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REGULATORY CONTROL AND FUNCTION OF ALANINE DEHYDROGENASE FROM A THERMOPHILIC BACILLUS

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Summary

L-alanine dehydrogenase, (L-alanine:NAD^+ oxidoreductase (deaminating), EC 1.4.1.1) synthesis in a thermophilic bacillus was found to be subjected to regulatory control. Addition of L- and D-alanine and L-serine to cultures growing in the presence of either succinate or pyruvate, induced an accelerated synthesis of the alanine dehydrogenase enzyme. Synthesis of the enzyme was dependent on the presence of inducer during growth and was arrested by addition of glucose. Catabolite repression by glucose was abolished by limiting the ammonium concentration during growth. The apparent K_m values of the substrates involved in alanine dehydrogenase activity are as follows (M): NH_4^+, $4 \cdot 10^{-2}$; pyruvate, $5 \cdot 10^{-4}$; NADH, $6 \cdot 10^{-5}$; L-alanine, $3.1 \cdot 10^{-3}$ and NAD, $2 \cdot 10^{-4}$. Alanine dehydrogenase activity was measurable at temperatures below the minimal growth temperature (at 25°C) and the highest activity was found at 65°C; heat denaturation occurred at 80°C.

Introduction

Alanine dehydrogenase (L-alanine:NAD^+ oxidoreductase (deaminating), EC 1.4.1.1) was studied in several mesophilic microorganisms [1—8]. The enzyme was found in vegetative cells [9], as well as in spores [8]. In vitro bacterial alanine dehydrogenase was found to catalyze both the reductive amination of pyruvate to L-alanine (in the presence of NH_4^+ and NADH; anabolic reaction) and the reversible reaction, i.e. the oxidative deamination of L-alanine to pyruvate (in presence of NAD; catabolic reaction). In situ, however, regardless whether in vegetative cells or in spores, the catabolic nature of the enzyme was apparent [10]. Alanine dehydrogenase formation in various

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mesophilic bacteria was found to be repressed in the presence of glucose [9] and formation of the enzyme was shown to be inducible by L- and D-alanine as well as by certain other L- and D-amino acids [11]. On the other hand, in an alanine racemase-negative mutant of *Bacillus subtilis* only D-alanine and a few other D-amino acids were able to induce alanine dehydrogenase formation, whereas L-alanine and some other L-amino acids failed to do so; thus, D-alanine and not the L-isomer seemed to be the alanine dehydrogenase inducer [10].

This report deals with the regulatory properties of the alanine dehydrogenase of a thermophilic bacillus, the inducibility of the enzyme, its sensitivity to catabolite repression and conditions permitting escape from this control.

Materials and Methods

Cultivation of the microorganism. The previously described thermophilic bacillus [12] was grown in a minimal salt medium to which the appropriate carbon source (glucose, succinate, pyruvate, or others as mentioned in the text) was added aseptically at a final concentration of 0.5%. Growth conditions, unless otherwise stated, were similar to those described earlier [13].

Preparation of the cell-free extract. The technique used was described previously [13]. Protein was estimated by the method of Lowry et al. [14].

Alanine dehydrogenase activity. Alanine dehydrogenase activity was measured by recording the changes in absorbance at 340 nm due to the oxidation of NADH or reduction of NAD (and using a Varian Techtron UV-VIS spectrophotometer Model 635). Unless otherwise specified, the compositions of the reaction mixtures for the reductive amination of pyruvate were as follows (μmol/0.9 ml): HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethansulfonic acid)/potassium hydroxide (pH 8.0), 50; NH_4Cl, 100; pyruvate, 20; and NADH, 0.2. For oxidative deamination of L-alanine (μmol/0.9 ml): HEPES/KOH buffer (pH 9.4), 50; L-alanine, 50; and NAD, 0.33. After equilibration at the specified temperature, 0.1 ml of the appropriately diluted enzyme preparation was added to start the reaction. In the reference beam, the substrate (L-alanine or pyruvate, respectively) was omitted from the reaction mixture. One alanine dehydrogenase unit catalyzes the oxidation of 1 μmol NADH per min at 55°C under the assay conditions. Specific activities are given in units per mg protein.

