coli* BL21(DE3). The cytoplasm was applied twice onto a column containing 2 mL of bed volume of Ni^{2+}-NTA agarose (Qiagen, Basel, Switzerland) pre-equilibrated with 10 mL of a wash buffer (20 mM Tris HCl and 500 mM NaCl (pH 7.9)). The column was washed with 30 mL of a wash buffer containing 5 mM imidazole and subsequently with a wash buffer containing 60 mM imidazole. Pure protein was eluted with 10 mL of a wash buffer containing 150 mM imidazole.

Purification of the C-Terminal Domain of OadA. The C-terminal domain of the α subunit was synthesized by expression of plasmid pET24bαC in *E. coli* BL21(DE3). The protein was purified from the cytoplasm by affinity chro-

matography on a SoftLink monomeric avidin-sepharose column (Promega) (*19*) equilibrated with buffer A (20 mM Tris HCl and 50 mM KCl (pH 8.0)). After application of the cytoplasm onto the column and being washed with 6 volumes of buffer A, pure protein was eluted with 2 volumes of buffer A containing 5 mM biotin.

Purification of Domains Fused to Maltose Binding Protein (MBP). Cytoplasm containing MBP fusion proteins was loaded onto a 5 mL amylose column, pre-equilibrated with a TK buffer (50 mM Tris HCl and 200 mM KCl (pH 7.5)) The column was washed with 20 mL of TK buffer, and pure protein was eluted with 10 mL of TK buffer containing 10 mM maltose.

Determination of Zn^{2+} Content in Oxaloacetate Decarboxylase Mutants. The Zn^{2+} content of different mutants in the γ subunit of oxaloacetate decarboxylase was measured as described (*7*). As we purified all of the mutants with Chelex water, there was no need to dialyze the samples prior to the measurements. The buffer alone contained <0.05 μM Zn^{2+}.

Labeling of the C-Terminal Domain of OadA with $^{14}CO_2$ from [4-^{14}C]Oxaloacetate. [4-^{14}C]oxaloacetate, prepared from [4-^{14}C]L-aspartate and α-ketoglutarate with glutamate/oxaloacetate transaminase was used to measure the transfer of the radioactive carboxyl residue to biotin bound to the C-terminal domain of OadA (*13*). The radiolabeled protein was separated from excess substrates by Sephadex G-25 column chromatography in a 100 mM Tris HCl buffer (pH 8.0). Fractions of 400 μL were collected, and the radioactivity was determined by liquid scintillation counting.

Analytical Procedures. The protein content of samples was determined according to Bradford (*20*) or using the BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin as a protein standard.

RESULTS

Assembly of the Oxaloacetate Decarboxylase Complex. Recent reports (*7*) indicated that a subcomplex of oxaloacetate decarboxylase consisting of the biotin-containing α subunit and the γ subunit was sufficiently stable for its isolation by avidin-sepharose affinity chromatography. To keep this intersubunit assembly together, the γ subunit could be bound to the N-terminal carboxyltransferase domain or to the C-terminal biotin domain of the α subunit. To discriminate between these possibilities, the N-terminal and the C-terminal domains of the α subunit were separately synthesized in *E. coli* as maltose binding protein fusions (αN-MBP and αC-MBP, respectively). Cells from either clone were mixed with *E. coli* cells synthesizing the γ subunit, and the MBP-tagged polypeptides were purified by amylose affinity chromatography. The results of Figure 2 show that the γ subunit was copurified with αC-MBP but not with αN-MBP, which indicates that the binding of the γ subunit to the α subunit occurs via its C-terminal biotin domain. These results were confirmed by complementary copurification experiments with alternate affinity tags; using Ni^{2+}-NTA chromatography, His_{10}-γ was copurified with αC but not with αN (both without tags), and using avidin-sepharose chromatography, the biotin-containing αC domain was purified as a complex with the γ subunit. In contrast, the interaction between αC and αN was too weak to isolate a complex of these two domains by avidin-sepharose chromatography.

