Molecular and biochemical characterisation of *Mycobacterium smegmatis* alcohol dehydrogenase C

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Abstract

The gene encoding an alcohol dehydrogenase C (ADHC) from *Mycobacterium smegmatis* was cloned and sequenced. The protein encoded by this gene has 78% identity with *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG ADHC. The *M. smegmatis* ADHC was purified from *M. smegmatis* and the kinetic parameters of this enzyme showed that using NADPH as electron donor it has a strong preference for aliphatic and aromatic aldehyde substrates. Like the *M. bovis* BCG ADHC, this enzyme is more likely to act as an aldehyde reductase than as an alcohol dehydrogenase. The discovery of such an ADHC in a fast-growing, and easily engineered mycobacterial species opens the way to the utilisation of this *M. smegmatis* enzyme as a convenient model for the study of the physiological role of this alcohol dehydrogenase in mycobacteria. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Alcohol dehydrogenases (ADHs) occur in a wide variety of organisms including animals, plants, yeasts and bacteria. ADHs have a wide range of substrate specificity and play several key physiological functions. They are usually grouped into three major categories. The first one, which includes the NAD(P)-dependent ADHs, is subdivided into three groups: (I) long-chain zinc-dependent enzymes (composed of the tetrameric and dimeric subfamilies, which in turn are divided into classes I–IV, C and P), (II) short-chain zinc-independent enzymes and (III) iron-activated enzymes. The second category of ADHs includes NAD(P)-independent enzymes, and finally those which catalyse essentially irreversible oxidation of alcohols fall into the third category [1,2].

Previous experiments showed that *Mycobacterium bovis* BCG grows poorly on zinc-deprived Sauton medium, and that it forms a thin, pale, unfolded pellicle that gets wet and sinks [3].

The reduced hydrophobic content of these cells, as well as the detection of an increased aldehyde content in the zinc-deficient culture filtrates, led to the hypothesis of a cause/effect relationship with a significant decrease in specific activity of a soluble zinc-dependent ADH. A *M. bovis* BCG zinc-dependent ADH was thus purified and characterised as a dimeric NADP-dependent enzyme belonging to the family of long-chain alcohol/polyol dehydrogenases, class C [4]. The *M. bovis* BCG ADHC (BCG-ADHC) encoding gene was cloned and later found to be identical to the *adhC* from *Mycobacterium tuberculosis* [5]. Its overexpression in *M. bovis* BCG enabled its purification and full biochemical characterisation. These studies suggested that BCG-ADHC might be involved in the biosynthesis of the free lipids required for the formation of the mycobacterial cell envelope [6,7]. Confirmation of this hypothesis would mean that this enzyme may be an interesting target for the development of new anti-mycobacterial agents.

To test this hypothesis, and due to the long generation time of *M. bovis* BCG and *M. tuberculosis* and to the inability to readily generate defined mutant strains in these mycobacteria [8], an easier approach is to first identify and characterise such an enzyme in a fast-growing and non-
pathogenic mycobacterium. In this study we show that Mycobacterium smegmatis contains an ADHC homologous to that of M. bovis BCG and M. tuberculosis, and we report the cloning and sequencing of its coding gene, the enzyme purification and its biochemical characterisation.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Escherichia coli MAX Efficiency STBL2® (Gibco BRL) and E. coli DH5α competent cells, respectively used for cloning and plasmid propagation purposes, were grown on solid or liquid Luria–Bertani medium containing 100 µg ml⁻¹ ampicillin or 25 µg ml⁻¹ kanamycin.

For protein extraction purposes, M. smegmatis mc²155 [9] was grown as surface pellicles for 3 days on Sauton medium [10] at 37°C. For all the other manipulations M. smegmatis mc²155 was grown in Nutrient Broth medium (Difco) supplemented with 0.05% (w/v) Tween 80 and 0.2% (w/v) glycerol, at 37°C and agitated at 150 rpm. Mycobacterial media were supplemented with 25 µg ml⁻¹ kanamycin for the recombinant strains.