Partial purification of the thermophilic alanine dehydrogenase. Using glucose-grown cells and the procedure employed for purification of the thermophilic glutamate dehydrogenase described earlier [13], a 28-fold purification of the thermophilic alanine dehydrogenase was achieved after the second $(NH_4)_2SO_4$ precipitation with recovery of 44%; activities of 2 μmol/mg protein per min were obtained.

Determination of heat stability of the alanine dehydrogenase. The partially purified (28-fold) enzyme preparation was incubated at temperatures and time intervals as indicated and the samples taken were cooled rapidly to 4°C prior to determination of activities.

Effect of pH and temperature on alanine dehydrogenase activity. The effect of pH (at the range between 6.4 and 10.4) and temperature (between 25 and 75°C) on the activity of the partially purified thermophilic alanine dehydrogenase was carried out using reaction mixtures (see alanine dehydrogenase assay

conditions) in which the buffer was substituted with a HEPES and PIPES (piperazine-*N*-*N*'-bis[2-ethansulfonic acid]) combination, 20 μmol each, and was titrated with KOH to the desired pH value.

Results

Effect of pH on alanine dehydrogenase activity. Using the partially purified alanine dehydrogenase, the effect of pH (in the range between pH 6.4 and 10.4) on the activity of the enzyme was tested. At 55°C reductive amination of pyruvate was maximal at pH values between 8.0 and 8.4, while the oxidative deamination of L-alanine was optimal in the much higher pH range of 9.8—10.2.

Heat stability of the partially purified alanine dehydrogenase. Almost no loss of alanine dehydrogenase activity was noticed after heating the enzyme at 70°C for as long as 180 min. At 80°C, however, complete destruction of the activity occurred after only 5 min (Fig. 1).

Effect of temperature on alanine dehydrogenase activity. Effect of temperature (between 25 and 75°C) and pH (between 6.4 and 10.4) on the activity of a partially purified preparation was tested. Amination of pyruvate by the purified thermophilic alanine dehydrogenase occurred even at temperatures as low as 25°C (the lowest temperature tested), and deamination of alanine could be detected already at 30°C. Thus, both activities could be demonstrated at temperatures below the minimal (approx. 40°C) and above the optimal (58°C) growth temperatures of the thermophilic bacillus. The influence of temperature on the amination of pyruvate and deamination of alanine by the thermophilic alanine dehydrogenase indicate respectively the following: (a) The optimal temperature for both reactions was at 65°C; (b) alanine dehydrogenase activity decreased at temperatures between 65 and 75°C although thermal inactivation started only near 80°C (Fig. 1).

Substrate specificity and apparent K_m values of the alanine dehydrogenase. The enzyme is specific for NADH (and NAD), while NADPH (or NADP) was inactive as coenzyme (2 μmol of NADPH per reaction in a 2-mm light path cuvette and 6 μmol of NADP per reaction were the highest concentrations test-

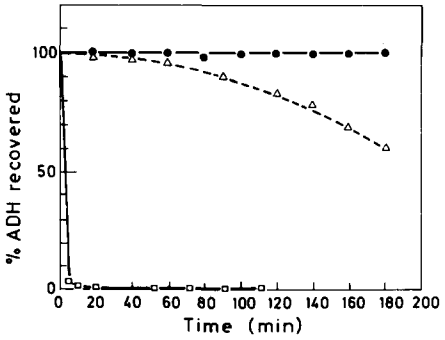

Fig. 1. Heat stability of the alanine dehydrogenase. The partially purified enzyme preparation was incubated in a HEPES/KOH buffer 0.02 M (pH 7.2) at the following temperatures: ●———●, 70°C; △———△, 75°C and □———□, 80°C. Samples of the heat-treated enzyme were taken at time intervals as indicated, cooled rapidly to 4°C and the residual alanine dehydrogenase activity was determined at 55°C.

