PURIFICATION OF A NOVEL ALDEHIDE-DEHIDROGENASE WITH WIDE SUBSTRATE SPECIFICITY

MARIUS MIHĂŞAN [1,*], VLAD ARTENIE [1], RODERICH BRANDSCH[2]

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Abstract: The pAO1 megaplasmid of *Arthrobacter nicotinovorans* encodes two different pathways: one for nicotine metabolism and a putative sugar catabolic pathway. An open reading frame, orf39, from the latter pathway was cloned, purified to homogenity and partially characterized. It consists of a monomeric NAD/NADP-dehidrogenase acting on various aldehyde as glutaraldehyde or butyraldehyde with an Cys residue in the active site. A possible catalytic mechanism is postulated.

INTRODUCTION

Plasmids are simple genetic elements, independent from the bacterial chromosome, involved both in vertical and horizontal-gene transfer. Most of the time, the plasmids encode different properties (resistance to antibiotics, to highly toxic compounds) which give the host cell an evolutionary advantage. The ability to un-common compounds as carbon and nitrogen sources is such an advantage, allowing the bacteria to be present in many environments as natural autochthonous microflora with a high potential for bioremediation of pollutants. Several plasmid-encoded pathways were described (ex: for metabolism of phthalate (10) or naphthalene (23)) but only few are completely elucidated.

The presence of the 165- kb pAO1 megaplasmid inside the cells of the gram positive soil bacteria *Arthrobacter nicotinovorans* allows this microorganism to use nicotine as sole carbon and nitrogen sources. The complete sequence of this plasmid was determined and two putative pathways could be described (15): on one hand the nicotine-degrading pathway, fully characterized by Brandsch (6) and on the other hand an yet unknown putative sugar-catabolic pathway. The overall GC content of the pAO1 plasmid indicates that nicotine-catabolism gene clusters are a new acquisition, being attached during the evolution to an older plasmid, containing the sugar-catabolic pathway. Recently shown analogies of the pAO1 encoded pathway for nicotine metabolism and the chromosome encoded one from *Nocardioides* sp. strain js614 (12) would suggest an horizontal gene transfer.

The sugar-catabolic pathway is comprised of several genes, among which a putative cellulase, an ABC-transporter system gene cluster and a cluster of several dehydrogenases and oxidoreductases. This last cluster probably encodes the last steps of the pathway, connecting it to the general metabolism of the cell. A part of this cluster is ORF39, a putative succinate-semialdehyde dehydrogenase and ORF40, a putative oxidoreducase. The ORF40 was found to encode an tetrameric sugar-dehydrogenase (20) containing Zn.

Our current study is focused on the ORF39 protein and its possible role in the cell. By cloning the gene in the expression vector pH_6EX_3 (4), we were able to express it as a recombinant His-tagged protein and to easily purify it to homogeneity.

MATERIAL AND METHODS

Isloation and cloning of orf39. The orf39 was isolated by PCR using the primers in table 1 and a suspension of *Arthrobacter nicotinovorans* cells as template. Directional cloning (24) of the fragment containing the *orf39* in the pH_6EX_3 vector was achieved by using *BamHI* şi *SalI* (NEB, U.K) enzymes and Rapid DNA ligation Kit, Roche). Transformed *E. coli XL1* Blue competent cells were selected on plates containing ampiciline (50 μg/ml) and the recombinant plasmid was checked for the presence of insert by restriction enzyme digestion.

Table 1. Oligo-nucleotides used for isolation of *orf39*

Primer	Sequence
Orf39forw	5´-CAG CCA TCG TGA TCA GCA ACA AGG-3´
Orf39rev	5´-GTT GAA GGG TCG ACG CTG AGG GTT AG-3´

Protein expression was done using auto-inducible medium as described elsewhere (19). **Protein purification** was achieved using standard IMAC techniques (3) on Fast-Flow Ni-chelating Sepharose (Amersham Biosciences, Sweden). All buffers used in the purification process had 10 mM β-mercaptoethanol final concentration. **Native molecular weight determination** was done using gel permeation chromatography on an HiLoad 16/60 Superdex 200 column connected to an AKTA Basic FPLC system. **Protein concentration** was assayed using the dye-binding method of Bradford (5). **SDS-**

PAGE was performed using the discontinuous system of Laemlli fallowing the procedure described by Sambrook, 1989(24).
Enzyme assay was developed by following the guidelines described by de Caballero et. al. (8), Marchal and Branlant(17), Farrbs et al. (11). The assay mixture was phosphate buffer 100 mM pH 8,4, 10 mM β-mercaptoethanol, 1 mM $NAD(P)^+$ and 20 µg purified enzyme The reaction was started by adding the substrate at 33 mM final concentration. The formation of NAD(P)H was monitored at 340 nm for 2 min. Enzyme activity was expressed as nanomoles NADH formed per minute per microgram enzyme (molar extinction coefficient for NAD^+ 6220 M-1*cm-1).
Tertiary structure predictions were performed with Jpred (9). For structure based sequence aligment the Seqoia program (7) was used. The pictures were generated from the aligments files using ESPript (13).