2.2. DNA techniques

Genomic DNA from M. smegmatis was isolated from a 100-ml culture grown to an OD₆₀₀ of 0.6. To achieve easier DNA extraction, we prepared protoplasts by the addition of 800 µl of a solution containing 0.2 M glycine, 60 µg ml⁻¹ d-cycloserine, 20 mM lithium chloride, 200 µg ml⁻¹ lysozyme and 5 mM EDTA. The culture was then reincubated for an additional 16 h prior to harvest, and after centrifugation, 3-mm glass beads were added to the pellet and the tube was vortexed for 3 min. The cells were then suspended in 8 ml of a solution containing 2.5 µg ml⁻¹ lysozyme, 25 mM Tris–HCl, 50 mM glucose, 10 mM EDTA, pH 8, and after the removal of the beads, 4 ml of 10% SDS was added and the mixture was incubated for 15 min at 65°C. 6 ml of 3 M sodium acetate pH 5.2 was then added, and phenol-chloroform extractions were performed followed by isopropanol precipitation and dissolution in TE (10 mM Tris, 1 mM EDTA, pH 8). Genomic DNA (2 µg) was digested with BamHI and DNA fragments were separated by electrophoresis on a 1% agarose gel. Fractionated DNA fragments were denatured by incubating the gel in a 0.5 M NaOH, 1.5 M NaCl solution. The gel was then neutralised by incubation in a 1.5 M NaCl, 0.5 M Tris–HCl (pH 7.2), 1 mM EDTA solution, and DNA was transferred by capillarity to a Hybond-N nylon membrane (Amersham). Southern hybridisations of genomic DNA were performed using as a probe a 569-bp fragment (P600) obtained by PCR amplification of M. smegmatis genomic DNA. This fragment was obtained using the primers G344 (5’-GGGCGGCTTGTGACGCGGGGC-3’) and G345 (5’-GTCGATGACGAAGCGGGAC-3’). Tag DNA polymerase (Promega) was used and PCR conditions were as follows: 1 min at 95°C, 30 cycles of 1 min at 95°C, 1 min at 60°C, 1 min at 72°C ending with a 10-min extension period at 72°C. The amplified fragments were purified from an agarose gel and were labelled by random priming with [α-³²P]dCTP, using a random priming kit (Amersham). The hybridisation was conducted in the Rapid-Hyb buffer of Amersham according to the instructions of the manufacturer. After hybridisation, the membrane was washed at high stringency (final wash at 65°C in a 0.1% SDS, 0.1× SSC buffer) and autoradiographed at −70°C. The 3.3-kb hybridised BamHI DNA fragments were isolated and purified from a preparative 1% agarose gel, subcloned into a BamHI-digested pUC19 vector and transformed into E. coli MAX Efficiency STBL2® competent cells (Gibco BRL), according to the manufacturer’s protocol. The resulting mini-library (about 360 colonies) was screened by PCR on plasmid pools of five colonies, using the primers G344 and G345 as described.

DNA was sequenced on both strands by Texas Red dye-primer cycle sequencing on an automatic sequencer using a Thermosequenase Core Sequencing Kit with 7-deaza-dGTP (Vistra DNA system, Amersham).

2.3. Enzyme purification and protein assays

M. smegmatis mc²155 was grown as a surface pellicle for 3 days and the ADH was extracted and purified as previously described [7]. Protein concentration was assayed by the Coomassie brilliant blue method [11] with the Bio-Rad reagent using bovine serum albumin as a standard. ADH specific activity was determined by measuring the rate of oxidation of 0.25 mM NADPH at 340 nm in 0.02 M KH₂PO₄/Na₂HPO₄ buffer pH 7.3 and in the presence of 50 µM benzaldehyde. This substrate was shown to be the best substrate for BCG-ADHC. Kinetic parameters were determined by direct spectrophotometric measurement using an Uvikon 930 spectrophotometer. Purified enzyme was assayed both for the reduction of the aldehydes (forward reaction), and for the oxidation of the corresponding alcohol (reverse reaction) as previously described [7]. SDS–PAGE was carried out as described [12] with 12% polyacrylamide gels on a MiniProtean II system (Bio-Rad Laboratories, Hercules, CA, USA), and proteins were stained with Coomassie brilliant blue. Proteins were transferred onto nitrocellulose filters with a LKB Multiphor II electrophoresis unit by semidy electroblotting. ADH was detected by incubation with a murine monoclonal antibody raised against BCG-ADHC (4B5) [6] and revealed with the Protoblot Western blot alkaline phosphatase system (Promega), according to the instructions of the manufacturer.
Fig. 1. \textit{M. smegmatis} ADHC. A: Partial restriction map of the 3.3-kb \textit{Bam}HI genomic DNA fragment present in pAGA5. Shaded and black fragments correspond to the sequence shown below. B: Nucleotide sequence and deduced amino acid sequence of the \textit{M. smegmatis} ADHC. This sequence has been deposited in the EMBL database under accession number AJ291708. Potential promoter regions and the putative Shine–Dalgarno AAGAGG, located six nucleotides upstream of the translational start codon, are indicated by boldface letters. The sequences AGTAAT and GTGAAC resemble the 310 and 335 hexamers of the \textit{E. coli} c70 consensus, respectively. The locations of the primers G344 and G345 are underlined. The 14 N-terminal residues determined by automated Edman degradation are also underlined.
2.4. Analysis of the N-terminal amino acid sequence

