Properties of the 40 kDa antigen of *Mycobacterium tuberculosis*, a functional L-alanine dehydrogenase

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The 40 kDa antigen of *Mycobacterium tuberculosis* is the first antigen reported to be present in the pathogenic *M. tuberculosis*, but not in the vaccine strain *Mycobacterium bovis* BCG. It is a functional L-alanine dehydrogenase (EC 1.4.1.1) and hence one of the few antigens possessing an enzymic activity. This makes the 40 kDa antigen attractive for potential diagnostic and therapeutic interventions. Recently, we developed a strategy to purify quantities of the recombinant protein in active form, and here we describe the biochemical properties of this enzyme. In the oxidative-deamination reaction, the enzyme showed K_m values of 13.8 mM and 0.31 mM for L-alanine and NAD^+, respectively, in a random-ordered mechanism. $K_{m,app}$ values in the reductive-amination reaction are 35.4 mM, 1.45 mM and 98.2 μM for ammonium, pyruvate and NADH, respectively. The enzyme is highly specific for all of its substrates in both directions. The pH profile indicates that oxidative deamination virtually may not occur at physiological pH. Hence L-alanine most likely is the product of the reaction catalysed *in vivo*. The enzyme is heat-stable, losing practically no activity at 60 °C for several hours.

Key words: heat-stable enzymes, oxidative deamination, peptidoglycan biosynthesis, reductive amination, tuberculosis.

INTRODUCTION

Tuberculosis is still a major threat to global public health and has in fact re-emerged in recent years, even in the developed countries [1]. Although research on tuberculosis has been reinforced in the last years, rapid and reliable diagnostic tools are still unavailable [2]. No new drugs have been developed in recent years. The antibiotics prescribed in combination therapy require long-term treatment and are drugs developed about 20 years ago [3]. Some of them have become less effective due to the widespread emergence of antibiotic resistance. Therefore better understanding of molecular processes involved in the disease is urgently needed to develop novel tools for diagnosis, treatment and vaccine development.

Proteins present in the culture filtrate of *Mycobacterium tuberculosis* are the primary targets of the immune response [4]. Recently, research has focused on these protein antigens, since they are capable of inducing an immune reaction in a primary or a recall infection [5]. They can also prime T cells for a long-lived memory-immune response [6]. The 40 kDa antigen is one of the earliest proteins detectable in the culture medium of *M. tuberculosis*, present in as early as 4-day-old cultures [7]. The gene product is present in the pathogenic *M. tuberculosis*, but absent in the strain used for vaccines *Mycobacterium bovis* BCG. However, the latter gives signals on Southern blots, indicating the presence of the corresponding gene. A monoclonal antibody HBT-10 is highly specific for the 40 kDa antigen and is capable of discriminating between these two strains [8].

Based on sequence similarity, the enzymic function of the 40 kDa antigen as an L-alanine dehydrogenase (EC 1.4.1.1) was predicted and later confirmed by enzymic assays [7,9]. This makes it one of the first antigens with a well-defined enzymic function. The protein consists of six identical subunits with a predicted relative molecular mass (M_r) of 38988 for the monomer. The properties of the enzyme are important in deciphering the physiological function of this antigen. In addition, the enzymic nature of this antigen and its expression primarily in the pathogenic *M. tuberculosis* make it a potential target for therapeutic intervention.

We used a recently developed purification method to obtain quantities of the nearly homogeneous recombinant protein [9]. All of the enzymic parameters that were measured support a proposed function for the antigen in peptidoglycan biosynthesis [7]. Interestingly, this appears to be an exceptional case of an L-alanine dehydrogenase mainly involved in an anabolic pathway.

MATERIALS AND METHODS

Preparation of protein extracts

The soluble cell extract of the heat-induced strain *Escherichia coli* CAG629 (pMSK12) was prepared as previously described [9]. The recombinant L-alanine dehydrogenase accumulates to as much as 5% (w/w) of the total soluble protein. Apparently homogeneous enzyme was prepared by chromatography on an anion-exchange column, followed by affinity chromatography on Blue Sepharose as previously described [9].