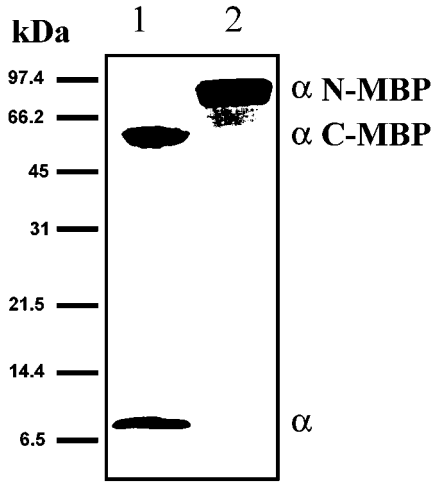

FIGURE 2: Specific binding of the γ subunit to the C-terminal domain of the α subunit of oxaloacetate decarboxylase. Recombinant *E. coli* cells synthesizing the γ subunit were grown and mixed with *E. coli* cells synthesising the C-terminal domain of the α subunit fused to maltose binding protein (αC-MBP, lane 1) or with cells synthesizing the N-terminal domain of the α subunit (αN-MBP, lane 2). Membranes were isolated from these cell mixtures and solubilized with Triton X-100. The maltose binding protein conjugates were subsequently affinity purified on amylose columns and subjected to SDS−PAGE. The gel was stained with silver.

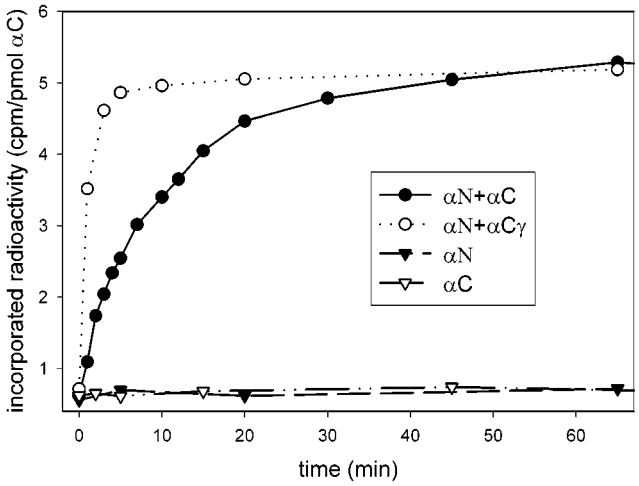

FIGURE 3: Kinetics of the carboxyl-transfer reaction with [4-^{14}C]-oxaloacetate and the C-terminal biotin domain of the oxaloacetate decarboxylase α subunit (αC) (7.3 μg) as substrates as catalyzed by 1.1 μg of the N-terminal domain of the α subunit (αN) (●). The kinetics were accelerated if the biotin-domain substrate was replaced by a biotin-domain−γ complex (αCγ) (12.6 μg), which was isolated by avidin-sepharose chromatography (○). Also shown are controls without the biotin-domain of the α subunit (▼) or without the N-terminal carboxyltransferase domain (▽).

To test whether the two isolated domains were still catalytically active, we measured the carboxyl transfer from [4-^{14}C]oxaloacetate to the biotin prosthetic group present in αC. The results of Figure 3 show that the protein-bound radioactivity increased with time to reach a final level after about 40 min if both α-subunit domains were present, whereas no incorporation of radioactivity was found with either domain alone. If in the carboxyl-transfer assay αC was replaced by an equivalent amount of the purified αC-γ subcomplex, the initial rate was accelerated more than 6-fold. Labeling was complete after less than two minutes in this case. The same final level of labeling was obtained with αC