The purified protein was separated by SDS–PAGE and electroblotted onto a PVDF membrane by the method of Matsudaira [13], and subjected to N-terminal amino acid sequence analysis by Edman degradation in an automated sequencer at the Department of Biological Chemistry, Faculty of Sciences, University of Mons, Belgium.

3. Results and discussion

3.1. Cloning and sequencing of an adhC gene from M. smegmatis

A previous search of similarity between BCG-ADHC and proteins deposited in sequence databanks indicated that BCG-ADHC is similar to bacterial ADHs from three species (Bacillus subtilis, E. coli 2 and Helicobacter pylori) and to a large group of plant cinnamyl dehydrogenases [7].

An alignment of 15 ADH amino acid sequences from these organisms allowed the identification of two regions from BCG-ADHC that are conserved in all analysed sequences. In these regions we designed two primers, G344 and G345, to amplify a homologous DNA fragment from the M. smegmatis genome. G344 and G345 allowed the amplification of the 569-bp DNA fragment (P600) which was then used as a probe to identify the ADHC gene on a Southern blot of M. smegmatis DNA. P600 was found to hybridise to a BamHI DNA fragment of 3.3 kb. DNA fragments of this size were isolated from an agarose gel and cloned into the BamHI site of plasmid pUC19 (results not shown). This resulted in a mini-library composed of 360 E. coli recombinants which was screened by PCR using primers G344 and G345. One positive clone was found. The recombinant plasmid of this clone, pAGA5, contains a 3329-bp insert which hybridises to the P600 probe (results not shown). This M. smegmatis DNA inserted in pAGA5 was then sequenced on both strands, and a 1044-bp open reading frame (ORF) encoding a 348-amino acid protein was identified (Fig. 1). Its nucleotide sequence was found to be similar to the published sequences of the ADHC from M. tuberculosis and M. bovis BCG (77% identity), though neither of the two flanking genes of M. tuberculosis adhC (fecB and Rv3046c) were found upstream or downstream of M. smegmatis adhC. Actually, the flanking sequences of M. smegmatis adhC did not share notable homology with any sequence available in databases.

The predicted 348-amino acid sequence of M. smegmatis...
ADHC (Ms-ADHC) has a calculated isoelectric point of 5.05 and a calculated molecular mass of 37.1 kDa.

The alignment of the amino acid sequences deduced from the available genomic sequences from other mycobacteria with that of Ms-ADHC showed that the *M. smegmatis* protein shares a strong degree of identity not only with the *M. bovis* BCG and *M. tuberculosis* ADHCs (78%), but also with the ones from *M. avium* and *M. paratuberculosis* (76%), and with *M. leprae* (75%) (Fig. 2). All the known mycobacterial ADHC sequences were found to share identity with members of the family of long-chain zinc-dependent ADHs. Horse liver ADH, the archetypal enzyme of this family, binds two zinc atoms per enzyme subunit, with a catalytic zinc atom bound at the active site to the residues Cys46, His67 and Cys174, and a structural zinc atom bound to the cysteine residues 97, 100, 103 and 111 [2]. These zinc-binding residues were found to be conserved in all the mycobacterial ADHCs (Fig. 2).

### 3.2. Analysis of cell-free extracts and purification of *M. smegmatis* ADHC

In order to purify Ms-ADHC, we first checked, by Western blotting, whether this enzyme could be detected in the filtrate of young cultures, as previously reported for *M. bovis* BCG [7]. However, no ADH was detected in the filtrate of young static *M. smegmatis* cultures (2-day culture), which indicates that this enzyme is an intracellular protein (data not shown).

