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Cellular localisation by immunolabelling and transmission electron microscopy of oxaloacetate decarboxylase or its individual subunits synthesised in *Escherichia coli*

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Abstract

The genes *oadGAB* encoding the oxaloacetate decarboxylase γ, α and β-subunits from *Klebsiella pneumoniae* were expressed in *Escherichia coli*. Using different expression vectors, the entire enzyme or its individual subunits were synthesised. The expression was evidenced immunologically in whole cells with polyclonal antibodies raised against the purified oxaloacetate decarboxylase. The expressed α-subunit or a combination of α and β-subunits were shown to reside in the cytoplasm, while the entire oxaloacetate decarboxylase or a $\gamma\alpha$-complex were located mostly in the cytoplasmic membrane. Interestingly, overexpression of the $\gamma\alpha$-complex or the entire oxaloacetate decarboxylase in *E. coli* led to a significant immunogold labelling in the cytoplasm, indicating that the α-subunit was not completely complexed to the membrane-bound γ or $\beta\gamma$-subunits.

Keywords: *Klebsiella pneumoniae*; Oxaloacetate decarboxylase; Sodium pump; Expression of membrane proteins; Immunogold labelling

1. Introduction

The Gram-negative, facultatively anaerobic enterobacterium *Klebsiella pneumoniae* synthesises oxaloacetate decarboxylase during its anaerobic growth on citrate as sole carbon source. This enzyme plays an essential role in the anaerobic citrate fermentation pathway since it takes advantage of the exergonic decarboxylation reaction to maintain an electrochemical Na^+ potential across the membrane. This potential is exploited by the Na^+-dependent citrate carrier (CitS) to take up citrate in a co-transport mechanism [1,2] as well as by the Na^+ translocating NADH:ubiquinone oxidoreductase, which utilises this energy for the formation of reducing equivalents needed for the synthesis of cell materials [3]. Oxaloacetate decarboxylase consists of three different subunits, α, β and γ with M_r 63 600, 44 900 and 8900, respectively [4–6]. The biotin-containing α-subunit is a water-soluble protein which is associated with the membrane-bound $\beta\gamma$-subunits.

The decarboxylation of oxaloacetate can be divided into two separate half-reactions. In the first half-reaction, the carboxyl group from oxaloacetate

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is transferred to the prosthetic group biotin on the α-subunit. This carboxyltransferase activity is independent of Na^+ ions and resides on the isolated α-subunit [7]. However, this reaction is markedly accelerated in the presence of the γ-subunit, which was shown to bind the cofactor Zn^{2+} [8]. The second half-reaction, i.e. the decarboxylation of the formed carboxybiotin enzyme, requires the presence of Na^+ ions and of the $\beta\gamma$-subunits.

The *oadGAB* genes encoding the oxaloacetate decarboxylase γ, α and β-subunits represent only a part of the citrate regulon, a large gene cluster harbouring additional genes specifically required for the anaerobic citrate metabolism, i.e. those for citrate lyase (*citDEF*), citrate lyase ligase (*citC*) and the Na^+-dependent citrate carrier (*citS*) [9,10]. The transcription of these genes is controlled by a two-component regulatory system (*citAB*) whose genes are located on the same regulon, downstream to the *oadB* gene [11]. In a recent work, we expressed the oxaloacetate decarboxylase genes in *Escherichia coli* and characterised further the function of the individual subunits [8]. In this report we visualise the expression of the oxaloacetate decarboxylase genes by electron microscopy with immunological techniques using polyclonal antibodies which were labelled with protein A-gold after binding to the antigenic α-subunit. The results confirm those obtained with the biochemical characterisation.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this work are listed in Table 1. *Klebsiella pneumoniae* was grown anaerobically in citrate minimal medium as described [12]. For the expression of the entire oxaloacetate decarboxylase or the $\gamma\alpha$-subunits 5 ml LB cultures containing 100 μg ml^{-1} ampicillin were inoculated with a single colony of freshly transformed *E. coli* DH5α carrying the expression plasmid pSK-GAB or pSK-GA2. The cells were grown overnight at 37°C and centrifuged at the late exponential growth phase (after about 12 h). *E. coli* BL21(DE3)pLysS cells expressing the α- or $\alpha\beta$-subunits were also inoculated with freshly transformed single colonies and grown in 5 ml LB medium containing 100 μg ml^{-1} ampicillin and 40 μg ml^{-1} chloramphenicol. The expression of the oxaloacetate decarboxylase subunits α or $\alpha\beta$ was induced with 0.4 mM IPTG after the cultures reached an OD_{600} = 0.6–0.8. The induced cultures were grown for another 3 h and harvested by centrifugation. The sedimented cells were directly prepared for the subsequent immunological and microscopical steps.

