Increased alanine dehydrogenase activity during dormancy in
Mycobacterium smegmatis

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Abstract

The aerobic fast-growing Mycobacterium smegmatis has, like its slow-growing pathogenic counterpart M. tuberculosis, the capability to adapt to anaerobiosis by shifting down to a drug resistant dormant state. Here, we report the identification of the first enzyme, L-alanine dehydrogenase, whose specific activity is increased during dormancy development in M. smegmatis. This mycobacterial enzyme activity was previously identified as the 40-kDa antigen in M. tuberculosis and shows a preference for the reductive amination of pyruvate to alanine at physiological pH. The determination of the temporal profile of alanine dehydrogenase activity during dormancy development showed that the activity stayed at a low baseline level during the initial aerobic exponential growth phase (0.7 mU mg⁻¹ min⁻¹). After termination of aerobic growth, alanine dehydrogenase activity increased rapidly 5-fold. As oxygen becomes more and more limiting, the enzyme activity declined until it reached a level about two-third that of the peak value. The strong induction immediately after deflection from aerobic growth suggests that alanine might be required for the adaptation from aerobic growth to anaerobic dormancy. As alanine synthesis is coupled to NADH oxidation, we propose that the induction of alanine dehydrogenase activity might also support the maintenance of the NAD pool when oxygen as a terminal electron acceptor becomes limiting. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Mycobacterium tuberculosis is a pathogen capable of causing both an acute disease and an asymptomatic latent infection. In the latent infection, dormant tubercle bacilli persist for years before reviving and resulting in reactivation of tuberculosis. Our current arsenal of drugs for treating active tuberculosis is relatively ineffective against the latent form ([1], for review). Lawrence Wayne has conducted pioneering studies of the dormancy of M. tuberculosis ([2] and references therein). In the Wayne model, cultures of M. tuberculosis are subjected to gradual oxygen depletion by incubation in sealed containers with controlled agitation. Growth under such conditions leads to a physiologically well-defined anaerobic, drug resistant, non-replicating synchronised state of the tubercle bacillus [3]. The Wayne model has clinical correlations with human anaerobic latent lesions containing dormant bacilli [4].

Recently, we demonstrated that the physiological
behaviour of the fast-growing saprophyte *M. smegmatis*, under the oxygen-depletion conditions of the Wayne model, is strikingly similar to that shown by the slow-growing pathogenic *M. tuberculosis* [5]. Furthermore, pulse labelling experiments indicated that the bacilli in the dormant state show low level RNA [5] and protein synthesis (Dick, unpublished observations). This indicates that the dormant bacilli are not metabolically inactive but maintain a low level metabolism.

We are interested in the molecular mechanisms of oxygen depletion-induced dormancy and we use *M. smegmatis* as a fast-growing non-pathogenic model. Our present knowledge of the machinery involved in dormancy is limited [6-9]. One of the central questions is how the bacilli recycle NADH, derived from their catabolic activities, under oxygen limiting conditions. *M. tuberculosis* L-alanine dehydrogenase (EC 1.4.1.1, 40-kDa antigen [10,11]) catalyses the reductive amination of pyruvate to alanine under physiological pH [12,13]. We report here that the specific activity of alanine dehydrogenase during dormancy development was increased. We propose that the enzyme, whose suggested function is the generation of alanine for protein and peptidoglycan synthesis, may play an additional role in maintaining the NAD pool under oxygen limiting conditions.

2. Materials and methods

2.1. Strain and cultivation

All experiments were conducted with *M. smegmatis* mc²155 [14] in Dubos Tween-albumin broth (Difco) at 37°C. Anaerobic dormant and aerobic growing *M. smegmatis* cultures were prepared and monitored as described previously [5,3]. Briefly, screw-cap test tubes, 20 × 125 mm, with a total fluid capacity of 25.5 ml were used as containers. An early exponential phase preculture was diluted to 10⁶ c.f.u. ml⁻¹ in a total volume of 17 ml. For sealed dormancy cultures, solid caps with latex liners were tightly screwed down and the tubes were aerated by vigorous shaking at 250 r.p.m. until they reached early exponential phase (2 × 10⁷ c.f.u. ml⁻¹). Growth and survival of the bacterial populations were monitored by viable count measurements. Microscopic examination of the culture samples before plating showed that significant clumping affecting c.f.u. determination did not occur.

2.2. Preparation of extracts, assay of protein, in situ and photometric enzyme assays

Protein extracts were prepared according to Basu et al. using glass beads to break up the cells [15]. Protein concentrations were determined using a Bio-Rad kit as recommended by the supplier. Alanine dehydrogenase activity was visualised in non-denaturating polyacrylamide gels using nitroblue tetrazolium and phenazine methosulfate as described previously [11]. For the detection of other NAD-dependent amino acid dehydrogenase activities, gels were incubated as described for the alanine dehydrogenase with the respective amino acid, in a buffer of 20 mM Tris-HCl, pH 8.0 [16]. The photometric alanine dehydrogenase assay is based on measurement of the rate of reduction of NAD to NADH that accompanies the oxidative deamination of alanine to pyruvate. Units of specific activity of alanine dehydrogenase are expressed as micromoles of NAD reduced per min per milligram of protein [17]. For the photometric determination of the reductive amimation of pyruvate to alanine the reaction mixture contained 20 mM Tris-HCl, pH 8.0, 20 mM pyruvate and 0.5 mM NADH. In order to determine the pH dependence of the reactions the following buffers were used: 40 mM Na₂HPO₄/NaH₂PO₄, pH 7.0; 20 mM Tris-HCl, pH 8.0; 20 mM Tris-HCl, pH 9.0; 125 mM glycine-KOH, pH 10.2. For the photometric measurement of proline dehydrogenase activity alanine was substituted by proline and 20 mM Tris-HCl, pH 8.0, was used as buffer. To detect glycine dehydrogenase activity, an assay based on optical measurement of the rate of oxidation of NADH to NAD that accompanies the reductive amination of glyoxylate to glycine was used [18,19]. The activities of all amino acid dehydrogenases were measured against a cuvette containing the reaction mixture without the respective substrate.
3. Results and discussion