Measurement of L-alanine dehydrogenase activity

Photometric determination of alanine dehydrogenase activity was accomplished by measuring the rate of the production of NADH that accompanies the conversion of alanine into pyruvate

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in the oxidative deamination. The reaction mixture consisted of 125 mM glycine/KOH (pH 10.2), 100 mM L-alanine and 1.25 mM NAD^+ [10]. For the reductive amination the mixture consisted of 1 M NH_4Cl/NH_4OH (pH 7.4), 20 mM pyruvate and 0.5 mM NADH, and the conversion of NADH into NAD^+ was observed photometrically. One unit of L-alanine dehydrogenase activity is defined as the turnover of 1 μmol of NAD^+/NADH per minute [11]. Reactions were carried out at 37 °C in a heat-controlled Ultrospec K 4053 spectrophotometer (Pharmacia). For the determination of the kinetic parameters, the concentration of the substrates were varied. To determine the pH profile, the following buffers were used: 1 M NH_4Cl/NH_4OH for pH 5.3–7.8, 12.5 mM Tris/HCl for pH 8.0–8.8 and 125 mM glycine/KOH for pH 9.1–12.3. The pH was checked before and after each reaction. For thermodynamic measurements, the cuvettes and/or the reaction mixture were pre-incubated at the desired temperatures. Additional substrates and effectors were added to the mixture either in addition or to substitute for one of the substrates. Details are given in the text.

RESULTS AND DISCUSSION

Kinetic properties

The pH profiles were determined under substrate saturating conditions. Strikingly, the pH optimum for the oxidative deamination (pH 10–11) was about three pH units above the optimum for the reductive amination (pH 7–7.5) (Figure 1). Since the physiological environment of *M. tuberculosis* has a neutral pH [12], it can be assumed that the enzyme catalyses exclusively the formation of L-alanine *in vivo*. Michaelis–Menten constants were determined at the optimum pH for each direction. K_m values determined for the oxidative deamination were 13.8 mM for L-alanine and 0.31 mM for NAD^+. The primary

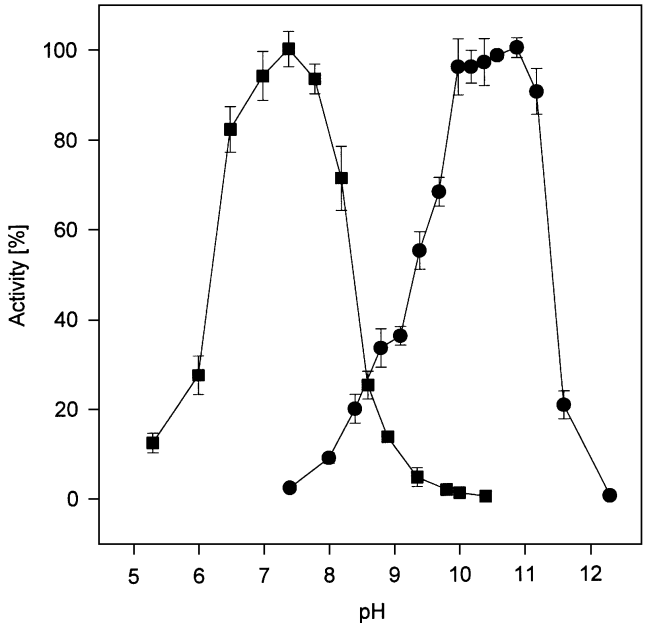

Figure 1 pH dependency

Mean values and standard deviations are shown from two experiments. Squares (■) symbolize the reductive amination and circles (●) symbolize the oxidative deamination. The pH at which maximum activity was observed for each direction was set as 100%. The buffers used for the various pH ranges are listed in the Materials and methods section.