Table 1
Bacterial strains and plasmids used in this work

Strain/plasmid	Genotype/description	Source/reference
K. pneumoniae		Boehringer Mannheim
E. coli		
DH5α	*supE44 lacU169(80lacZM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Bethesda Research Laboratories
BL21(DE3)pLysS	Cm^R; *hsdS gal* (λcI*ts857 ind1 Sam7 nin5 lacUV5-T7* gene *1*), T7 lysozyme gene in pACYC184	[17,18]
Expression plasmids		
pSK-GAB	Ap^R (pBluescript); *oadGAB*	[8]
pSK-GA2	Ap^R (pBluescript); *oadGA*	[8]
pT7-AL	Ap^R (pT7-7); *oadA birA*	[8]
pT7-AB	Ap^R (pT7-7); *oadAB*	[8]
pT7-BXY	Ap^R (pT7-7); *oadB*	[8]

Abbreviations: Ap^R, ampicillin resistance; Cm^R, chloramphenicol resistance.

2.2. Verification of the expression

To be sure that the oxaloacetate decarboxylase subunits were indeed synthesised in the *E. coli* cells which were further investigated by electron microscopy, the expression of the genes was analysed either by SDS-PAGE according to [13] or by reconstitution of the oxaloacetate decarboxylase activity after recombining the different subunits as follows: *E. coli* DH5α/pSK-GAB cells expressing the complete enzyme complex were disrupted with a French Press and the decarboxylation activity of the crude

Fig. 1. Immunolabelling and transmission electron microscopy of oxaloacetate decarboxylase expressed in *K. pneumoniae* (**A**) or *E. coli* (**B**). The black spots indicate the immunogold-labelled enzyme complex. Bars represent 0.5 μm.

extract was measured as described [12]. The expression of the $\gamma\alpha$-subunits was verified by reconstituting oxaloacetate decarboxylation activity after incubation of the solubilised membrane vesicles of *E. coli* DH5α/pSK-GA2 and *E. coli* BL21(DE3)pLysS/pT7-BXY. The expression of the α, β and $\alpha\beta$-subunits from *E. coli* BL21(DE3)pLysS containing the expression plasmid pT7-AL, pT7-BXY and pT7-AB, respectively, were visualised by SDS-PAGE of cell lysates as described [8].

2.3. Immunolabelling for transmission electron microscopy

The harvested cells were subjected to high pressure freezing in cellulose capillary tubes [14,15]. The capillary tubes, containing the frozen bacterial suspensions, were freeze-substituted in ethanol containing 0.5% uranyl acetate. Samples were kept in the freeze-substitution medium for 9 h at −90°C, 6 h at −60°C, 3 h at −30°C, 1 h at 0°C, and subsequently Epon/Araldite embedded according to Hohenberg et al. [15].

Polyclonal rabbit antibodies against the purified oxaloacetate decarboxylase were diluted 1:400, applied to the thin-sections and incubated for 2 h. The bound antibodies were then incubated with protein A coupled to 10 nm colloidal gold for 1 h. Previous masking of unspecific protein binding sites, washing and post-staining was performed according to Schwarz and Humbel [16].

3. Results and discussion

Since many years it is known that oxaloacetate decarboxylase from *Klebsiella pneumoniae* is a membrane-bound enzyme. This location of the protein in the cytoplasmic membrane is shown by immunogold labelling with antibody that reacted specifically with the peripheral membrane-bound α-subunit of the decarboxylase (Fig. 1A). Remarkably, almost all of the labelled enzyme is visualised on the surface of the cells. In contrast, after synthesis of the complete enzyme in *E. coli* (DH5α/pSK-GAB), a moderate part of the labelling is also located in the cytoplasmic volume (Fig. 1B). The specific oxaloacetate decarboxylase activity of the cytoplasmic fraction from the *E. coli* expression clone, however, was not higher than the specific activity of the corresponding fraction from *K. pneumoniae* cells (data not shown), indicating that the elevated cytoplasmic labelling probably derived from the isolated α-subunit. A reason for the cytoplasmic labelling could therefore be the higher expression (on translational level) of the *oadA* gene in *E. coli*, as compared to the *oadGB* genes, and/or a less effective complex formation between the α- and $\beta\gamma$-subunits due to overexpression and partial degradation of the membrane-bound components. Furthermore, it was observed that not all *E. coli*/pSK-GAB cells were labelled, or only weakly labelled by the antibody protein-A–gold complex, in contrast to the *K. pneumoniae* cells which all showed labelling on their cytoplasmic membrane (data not shown). This was indeed expected since the decarboxylase was not an essential enzyme for the growth of the *E. coli* cells, which may therefore have been overgrown by faster growing cells that had lost the plasmid during the late exponential phase, in which ampicillin is thought to be completely degraded. The fact that there was a part of *E. coli*/pSK-GAB cells without labelling also indicates that the cytoplasmic immunolabelling was not due to a possible cross-reaction with acetyl-CoA carboxylase, the only biotin-containing enzyme of *E. coli* which shows homologies to the oxaloacetate decarboxylase α-subunit [4,19].