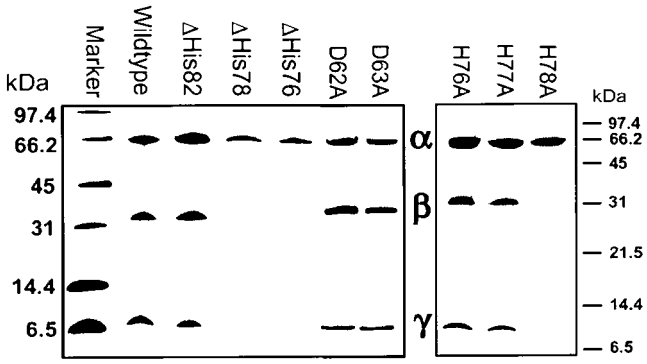

FIGURE 4: Stability of oxaloacetate decarboxylase complexes with mutations in OadG. Oxaloacetate decarboxylases with the mutations in OadG indicated were synthesized in *E. coli* and purified by avidin-sepharose chromatography. The proteins specifically eluted with biotin were analyzed by SDS−PAGE. Polyacrylamide gels (10%) were used in the left box, and polyacrylamide gels (13%) were used in the right box. Also shown are the wild-type enzyme and the mobilities of marker proteins. The gels were stained with silver.

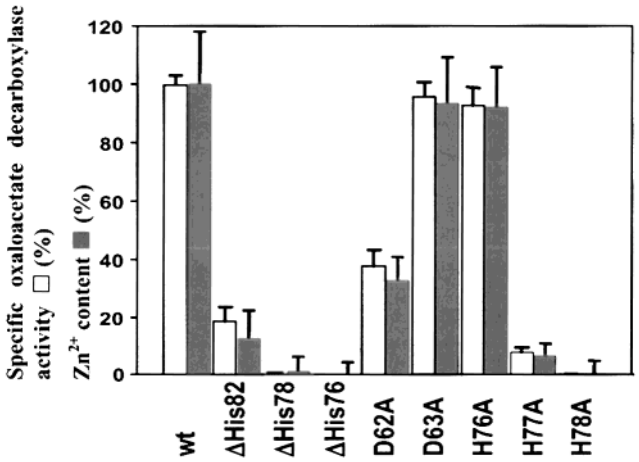

FIGURE 5: Correlation between specific oxaloacetate decarboxylase activities (open bars) and Zn^{2+} content (filled bars) in the wild-type enzyme (wt) and various mutants in OadG (indicated at the bottom line). The mutant enzymes were synthesized in *E. coli* and purified to apparent homogeneity by avidin-sepharose chromatography. The data recorded for the mutants represent percentages of specific oxaloacetate decarboxylase activities and Zn^{2+} content with respect to the wild-type (100%). The wild-type enzyme contains 1 mol of Zn^{2+}/mol of enzyme and has a specific activity of ~26 units/mg of protein. Each bar represents the mean ± S.D. of at least nine measurements.

or αC-γ, as expected from the equivalent amounts supplied in both cases. These results indicate that the isolated αN domain is a catalytically active carboxyltransferase. The isolated αC domain acts as an acceptor for the carboxyl groups stemming from oxaloacetate and the γ subunit, which forms a strong complex with αC accelerates this reaction significantly. We observed in other experiments that this rate acceleration did not require the entire γ subunit but only the soluble C-terminal domain. The N-terminal hydrophobic domain of the γ-subunit, therefore, anchors the γ-subunit in the membrane but has no catalytic function.

The role of the C-terminal portion of the γ subunit for the assembly of a stable oxaloacetate decarboxylase complex was investigated by deletion mutagenesis. Oxaloacetate decarboxylases with C-terminal deletions of the γ subunit of 2 (γΔH82), 6 (γΔH78), or 8 (γΔH76) residues were synthesized in *E. coli* and purified by avidin-sepharose chromatography as usual. The α, β, and the trimmed γ subunits were synthesized in all three cases, but a stable oxaloacetate decarboxylase complex could only be isolated from the γ-subunit mutant with two deleted amino acids (Figure 4). The γ subunits with deletions of 6 or 8 amino acids were, on the other hand, unable to associate properly to a complex with the α subunit; therefore, this biotin-containing protein was isolated alone on the avidin-sepharose column. These results indicate that the four amino acids at position 78−81 of the γ subunit play a significant role in complex formation with the α subunit. These results were corroborated by point mutations; stable complexes were isolated after mutating γH76 or γH77 to A, but after mutating γH78 to A, the complex was unstable, and subunit α was purified alone on the affinity column. Hence, γH78 appears to be crucial for the formation of a stable oxaloacetate decarboxylase complex. This is in remarkable agreement with the previous hypothesis that protonation or deprotonation of a histidine probably promotes the dissociation or assembly of the complex (*12*).