3.1. Alanine dehydrogenase activity is upregulated during dormancy

To assess whether L-alanine dehydrogenase activity can be detected in *M. smegmatis* and whether there is a difference in the enzyme levels between aerobic growing and anaerobic dormant cultures, protein extracts from both cultures were analysed by an in situ activity assay after electrophoresis in non-denaturing polyacrylamide gels. Fig. 1B (lanes 1 and 2) shows that alanine dehydrogenase activity was detectable in the extract from the aerobic growing culture and that the activity was increased in the extract from the anaerobic dormant culture. Fig. 1A shows an identical gel incubated in the substrate solution without alanine. No reaction was observed, showing that the bands detected in Fig. 1B are alanine dependent. These results demonstrate that *M. smegmatis* contains alanine dehydrogenase and that this activity is upregulated in the anaerobic non-replicating persistent culture.

In order to analyse the temporal profile of alanine dehydrogenase activity during dormancy development, we determined the enzyme activity under sealed dormancy culture conditions at various time points. Fig. 2 shows that the activity stayed at a low baseline level during the initial aerobic exponential growth phase (0.7 mU mg⁻¹ min⁻¹). After termination of aerobic growth, alanine dehydrogenase activity increased rapidly to a level 5-fold greater than the baseline level. As oxygen became more and more limiting, the enzyme activity declined until it reached a level about two-third that of the peak value. These results show that alanine dehydrogenase activity is strongly upregulated immediately upon oxygen limitation-induced termination of growth. Then the level declines but stays 3.5-fold above the baseline level seen during aerobic growth. The strong induction
immediately after deflection from aerobic growth – compared to the less pronounced induction during anaerobic dormancy – suggests that the enzyme might play a role in the adaptation from aerobic growth to anaerobic dormancy. What might this physiological role be?

Alanine dehydrogenase from \textit{M. tuberculosis} catalyses the reductive amination of pyruvate at physiological pH [12,13]. To demonstrate that the \textit{M. smegmatis} enzyme shows similar pH dependence, activity measurements at various pHs (pH 7.0–10.2) were carried out. These analyses showed that the velocity of oxidative deamination of alanine at physiological pH was only about 10% compared to the velocity at pH 10.2. In contrast, the pH optimum for the reductive amination of pyruvate was pH 7–8. This suggests that the metabolic function of the enzyme is the generation of alanine. Alanine is required for protein and peptidoglycan synthesis. Therefore, the induction of alanine dehydrogenase activity might indicate the requirement of protein and/or peptidoglycan synthesis for the development of the persistent dormant form of the bacilli. The generation of alanine is accompanied by the oxidation of NADH. This aspect of alanine synthesis might play a role under the oxygen limiting conditions encountered after deflection from aerobic growth and later during anaerobic dormancy. Therefore, we propose that the induction of alanine dehydrogenase activity might also support the maintenance of the NAD pool when oxygen as terminal electron acceptor becomes limiting.

3.2. Proline dehydrogenase activity is growth phase independent and glycine dehydrogenase activity is not detectable

In addition to alanine dehydrogenase, two other NAD-dependent amino acid dehydrogenases have been identified in \textit{M. tuberculosis}. A putative proline dehydrogenase gene (EC 1.5.99.8) was discovered in the genome project [20]. Glycine dehydrogenase activity (EC 1.4.1.10) was discovered by Goldman and Wagner [18]. To assess whether proline and glycine dehydrogenase activities can be detected in \textit{M. smegmatis}, extracts from aerobic cultures and anaerobic dormant cultures were analysed in non-denaturing polyacrylamide gels. In addition the extracts were tested for NAD-dependent dehydrogenase activities for the other 17 amino acids found in proteins. The only amino acid dehydrogenase detectable was proline dehydrogenase showing the same staining in growing and dormant culture (Fig. 1C). Photometric quantification of proline dehydrogenase revealed a growth phase independent activity of 0.3 mU min$^{-1}$ mg$^{-1}$. Photometric glycine dehydrogenase assays confirmed the absence of glycine dehydrogenase activity in \textit{M. smegmatis}.

Wayne and coworkers demonstrated recently an increase of glycine dehydrogenase activity during oxygen depletion induced dormancy in \textit{M. tuberculosis} and the authors proposed that the reductive amination of glyoxylate to glycine could provide one of the mechanisms used by the bacilli to recycle their NADH under oxygen limiting conditions [3,19]. In this work we demonstrate the upregulation of alanine dehydrogenase activity during oxygen depletion in \textit{M. smegmatis}. Whether alanine dehydrogenase activity is increased during dormancy in \textit{M. tuberculosis} or whether this enzyme activity substitutes the missing glycine dehydrogenase in \textit{M. smegmatis} during oxygen limitation remains to be elucidated.

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References