In a recent study [8], we demonstrated the expression of the $\gamma\alpha$ and $\alpha\beta$-subunits in *E. coli*. We were able to purify $\gamma\alpha$-complexes from the membrane fraction of the cells, while it was not possible to isolate $\alpha\beta$-complexes neither from the cytoplasmic fraction nor from membrane vesicles. The purification protocol of the oxaloacetate decarboxylase subunits involved cell disruption with a French Press. Thereby, we could not exclude that a loosely bound $\alpha\beta$-complex dissociated during the preparation. Immunolabelling and the subsequent analysis by electron microscopy allowed us in this study to localise the expressed products under less harsh conditions. From various immunoblot analysis we knew that only the soluble α-subunit reacted with antibodies raised against the purified oxaloacetate decarboxylase from *K. pneumoniae* (unpublished results) and therefore renounced to further expand this study for

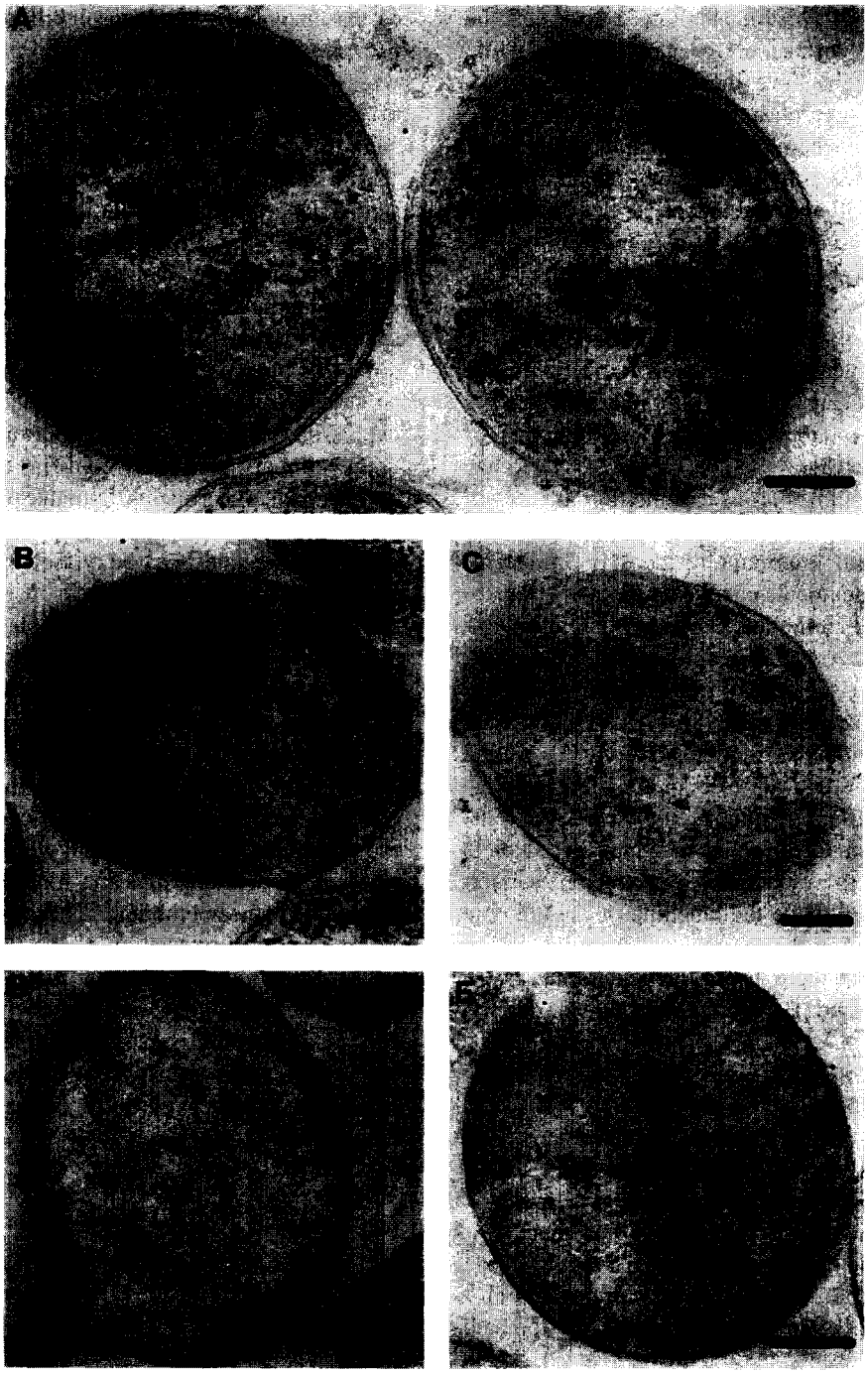

Fig. 2. Expression of the oxaloacetate decarboxylase subunits in *E. coli* and immunolabelling of the synthesised products. α-Subunit (**A**), $\alpha\beta$-complex before (**B**) and after (**C**) induction with IPTG, $\alpha\gamma$-complex poorly (**D**) or highly expressed (**E**). Bars represent 0.1 μm.

the localisation of the separately expressed hydrophobic β and γ-subunits, for which no antibodies are available. In Fig. 2 it is clearly visible that $\gamma\alpha$-expressing *E. coli* cells show a part of the labelling on the surface of the cells (D, E), while $\alpha\beta$-expressing cells have the labelling spread randomly into the cytoplasmic compartment (B, C), confirming the biochemical data obtained in the previous study [8]. *E. coli*/pT7-A cells overexpressing the α-subunit of the oxaloacetate decarboxylase complex show, as expected, a labelling only within the cytoplasm (Fig. 2A). The overexpression of this subunit as well as the overexpression of a possible $\alpha\beta$-complex from clone *E. coli*/pT7-AB using the strong T7 promoter did not lead to the formation of inclusion bodies (Fig. 2B, C), although considerable amounts of the α-subunit (50 mg (l cell culture)$^{-1}$) could be purified [8].

Mutagenesis studies let us assume that the β-subunit contains a carboxybiotin binding pocket, which may interact with important residues of the membrane-spanning segments catalysing the Na^+ translocation across the membrane (Di Berardino and Dimroth, submitted). It was also shown that the overexpression of the α-subunit led to the synthesis of only partially biotinylated enzyme [8]. Fig. 2B shows that the missing complex formation between the α and β-subunit is not due to low biotinylation of the α-subunit in $\alpha\beta$-expressing *E. coli*/pT7-AB cells, since even not induced cells, in which a complete biotinylation of the moderately expressed α-subunit should be ensured, have the labelling of the epitopic α-subunit located in the cytoplasm. From this result we conclude that there is no sufficient interaction between the α- and β-subunits to attain the formation of a complex.