TABLE I

APPARENT K_m VALUES FOR SUBSTRATES OF THE ALANINE DEHYDROGENASE FROM THE THERMOPHILIC BACILLUS

K_m values were calculated according to the method of Lineweaver and Burk [15].

Substrate	K_m (M)
NH_4^+	$4.0 \cdot 10^{-2}$
Pyruvate	$5.0 \cdot 10^{-4}$
NADH	$6.0 \cdot 10^{-5}$
L-alanine	$3.1 \cdot 10^{-3}$
NAD	$2.0 \cdot 10^{-4}$

ed). The apparent K_m values of the substrates involved in the alanine dehydrogenase reaction (at 55°C), listed in Table I, were calculated from the double reciprocal plot according to the method of Lineweaver and Burk [15]. The K_m values for L-alanine and NAD were about 10 and 3 times greater than for pyruvate and NADH, respectively; the K_m value for NH_4^+ was found to be very high ($4 \cdot 10^{-2}$ M, Table I). Nevertheless, at all temperatures tested (between 25 and 75°C) the maximal velocity (V) of the reductive amination of pyruvate was 10 times greater than that of the oxidative deamination. These findings will be discussed with regard to the physiological role of the alanine dehydrogenase.

Induction of alanine dehydrogenase by L-alanine. In the absence of added inducer, very low alanine dehydrogenase activity could be detected in cultures of

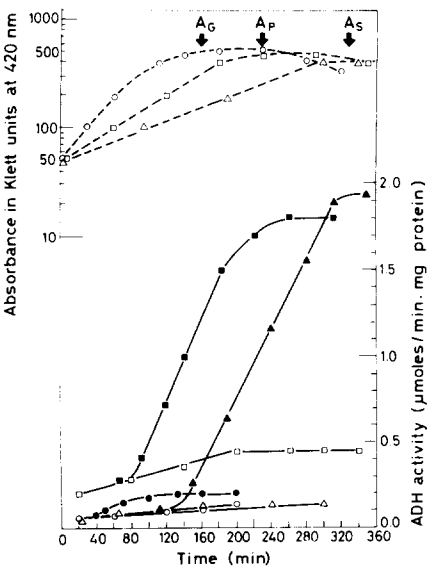

Fig. 2. Effect of medium composition on alanine dehydrogenase induction. The thermophilic bacillus was grown in a minimal salt medium containing NH_4^+ ($1 \cdot 10^{-2}$ M) as the nitrogen source and one of the following carbon sources ($5 \cdot 10^{-3}$ M): glucose (○———○), succinate (△———△), and pyruvate (□———□). As the cultures reached a turbidity of 100 Klett units (broken line), L-alanine ($1 \cdot 10^{-2}$ M) was added to the various cultures containing glucose (●———●), succinate (▲———▲) and pyruvate (■———■). At times indicated by arrows the concentration of the respective carbon sources was doubled. At intervals, samples were taken from each of the cultures, and alanine dehydrogenase activity was determined.

TABLE II

EFFECT OF VARIOUS COMPOUNDS ON THE INDUCTION OF THE THERMOPHILIC ALANINE DEHYDROGENASE

The cells were grown at 58°C in the presence of succinate and NH_4^+ as sole carbon and nitrogen sources, respectively. ADH concentration was determined 60 min after addition of the listed compounds to the culture and before maximal induction was obtained.