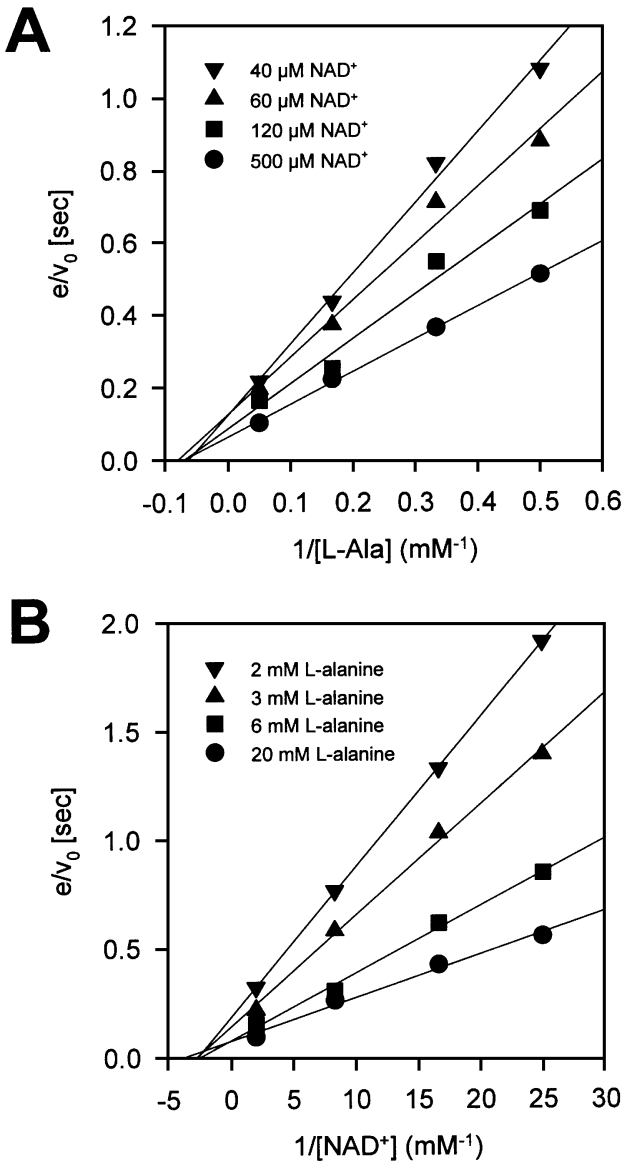

Figure 2 Kinetic analysis of the oxidative deamination

Lineweaver–Burk plots of the oxidative deamination are shown. In panel (**A**) the NAD^+ concentration was kept constant and the L-alanine concentration was varied. In panel (**B**) the L-alanine concentration was kept constant and NAD^+ concentration was varied. In both panels the straight lines intersect at one point ($1/K_m$) on the *x*-axis, indicating a random-order mechanism. The *y*-axis parameters are e, enzyme concentration, and v_0, initial velocity.

plots are shown in Figure 2. Apparent K_m values in the reductive amination were 35.4 mM, 1.45 mM and 98.2 μM for ammonium, pyruvate and NADH, respectively. Maximum velocity (V_{max}) is slightly higher in the reductive amination (31.8 units/mg) than in the oxidative deamination (23.7 units/mg).

Substrate specificity and effectors

The specificity of many L-alanine dehydrogenases is fairly weak, and often compounds structurally similar to L-alanine and pyruvate are converted into their corresponding products. L-Alanine can be replaced by D-alanine, L-serine or L-α-aminobutyrate for some enzymes [13–16]. Replacements for pyruvate are even more numerous and include β-hydroxypyruvate, α-

Table 1 Substrate specifity

Maximal activities measured with the natural substrates in the standard assays were set as 100%. Analogues were used to substitute for the natural substrates in the same molar concentrations (100 mM for analogues of L-alanine; 1.25 mM for NAD^+; 20 mM for pyruvate and 0.5 mM for NADH). The activities are the mean values of three independent experiments.

Oxidative deamination		Reductive amination	
Substrate	Activity (%)	Substrate	Activity (%)
L-Alanine	100	Pyruvate	100
D-Alanine	0	β-Hydroxypyruvate	1.2
L-Serine	0	α-Oxobutyrate	1.2
L-2-Aminobutyrate	0.7	α-Oxocaproate	1.1
		α-Oxoisocaproate	1.5
NAD^+	100	NADH	100
$NADP^+$	2.5	NADPH	1.6

oxobutyrate and derivatives of valerate and caproate (= hexanoate) [11,15,17,18]. NAD^+ can be replaced by $NADP^+$ [19]. In contrast, the recombinant L-alanine dehydrogenase of *M. tuberculosis* was highly specific for all of its substrates, and no significant conversion of any analogue tested was observed (Table 1). Also, glutamine and aspargine were unable to donate an ammonium group in the reductive amination. In fact, we have not found any substrate that is accepted apart from the natural substrates L-alanine and pyruvate. This specificity is in contrast with many other well-characterized alanine dehydrogenases [11,17] and may be interesting for the technical use of the enzyme in the production of L-alanine [20,21] or in medical diagnostics for the assays of dipeptidase [22], γ-glutamyltransferase [23] or γ-glutamylcyclotransferase [24].