As with *E. coli*/pSK-GAB cells synthesising the complete decarboxylase, $\gamma\alpha$-expressing cells (*E. coli*/pSK-GA2) also showed inhomogeneous labelling of the expressed α-subunits due to the differential loss of the dispensable expression plasmid. In cells in which a high expression of the genes was observed, the labelling was distributed over the whole cells, whereas cells expressing only minor amounts of this complex were mostly labelled on the cell surface. These results may indicate that, beside a possible higher expression of the α-subunit alone, the interactions between the γ and α-subunit were only weak and, consequently, that part of the complex was in a dissociated state. Moreover, it was not possible to reconstitute the decarboxylation activity of the enzyme by mixing solubilised membranes of β-expressing *E. coli* (BL21(DE3)pLysS/pT7-BXY with the cytoplasm of *E. coli*/pSK-GA2 [8]. Since in a similar experiment with a membrane extract from *E. coli*/pSK-GA2 (containing subunits α and γ) the catalytically active decarboxylase was reconstituted, the labelling in the cytoplasm was only due to the α-subunit. From the previous study we must assume that the γ-subunit is subjected to proteolytic digestion when expressed alone [8]. The degradation of a significant part of the γ-subunit, which is required to bind the α-subunit to the membrane, may be another reason for the substantial labelling of the expressed enzymes in the cytoplasm.

In summary, this study confirms the results of our biochemical experiments. Notable differences were observed by comparing the expression of the complete oxaloacetate decarboxylase in *E. coli* and *K. pneumoniae*. The α-subunit in *K. pneumoniae* resides almost entirely at the cytoplasmic membrane, consistent with the existence of a membrane-bound oxaloacetate decarboxylase complex. Overexpression of the enzyme in *E. coli*, however, also produced moderate amounts of the α-subunit in the cytoplasm. Similarly, *E. coli* cells expressing the $\gamma\alpha$-subunits in elevated amounts show a significant proportion of the α-subunit in the cytoplasm, whereas under conditions of moderate expression most of the α-subunit is bound to the membrane. These results may indicate a preferred translation of the *oadA* gene within the *oadGAB* cluster and/or that the formation of membrane-bound oxaloacetate decarboxylase or of $\alpha\gamma$ subcomplexes is partially impaired under high level expression in *E. coli*, perhaps because the γ-subunit becomes partially degraded under these conditions.

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