Compound added ($5 \cdot 10^{-3}$ M)	Alanine dehydrogenase concentration (units *)
None	15
Pyruvate	16
L-alanine	103
D-alanine	167
L-serine	87
D-serine	14
2-methyl α-aminopropanol	15
Propionamide	15
α-Amino isobutyrate	16
β-Alanine	15
N-Benzyloxycarbonyl L-alanine	15
N-Benzyloxycarbonyl D-alanine	18
N-Benzyloxycarbonyl L-serine	16

* One alanine dehydrogenase unit was defined as the amount of alanine dehydrogenase enzyme which catalyzes the oxidation of 1 μmol of NADH per min in the presence of pyruvate ($2 \cdot 10^{-2}$ M) and NH_4Cl ($1 \cdot 10^{-1}$ M), pH 8.0 at 55°C.

the thermophilic bacillus grown in presence of glucose or succinate (Fig. 2). On the other hand, a noticeable alanine dehydrogenase level was found in the pyruvate-grown culture. Addition of L-alanine ($1 \cdot 10^{-2}$ M) to cultures, grown on NH_4^+ and either succinate or pyruvate, induced rapid increase of alanine dehydrogenase synthesis which was seen as soon as half a generation after addition of inducer. A 20-fold increase in the specific activity of the enzyme was noted using succinate-grown cultures, whereas only a 2- to 5-fold increase was seen in glucose- and pyruvate-grown cultures, respectively. Irrespective of the carbon source used (glucose, succinate, pyruvate) the specific activity of the alanine dehydrogenase increased simultaneously with growth, and reached maximal concentration as the culture approached stationary phase (Fig. 2). The same results were obtained on doubling the concentration of the respective carbon sources during stationary phase (to avoid carbon starvation).

Catabolite repression of the thermophilic alanine dehydrogenase. Among the substances tested (Table II), only L-alanine, D-alanine and L-serine induced alanine dehydrogenase synthesis by succinate-grown cultures; in contrast to L-alanine the latter two did not serve as substrates for alanine dehydrogenase. No unmetabolizable inducer of the alanine dehydrogenase system was found (Table II); neither β-alanine nor *N*-benzyloxycarbonyl derivatives of L- and D-alanine and L-serine were effective as inducers. Also ineffective were propionamide, 2-methyl-2-amino propanol, and α-aminoisobutyrate, the latter of which is known to permeate into the thermophilic bacillus through the same transport system as L-alanine [16]. Among the compounds which induced alanine dehydrogenase synthesis (L-alanine, D-alanine and L-serine), D-alanine was at least as effective as L-alanine, and in concentrations as low as $1 \cdot 10^{-3}$ M it in-

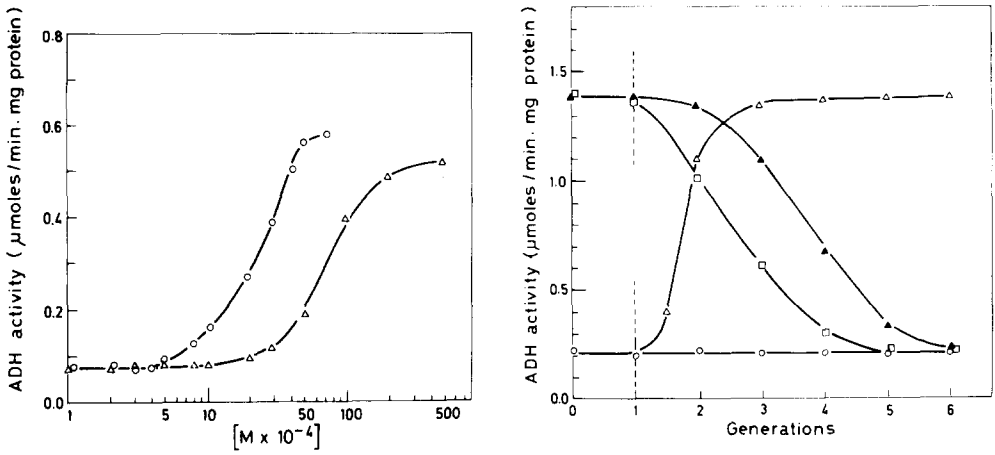

Fig. 3. Effect of L- and D-alanine on the induction of the thermophilic alanine dehydrogenase. Cells were grown in a minimal salt medium containing succinate (0.5%) and NH_4Cl ($1 \cdot 10^{-2}$ M). As the cultures reached a turbidity of 100 Klett units (420 nm) either L-alanine (△———△) or D-alanine (○———○), at the specified concentrations were added to the cultures. 60 min after addition of the inducer, 5-ml samples from each culture were filtered through membrane filters and alanine dehydrogenase activity was determined.