Identification of Putative Zinc-Binding Residues on the Oxalacetate Decarboxylase γ Subunit. The results described here and elsewhere (*13*) clearly show a dual role of the γ subunit (i.e., in the assembly of the oxaloacetate decarboxylase complex and in accelerating the carboxyltransfer reaction). The catalytic function of γ has been attributed to the presence of a tightly bound Zn^{2+} metal ion that cannot be removed with EDTA or 1,10-phenanthroline (data not shown). This Zn^{2+} is assumed to act as a Lewis acid to polarize the carbonyl oxygen bond of oxaloacetate (*3*). A literature search of Zn^{2+}-containing enzymes with known structures revealed that cysteine, histidine, aspartate, or glutamate are most frequently involved in the coordination of a single, catalytic Zn^{2+} ion (*21*−*23*). On the basis of this knowledge, the C-terminal water-soluble part of the γ subunit contains six potential candidates for Zn^{2+} coordination: D62, D63, H76, H77, H78, and H82. To investigate the role of these residues as ligands for Zn^{2+} binding, we substituted each of these residues with alanine using site-directed mutagenesis. Mutant decarboxylases were synthesized in *E. coli* and purified by avidin-sepharose chromatography. The results of Figure 4 show that stable three-subunit complexes were obtained for the mutants γD62A, γD63A, γH76A, γH77A, and the γΔHis82 deletion mutant, where P83 and H82 were deleted from the C terminus.

The results of Figure 5 show the specific oxaloacetate decarboxylase activities and the Zn^{2+} contents of the purified mutant enzymes. Similar values as for the wild-type enzyme were obtained for the γD63A and γH76A muants, indicating that these two residues are not involved in Zn^{2+} binding. In contrast, dramatic losses of oxaloacetate decarboxylase activities to 5−35% of the wild-type enzyme were observed for the γD62A, γH77A, and the C-terminal deletion mutant γΔHis82. The results of Figure 5 also show a very good quantitative correlation between the activity drop and the loss of Zn^{2+} binding to the enzyme. As from the γH78A mutant and from the deletion mutants γΔHis78 and γΔHis76, only the α subunit was isolated on the affinity column (Figure 4); neither oxaloacetate decarboxylase activity nor Zn^{2+} was found in these samples (Figure 5). In summary, these results indicate that, for the binding of Zn^{2+}, the γ-subunit residues

D62, H77, and H82 are required and that the bound Zn^{2+} ion is essential for the oxaloacetate decarboxylase activity.

DISCUSSION

The Na^+ transport decarboxylase family of enzymes shares a number of common features with respect to structure and mechanism. These are multisubunit membrane-bound complexes usually composed of a water-soluble carboxyltransferase of about 60 kDa (α), a membrane-intrinsic carboxybiotin decarboxylase of about 40 kDa (*β*), an additional membrane-bound subunit of about 10−15 kDa (*γ*), and a water-soluble biotin carboxyl carrier protein subunit of about 10−20 kDa (*δ*). Oxaloacetate decarboxylase of *K. pneumoniae* consists of three subunits only because the carboxyltransferase and the biotin carboxyl carrier protein subunits are fused via a proline/alanine-rich linker peptide (*1*−*4*).