RESULTS AND DISCUSSIONS:

***ORF39* encodes a monomeric protein.** The recombinant protein obtained by cloning *orf39* in pH6EX3 has the N-terminal sequence as follows: HHHHHLVPRGSATRSI**M**, where the methionine in bold is the native START codon. This allowed for an one step purification process of the protein from the *E.coli* cell lysate using mobilized metal affinity chromatography. The purified enzyme had a relative molecular weight of 51, 44 kDa, in good accordance with the theoretical mass. The purity of our preparations was very high (over 95% on SDS-PAGE, fig. 1).

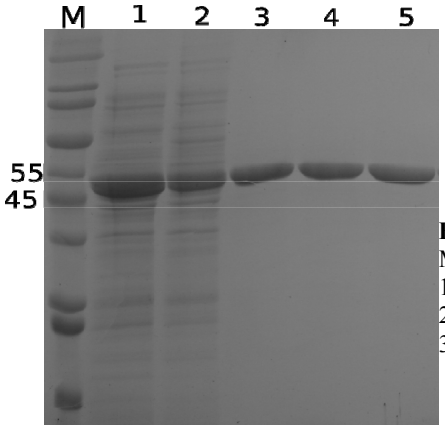

Figure 1. *Orf39* encoded protein was purified to homogeneity.
M –Molecular Weight Marker Sigma Wide Range
1 – Crude extracts (25 µg)
2 – Cell free extract (25 µg)
3, 4, 5– Purified protein (10 µg)

A BLAST search performed at the NCBI servers has shown that the ORF39 is similar at the sequence level with several enzymes belonging to the aldehyd-dehydrogenases family. Class I and II of this family is comprised of dimer and tetramer proteins, while class III enzymes are trimers (2). In order to establish native state of this enzyme in solution, a gel permeation chromatography was performed. Approximately 1.6 mg purified ORF39 were injected on a HiLoad 16/60 Superdex 200 column. The protein eluted as a single peak at with a molecular weight of 64.8 kDa. This would indicate that the enzyme is a monomer. This is the first report of a monomeric aldehyde-dehydrogenase (see below), as all known enzymes with aldehyde-dehydrogenase activity described are dimers, trimers or tetramers.
ORF39 is an aldehyde-dehidrogenase. The purified enzyme was used for functional tests, in order to establish its physiological role. Several substrates were tested and are listed in table 1. The enzyme is able to dehydrogenate several aliphatic and one aromatic aldehyde forming probably the corresponding acids. From the data presented in table 1, it can be see that the enzyme seems to prefer long-chain substrate.
Table 2. Dehydrogenase activity of ORF39 on various substrates

Substrates	**nmoli NADPH/min/mg proteină***
Butiraldehyde	229.1
Glyceraldehyde	93.78
Glutaraldehyde	77.71
Benzaldehyde	36.17
Formaldehyde	26.8
Succinic-semialdehyde	Not Detected

Dehydrogenase activity could be detected with both $NADP^+$ and NAD^+, but the speed of the reaction decreases with the

last co-enzyme (Table 3). Such dehydrogenases acting on aldehydes with both co-enzymes are common, being described also by Veladi et al., 1995 (25) and Ahvazi, 2000 (2). In conclusion, the enzyme encoded by the *orf39* from pAO1 megaplasmid is an NAD(P)- aldehyde-dehydrogenase (ALDH) and we will refer to it with this name from here after (18).

Table 3. Relative activity of ALDH with NAD^+ and $NADP^+$

Substrate (33 mM)	**Specific activity with NAD^+(nmoles NADH*min^{-1}*µg^{-1} enzyme)**	**Specific activity with $NADP^+$ (nmoles NADPH*min^{-1}*µg^{-1} enzyme)**
Gliceraldehyde	176.85	281.35
Glutaraldehyde	64.31	233.12
Butiraldehyde	24.12	458.2

ALDH has an Cys residue in the active site. A lot of time was lost in developing a purification procedure for this enzyme. Following common IMAC techniques and guidelines, the enzyme was always inactive and migrated on SDS-PAGE as a double band. As it can be see in figure 2, panel A, adding reducing agents as DTT or β-mercaptoethanol to this batches of purified inactive enzyme did not abolished this behavior.