Fig. 4. Effect of glucose and removal of D-alanine on alanine dehydrogenase synthesis. Cultures were grown at 58° C in a minimal salt medium containing succinate (0.5%) and NH_4Cl ($1 \cdot 10^{-2}$ M) without inducer (○———○), and in the presence of D-alanine ($5 \cdot 10^{-3}$ M) (▲———▲). At the time indicated by the vertical dashed line, D-alanine ($5 \cdot 10^{-3}$ M) was added to one culture (△———△) and removed by millipore filtration from another (□———□). Glucose (0.5%) (▲———▲) was added to a culture containing D-alanine. Samples, from each culture were taken at times indicated and the content of the alanine dehydrogenase was determined.

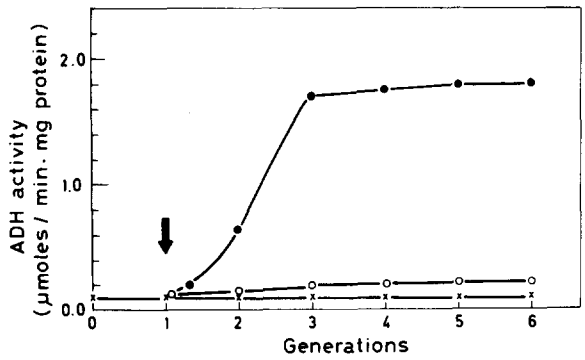

Fig. 5. Escape from catabolite repression by NH_4^+ limitation. Cultures of the thermophilic bacillus were grown (58° C) in a minimal salt medium containing glucose (0.5%) and NH_4Cl ($1 \cdot 10^{-2}$ M) as carbon and nitrogen sources, respectively, X———X. When the cultures reached a turbidity of 200 Klett units (420 nm), they were filtered through membrane filters and resuspended to a turbidity of 100 Klett units in a minimal salt medium containing the following: glucose (0.5%) and NH_4Cl ($1 \cdot 10^{-2}$ M) (X———X); glucose, NH_4Cl and L-alanine ($1 \cdot 10^{-2}$ M) (○———○); and glucose and L-alanine ($1 \cdot 10^{-2}$ M) but no ammonia (●———●). When the turbidity of each culture was doubled (after one generation) they were diluted to the initial turbidity (100 Klett units) and samples were taken for alanine dehydrogenase determination.

duced enzyme synthesis, while as much as $5 \cdot 10^{-3}$ M L-alanine was needed to produce the same effect (using succinate-grown cultures after 60 min at 58°C) (Fig. 3).

Addition of glucose to an induced culture arrested all further alanine dehydrogenase synthesis and the pre-existing enzyme was diluted among the progeny cells (Fig. 4). Similar results were obtained upon removal of the inducer from succinate-grown cultures.

Escape from catabolite repression in the absence of NH_4^+. Only negligible alanine dehydrogenase activity was noted when the thermophilic bacillus was grown in the presence of glucose (0.5%) and NH_4^+ ($1 \cdot 10^{-2}$ M) (Fig. 2). However, when the cells were resuspended (after filtration) in an ammonia-free medium, but containing L-alanine, an immediate acceleration in the differential rate of alanine dehydrogenase synthesis began, leading to a 20-fold increase in the specific activity (Fig. 5). Thus, an escape from catabolite repression by glucose was achieved by removal of the NH_4^+ from the growth medium.