There are clear phylogenetic relationships between carboxyltransferase subunits/domains of decarboxylases and other biotin enzymes acting on the same substrate (e.g., the N-terminal domain of the α subunit of oxaloacetate decarboxylase and the 5S subunit of transcarboxylase from *Propionibacterium shermanii* (*24*)). Profound sequence identities are also found among all membrane-bound *β* subunits of the various Na^+ translocating decarboxylases (*4*), and the biotin-carrier proteins of decarboxylases are related with those of other biotin enzymes (*5*). From sequence alone, a similar relationship is not apparent among the *γ* subunits from the various decarboxylases, but all of them share a similar domain structure (*1*−*4*). This consists of a very hydrophobic N-terminal domain which probably folds into an α helix by which the protein is anchored within the membrane. It follows a short sequence motif with a flexible proline/alanine linker and a more extended hydrophilic C-terminal portion. On the basis of this domain structure, the *γ* subunit appears to be well-suited as a mediator between hydrophilic and hydrophobic subunits, thus keeping the multisubunit assemblies together. The α and *β* subunits were, in fact, unable to stick to each other, whereas α*γ* and *βγ* subcomplexes have been described (*12*, *13*, *25*). This role of the *γ* subunit in the assembly of the complex is supported by features of the Na^+ translocating malonate decarboxylase of *Malonomonas rubra* (*26*). Unlike the other Na^+ translocating decarboxylases, this enzyme does not form a stable multisubunit complex. This can be attributed to the absence of a *γ*-subunit-like protein because a *γ*-subunit encoding gene has not been found within the malonate decarboxylase gene cluster (*27*).

In this paper, we show that the oxaloacetate decarboxylase *γ* subunit forms a strong complex with the C-terminal biotin-binding domain of the α subunit but not with its N-terminal counterpart. There is also clear evidence from deletion mutagenesis that the C-terminal portion of the *γ* subunit is important for the complex formation with the αC domain and, on the basis of the *γ*H78A point mutation which produces an assembly negative phenotype, one may conclude that *γ*H78 is a crucial residue for the assembly with αC. These results may be related to earlier dissociation and reconstitution experiments performed with oxaloacetate decarboxylase. A sharp decrease in dissociation and increase in reconstitution above pH 6.5 was described "indicating that a single ionizable group with a p*K* of about 6.5 (probably provided by a histidine residue) is involved in these processes" (*12*).

Besides its central role in the assembly of the oxaloacetate decarboxylase complex, the *γ* subunit also plays a profound role in the catalysis of the carboxyltransferase reaction in which its tightly bound Zn^{2+} metal ion is directly involved. Hence, localization of this metal site would provide hints as to which part of the *γ* subunit interacts with the carboxyltransferase catalytic site. In most zinc metalloenzymes whose structures are solved, the binding geometry is slightly distorted tetrahedral with the metal ion coordinated by three or four protein side chains (*23*). In catalytic sites, the zinc ion is usually exposed and bound to a water or substrate molecule, and over 70% of the metal ligands are His, >10% are Glu, >5% are Asp, and <5% are Cys (*22*). We reasoned that the C-terminal part of the *γ* subunit with its four histidine residues could contribute the Zn^+ binding ligands and, therefore, subjected this portion of the molecule to site-specific and deletion mutagenesis. The results of these analyses indicated severe reductions of the Zn^{2+} content in parallel with reductions of specific oxaloacetate decarboxylase activities if *γ*H82, *γ*H77, or *γ*D62 were mutated, indicating that these residues are probably Zn^{2+} binding ligands. The fourth zinc ligand of the oxaloacetate decarboxylase may be a water molecule, as found in 92% of all Zn^{2+}-containing catalytic sites from which structural data have been obtained (*22*).

A common feature of catalytic zinc sites is the specific arrangement of the ligands. Usually, a short spacer of only a few amino acids connects the first two ligands providing a zinc-binding nucleus. The third ligand, separated from the second ligand by a spacer of ∼20 to ∼120 amino acids, completes the coordination sphere. This long polypeptide loop further aligns protein residues around the zinc-binding site and, therefore, affects interactions with the substrate (*21*). This characteristic alignment of the ligands is present in the oxaloacetate decarboxylase *γ* subunit as well. The first ligand His82 is four amino acids apart from the second ligand His77, and the third ligand, Asp62, is separated from the second ligand His77 by 14 amino acids. Replacement of either of the two histidine residues affects the zinc-binding site to a greater extend than the replacement of Asp62, indicating that the histidine residues 77 and 82 forming the zinc-binding nucleus are more essential for the binding of the metal ion than the more distantly located Asp62 ligand.