Some metals are potent inhibitors of alanine dehydrogenases. However, their effect differs a lot depending on the origin of the enzyme. A metal ion that causes a strong inhibition of one enzyme may only slightly alter the activity of another form [25]. The recombinant L-alanine dehydrogenase of *M. tuberculosis* is sensitive to inhibition by $CuSO_4$ and $ZnCl_2$. $CuSO_4$ added to a final concentration of 1 mM inhibits the activity by 50.7%; at a concentration of 20 mM it inhibits by 96.3%. Inhibition by $ZnCl_2$ is slightly weaker, inhibiting the enzyme activity by 26.5% and 90.1% at 1 mM and 20 mM, respectively. Addition of EDTA in a 10-fold molar excess restores activity almost completely (92.9% for $CuSO_4$ and 99.7% for $ZnCl_2$).

Thermodynamic parameters

A feature of many alanine dehydrogenases is their elevated stability towards higher temperatures [14,26]. The recombinant enzyme of *M. tuberculosis* is also relatively stable, losing only approx. 25% of its total activity after 4 h at 60 °C. The melting point of the enzyme (the temperature at which the enzyme loses 50% of its total activity after an incubation period of 5 min) is 65.7 °C. Melting points of 86.0 °C and 63.0 °C were reported for the enzymes from *Thermus thermophilus* and *Bacillus subtilis*, respectively [27]. The temperature dependency is a parameter that characterizes the initial velocity of a reaction at a defined temperature. Since the velocity is highest at the very beginning of a reaction, inactivation that occurs later due to thermal instability has no impact on this term. The recombinant L-alanine dehydrogenase of *M. tuberculosis* is much more active at temperatures above 37 °C, particularly in the reductive amination. The velocity almost doubles at temperatures between 60–65 °C compared with 37 °C. Above 65 °C there is a sharp decrease in activity, due to thermal inactivation of the enzyme.

Alanine dehydrogenases are involved in a wide variety of metabolic processes (for a review see [28]). For some bacteria this enzyme is the link between carbon and amino acid metabolism, providing the cell with both nitrogen and/or energy [29–31]. In *B. subtilis* the L-alanine dehydrogenase is essential for normal sporulation [32]. Often the expression of this enzyme is correlated with entry into certain growth phases [33,34]. For *Mycobacterium smegmatis* an alanine dehydrogenase has been described that may be involved in the maintenance of the NAD pool during the shift to an anaerobic dormant state in which oxygen as a terminal electron acceptor becomes limited [35]. Another L-alanine dehydrogenase has been recently described for the *Mycobacterium* strain HE5 [36]. This enzyme, like the one from *M. smegmatis* [35], but in contrast with the enzyme described in the present paper, can be found intracellularly. It is characterized by its tetrameric structure, and its K_m values reflect its proposed function in ammonia assimilation during growth on morpholine and other heterocyclic xenobiotics [36]. In contrast, the enzyme characterized in the present paper is detectable early during aerobic growth and does not need any inducer. It is secreted into the culture medium, where it is present in large amounts. Hence it seems to be of different nature than the enzymes characterized in *M. smegmatis* and *Mycobacterium* strain HE5.

It has been proposed that the 40 kDa antigen of *M. tuberculosis* might be involved in peptidoglycan biosynthesis. Although direct evidence for this assumption is still missing, all the data so far support this hypothesis. The biochemical results suggesting that L-alanine is the product of the *in vivo*-catalysed reaction and the localization of the enzyme in the cell wall and the culture medium [8,37] make the hypothetical role of this enzyme in peptidoglycan biosynthesis seem quite reasonable. However, conclusive genetic evidence is still missing. In addition, it can be assumed that the enzyme is not essential, since other members of the *M. tuberculosis* Complex, like *M. bovis* and M. *bovis* BCG [7], lack a functional L-alanine dehydrogenase. *E. coli*, for example, has at least four pathways to synthesize L-alanine [38], none of them involving an L-alanine dehydrogenase. Thus, the L-alanine dehydrogenase of *M. tuberculosis* seems to be restricted for very special metabolic processes in this bacterium.

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