Discussion

Alanine dehydrogenase occupies an important role in the carbon and nitrogen metabolism of various microorganisms. It was therefore of interest to find out whether the alanine dehydrogenase of the thermophilic bacillus catalyzes the conversion of NH_4^+ into amino groups (anabolic function), or leads rather to the formation of pyruvate and its dissimilation (catabolic function). The following parameters were considered in an attempt to elucidate this question: (a) factors controlling alanine dehydrogenase synthesis; (b) the apparent K_m values and thus the availability of the various substrates of the alanine dehydrogenase-catalyzed reaction; (c) the optimal pH range required for the anabolic and catabolic reactions; and (d) rate constant relationship between the anabolic and catabolic reactions.

Most of the data brought in this study are in accordance with the view that the thermophilic alanine dehydrogenase serves a catabolic function, as was also found in various mesophilic microorganisms [1,2,7,9—11]. The pertinent findings are: (a) the susceptibility of alanine dehydrogenase synthesis in the thermophilic bacillus to catabolite repression by glucose (as was also found in mesophilic cells) [9]; (b) inducibility by L- and D-alanine and L-serine under conditions of weak catabolite repression (in succinate-grown cultures); (c) escape of the induced alanine dehydrogenase synthesis from catabolite repression (in presence of glucose) by NH_4^+ limitation; and finally (d) the very high apparent K_m value of $4 \cdot 10^{-2}$ M for NH_4^+ suggests that ordinarily the thermophilic enzyme does not participate in ammonia assimilation. Cells grown on glucose and ammonia show low alanine dehydrogenase activity, even in presence of inducer. On the other hand, formation of L-alanine should be favoured during growth on pyruvate and NH_4^+ as sole carbon and nitrogen sources. Under the latter conditions alanine dehydrogenase was indeed formed in presence of inducer, and limited synthesis occurred even in its absence. However, as neither pyruvate nor NH_4^+ induce alanine dehydrogenase formation, the limited synthesis in absence of added inducer is not fully understood.

The straightforward picture of formation of a catabolic alanine dehydroge-

nase is complicated when one considers the pH optima of the anabolic and catabolic reactions. Deamination of L-alanine requires a very high pH (9.8—10.2), whereas L-alanine synthesis is optimal in a more physiological range of pH values (8.0—8.4). Similar results were reported for alanine dehydrogenase from mesophilic cells [6,9]. If the differences in pH between in vitro and in situ conditions are not very great, one has to ascribe anabolic activity to the essentially catabolic alanine dehydrogenase. Moreover, the reaction rate for L-alanine synthesis was 10 times faster than for deamination of this amino acid. Hong et al. [1] ascribed an anabolic function to the alanine dehydrogenase enzyme found in a number of mesophilic bacilli with no detectable glutamate dehydrogenase activity. These bacilli, which were defective in their ability to synthesize some intermediate of the citrate cycle, could grow in a minimal medium containing NH_4^+ and glucose, pyruvate or glycerol, provided they were supplemented with α-ketoglutarate or di- or tricarboxylic intermediates [17].

Induction of L-alanine dehydrogenase activity by both L- & D-alanine can be explained by presence of an alanine racemase (EC 5.1.1.1) in the thermophilic bacillus, shown by inhibition of its growth by *O*-carbamyl D-serine (an inhibitor of the racemase [18—20]), in the absence, but not in the presence of D-alanine (experiments not shown). Similar results were also obtained in other microorganisms [11]. In an alanine racemase-deficient mutant of *B. subtilis*, on the other hand, D-alanine alone (and not the L-isomer) induced alanine dehydrogenase formation [10]. Whether D-alanine is the true inducer of alanine dehydrogenase formation also in the thermophilic bacillus remains to be seen.

Regardless of whether the function of the thermophilic alanine dehydrogenase is anabolic or catabolic, enzymatic activity can be demonstrated in vitro at a temperature below the minimum required for growth (40°C). About 10 and 1% of the alanine dehydrogenase activity (activity at 58°C taken at 100%) were found at 30 and 25°C, respectively.

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