In zinc proteases (*28*) and carbonic anhydrases (*29*), zinc ions serve as a powerful electrophilic catalyst by providing (i) an activated water molecule for nucleophilic attack, (ii) polarization of the carbonyl of the scissile bond, and (iii) stabilization of the negative charge in the transition state (*23*). In oxaloacetate decarboxylase, the Zn^{2+} ion is specifically required for the carboxyl transfer from oxaloacetate to protein-bound biotin. It appears that, in this case, the enhancement of the reaction rate by Zn^{2+} results from the third possibility (i.e., stabilization of the negative charge in the transition state). A model highlighting the role of Zn^{2+} in this catalysis is shown in Figure 6. It is anticipated that three regions of the protein contribute to the catalytic site for the carboxyl-transfer reaction: the C-terminal Zn^{2+}-binding region of the *γ* subunit, the biotin residue on the C-terminal domain of the α subunit, and amino acid residues on the N-terminal domain of the α subunit involved in

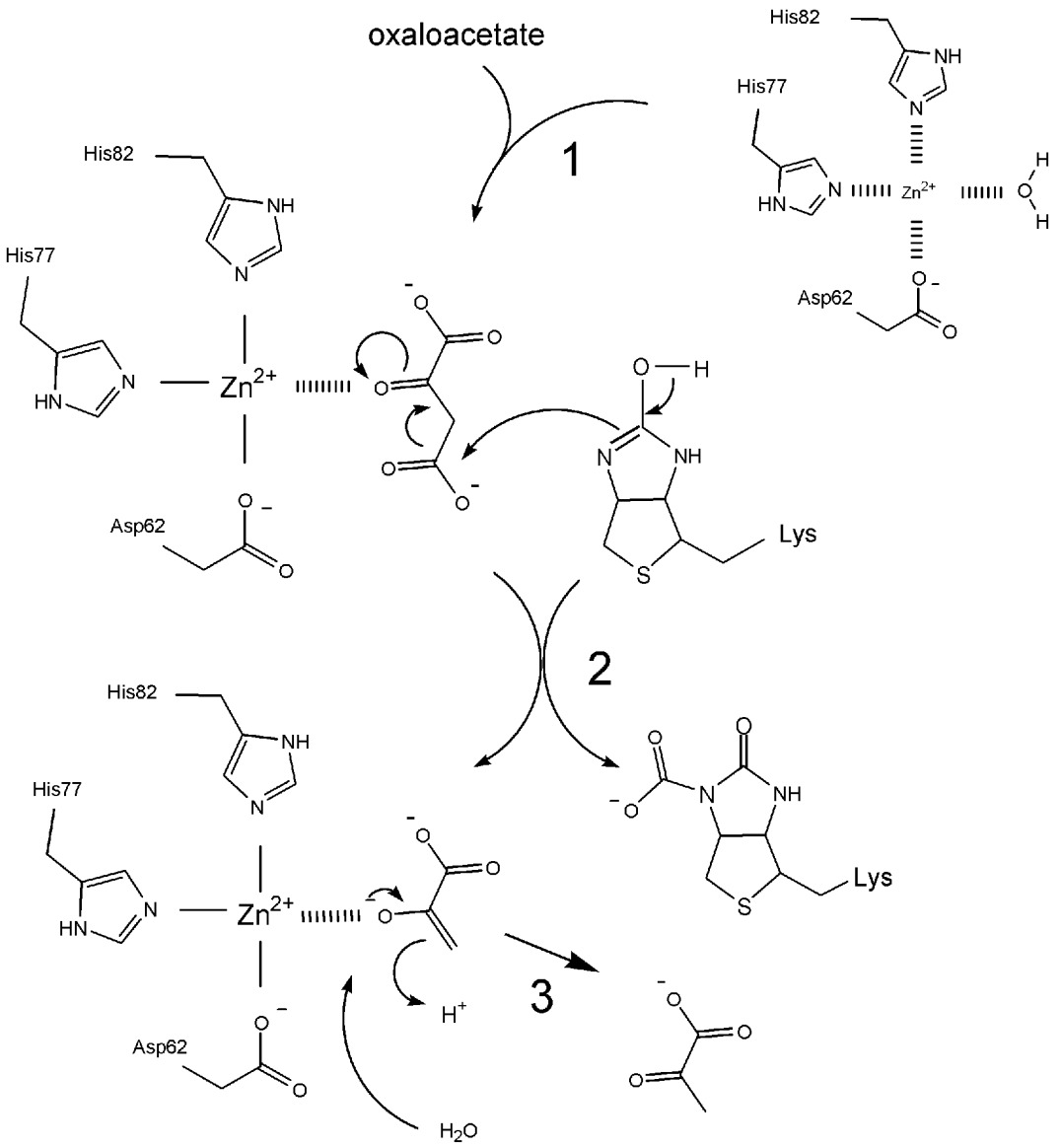

FIGURE 6: Model of the participation of the Zn^{2+} site in the carboxyltransferase mechanism. The Zn^{2+} metal ion is bound to the γ subunit, with Asp62, His77, His82, and a H_2O molecule as ligands. In the first step (1), oxaloacetate with its carbonyl oxygen replaces the water ligand. As a Lewis acid, the Zn^{2+} withdraws electrons from the carbonyl oxygen bond, which facilitates the carboxyl transfer from position 4 of oxaloacetate to biotin (2). The resulting enolpyruvate intermediate picks up a proton, and pyruvate is released (3). Simultaneously, the fourth coordination site of the Zn^{2+} is filled again with a water molecule. Note that the carboxy-transfer reaction additionally requires the N-terminal domain of the γ subunit.