Then we compared the ADH activity of *M. smegmatis* grown in static and agitated cultures. We found that the specific ADH activity of cell-free extracts from cultures grown as pellicles (265.8 nmol min\(^{-1}\) mg\(^{-1}\)) was eight-fold higher than that obtained from agitated cultures (32.6 nmol min\(^{-1}\) mg\(^{-1}\)). This might be due to a contribution of this protein to the synthesis of hydrophobic compounds of the cell envelope, which is known to be higher in cells from static cultures than in agitated ones. We thus used static cultures for the extraction and purification of Ms-ADHC. The ADH was purified from pellicle soluble extracts by a combination of ion exchange (DEAE Sephacel column) and affinity chromatography (2’,-5’-ADP affinity Sepharose column). After Coomassie staining the SDS-PAGE of the purified protein revealed a major protein with an apparent molecular mass of 38 kDa (Fig. 3). The purified enzyme had a specific activity of 24 000 nmol min\(^{-1}\) mg\(^{-1}\), which represents a 100-fold purification.

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The N-terminal amino acid sequence of the purified protein was determined up to the 14th residue and the obtained sequence, MSTVSAYAATSATE, corresponded to the amino acid sequence predicted from nucleotide sequence of the **adhC** ORF (Fig. 1), confirming that the purified enzyme corresponded precisely to the cloned ADHC gene.

### 3.3. Biochemical characterisation of *M. smegmatis* ADHC and comparison with the corresponding *M. bovis* BCG enzyme

From previous studies [7] it could be hypothesised that this enzyme is, in vitro, mainly an aldehyde reductase, processing alcohols far less efficiently than aldehydes. In order to compare the substrate specificity of the purified Ms-ADHC with BCG-ADHC, aromatic and aliphatic

![Table 1](https://academic.oup.com/femsle/article-abstract/196/1/51/474362/fig1?Rect=677|704|692|723)

**Table 1**

Substrate preference of purified *M. smegmatis* ADHC

<table>
<thead>
<tr>
<th>Substrate</th>
<th><em>Km</em> (µM)</th>
<th><em>kcat</em> (s(^{-1}))</th>
<th><em>kcat/Km</em> (mM(^{-1}) s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ms-ADHC</td>
<td>BCG-ADHC(^a)</td>
<td>Ms-ADHC</td>
</tr>
<tr>
<td>Octanal</td>
<td>30</td>
<td>9</td>
<td>37.8</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>53</td>
<td>11</td>
<td>32.1</td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>40</td>
<td>100</td>
<td>18.6</td>
</tr>
<tr>
<td>Butyraldehyde</td>
<td>215</td>
<td>200</td>
<td>16.6</td>
</tr>
<tr>
<td>Cinnamyl alcohol</td>
<td>500</td>
<td>7000</td>
<td>1.6</td>
</tr>
</tbody>
</table>

\(^a\)The values for BCG-ADHC are those presented in [7].
substrates previously used to characterise BCG-ADHC were tested (Table 1). Aldehyde reaction was assayed by measuring the rate of 0.25 mM NADPH oxidation by 1 μg of purified protein. According to the catalytic efficiencies ($k_{cat}/K_m$) determined for the aldehyde substrates, we concluded that octanal and benzaldehyde were the most specific for Ms-ADHC. From comparison with the values previously obtained for BCG-ADHC [7], it appears that the aldehydes which present the best catalytic efficiencies are similar for both proteins, though octanal has the best catalytic efficiency for Ms-ADHC. The cinnamyl alcohol oxidation was 145-fold less efficient than the cinnamaldehyde reduction. Like BCG-ADHC, in vitro, this enzyme seems to be mainly an aldehyde reductase.

The homology of *M. smegmatis* ADHC with *M. bovis* BCG and *M. tuberculosis* ADHCs demonstrated in this work strongly suggests that *M. smegmatis* can be used as a model to study the physiological role of the ADHCs in mycobacteria. This work provides the tools for the construction of an ADHC knock-out mutant of *M. smegmatis*. Such a mutant will be most useful for the evaluation of its in vivo significance as a target for anti-tuberculosis drugs.

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