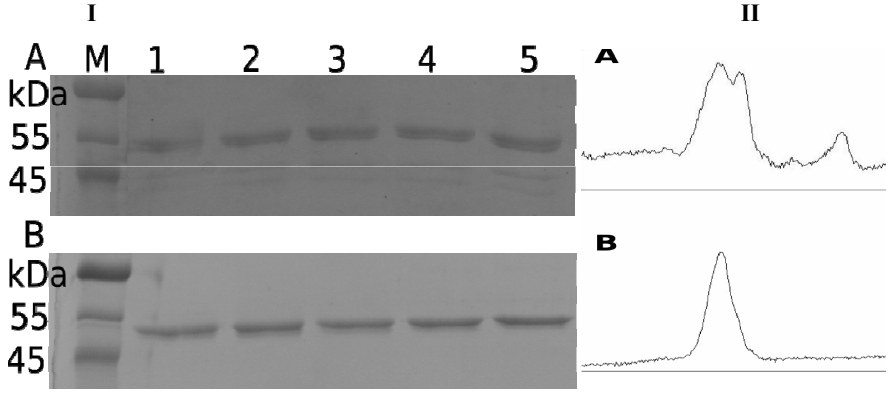

Figure 2. Different SDS-PAGE migration patterns of ALDH purified without (A) or with (B) β-mercaptoethanol.
I. SDS-PAGE with 1 microgram ALDH. Loading buffer has 1. o mM 2. 10 mM 3. 50 mM 4. 100 mM 5. 500 mM 2-mercaptoethanol final concentration II. Gel-densitometry of sample 3 (densitometry were realized with ImageJ (1)

This behavior is commonly explained in the literature by a di-sulfur bridge between two Cys residues. This would result in the formation of a loop on the amino acid chain of the protein, loop which is translated in a decrease of the relative molecular weight on SDS-gels with approximately 2 kDa (14).

Using from the starting of the purification process 10 mM β-mercaptoethanol in all the buffers has lead active enzyme. In this case, the protein migrated as a single band on SDS-PAGE (figure 2, B). So, without the reducing agent, the formation of a di-sulfur bridge is possible and leads to enzyme inactivation, either by changing the tertiary structure of the protein, by blocking the active residue or by both mechanisms. A sequence based alignment was performed with the computer generated model of ALDH and the crystal structures of two aldehyde-dehydrogenases, one from sheep (PDB id. 1bxs (21)) (30.4% identity) and one from human (PDB id. 1cw3, (22) (29.7% identity). The alignment (fig. 3) showed that the only highly conserved residue is Cys 266, which is involved in the catalytic mechanism of 1bxs and 1cw3.

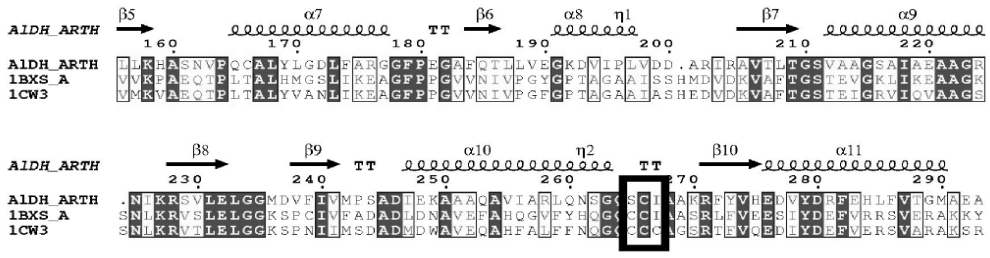

Figure. 3. Structure based alignment of ALDH of *Arthrobacter nicotinovorans* and aldehyde-dehydrogenases from sheep – 1bxs and from human - 1cw3 in the catalytic site region.
Probably the mechanism by which ALDH from *Arthrobacter dehydrogenates* the substrates is similar to that described for

other aldehyde-dehydrogenases by Marchal and Branlant,1999 (17) and Ki Ho (2005) (16). The Cys residue from the catalytic site is involved in the formation of a covalent intermediate during the catalytic process. Blocking this residue by a di-sulfur bridge would impair the formation of this intermediate, and those, render the enzyme inactive.

CONCLUSIONS

The ORF39 was cloned, expressed and purified to homogeneity. It consists of an novel aldehide-dehidrogenase of 52.44 kDa, which unlike the other enzymes of its class is an monomer in solution. Active enzyme preparations required the presence of 10 mM reducing agents in all the buffers. Without the reducing agent, the enzyme runs on SDS-PAGE as a double band typical for di-sulfur bridge formation. Structure based sequence alignments showed that Cys 266 is highly conserved, indicating its implication in the catalytic process and explaining the behavior of this enzyme. In order to fully establish the biotechnological potential of this enzyme, further investigations for characterization of the enzyme (heat stability, pH stability, Km, Kcat,) are underway.

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1. „Alexandru Ioan Cuza" University, Iaşi, Biology Faculty, Molecular and Experimental Biology Department
2. Institute for Biochemistry and Molecular Biology, Center for Biochemistry and Molecular Cell Research, Albert Ludwigs University, Freiburg, Germany
* marius.mihasan@uaic.ro