oxaloacetate binding. As a strong binary complex is formed only between the γ subunit and the C-terminal domain of the α subunit, the ternary complex which includes the N-terminal domain of the α subunit may exist only transiently during catalysis. We assume that oxaloacetate is bound to this site with its carbonyl oxygen atom contacting the Zn^{2+} metal ion, which probably involves the replacement of H_2O as the fourth ligand from the Zn^{2+} ion. Once bound in this way, electrons are withdrawn from the carbonyl oxygen bond of oxaloacetate leading to the decarboxylation of this β-ketoacid. The CO_2 reacts immediately with the biotin, liberating a proton while the enolate form of pyruvate is transiently stabilized by the Zn^{2+} until a proton adds and transforms it into pyruvate. This is subsequently released from the Zn^{2+} site by replacement with H_2O. Our proposal for the role of the tightly bound Zn^{2+} ion in polarizing the carbonyl oxygen bond of oxaloacetate rests primarily on the following observations: (i) oxaloacetate decarboxylase is the only Zn^{2+}-containing Na^+-translocating decarboxylase known (*1*), (ii) the Zn^{2+} is specifically required for the carboxyltransfer reaction, and (iii) carboxyltransferases of other biotin enzymes with oxaloacetate as substrate or product, such as the 5S subunit of transcarboxylase or pyruvate carboxylase, are phylogenetically related to the carboxyltransferase domain of oxaloacetate decarboxylase and are Zn^{2+}-containing (*30*) or other divalent metal-ion-containing enzymes (*31*, *32*) as well, whereas carboxyltransferases with thioester carboxylates are not. Instead of a metal ion, these carboxyltransferases may use two peptidic NH groups to stabilize the oxoanion intermediate, as was proposed recently for glutaconyl-CoA decarboxylase (*4*). Zn^{2+} is apparently not required for polarizing the carbonyl oxygen bond of biotin in the carboxyltransferase reaction because many of these enzymes operate without Zn^{2+} or another metal ion. Instead, amino acids have been reported to stabilize the enolate anion of biotin (*33*, *34*).

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