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An NAD$^+$-dependent alanine dehydrogenase from a methylotrophic bacterium

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A study was made of the NAD$^+$-dependent alanine dehydrogenase (EC 1.4.1.1) elaborated by the methylotrophic bacterium *Pseudomonas* sp. strain MA when growing on succinate and NH_4Cl. This enzyme was purified 400-fold and was found to be highly specific for NH_3 and NAD$^+$; however, hydroxypyruvate and bromopyruvate, but not α-oxoglutarate or glyoxylate, could replace pyruvate to a limited extent. The M_r of the native enzyme was shown to be 217000, and electrophoresis in SDS/polyacrylamide gels revealed a minimum M_r of 53000, suggesting a four-subunit structure. The enzyme, which has a pH optimum of 9.0, operated almost exclusively in the aminating direction *in vitro*. It was induced by NH_3 or by alanine, and was repressed by growth on methylamine or glutamate. It is suggested that this enzyme has two roles in this organism, namely in NH_3 assimilation and in alanine catabolism.

INTRODUCTION

The facultative methylotroph *Pseudomonas* sp. strain MA is able to utilize methylamine as a sole source of carbon, nitrogen and energy (Shaw *et al.*, 1966). Methylamine is converted first into *N*-methylglutamate, with concomitant liberation of NH_3, and the methyl group is subsequently oxidized to formaldehyde (Hersh *et al.*, 1971; Pollock & Hersh, 1971). Formaldehyde is assimilated into cell carbon via the isocitrate lyase variant of the serine pathway (Bellion & Hersh, 1972; Bellion & Woodson, 1975). A different facultative methylotroph, *Pseudomonas* sp. AM1, is also able to utilize methylamine; in this case a direct methylamine dehydrogenase (Eady & Large, 1968) liberates NH_3, and the resultant formaldehyde is incorporated into cell carbon by the isocitrate lyase-minus variant of the serine pathway (Large & Quayle, 1963; Salem *et al.*, 1973). Despite the different modes of oxidation of methylamine and carbon-assimilation pathways, the nitrogen of methylamine was shown to be assimilated into the cells in both species of bacteria via the sequential action of glutamine synthetase (EC 6.3.1.2) and glutamine:2-oxoglutarate aminotransferase (EC 1.4.1.13), that is by the 'GS/GOGAT' pathway (Bellion & Bolbot, 1983). This was the case whether methylamine was acting as both nitrogen and carbon source, or as nitrogen source only, with an alternative carbon source, such as succinate, present. However, when the cells were grown with NH_4Cl as sole nitrogen source, *Pseudomonas* AM1 elaborated an NADP$^+$-dependent glutamate dehydrogenase, whereas in *Pseudomonas* MA an NAD$^+$-dependent alanine dehydrogenase (EC 1.4.1.1) with pH optimum of 9.0 was elaborated also in high quantity. Paradoxically, the amount of biosynthetically active glutamine synthetase was diminished by only 50% in each cell type (Bellion & Bolbot, 1983), thereby creating apparent redundancy by having two enzymes present to perform the same task. This is in contrast with the situation in the enterobacteria, where the amount of biosynthetically active glutamine synthetase falls to essentially zero under similar conditions (Brenchley *et al.*, 1975; Tyler, 1978; Dalton, 1979). In an attempt to understand these phenomena more clearly, particularly the roles of the amino acid dehydrogenases in NH_3-grown cells, we embarked on a study of the various enzymes involved with nitrogen assimilation in methylotrophic organisms. We have previously described the results of our studies on the NADP$^+$-dependent glutamate dehydrogenase (EC 1.4.1.4) of *Pseudomonas* AM1 (Bellion & Tan, 1984), and we now report the results of our studies with the inducible NAD$^+$-dependent alanine dehydrogenase of *Pseudomonas* MA.

Alanine dehydrogenases have previously been studied in several *Bacillus* species in connection with the role of the enzyme in the sporulation process (Freese & Oosterwyk, 1963; McCormick & Halvorson, 1964; Yoshida & Freese, 1964, 1965; McCowen & Phibbs, 1974), but Meers & Pedersen (1972) found no evidence of involvement of the enzyme in primary NH_3 assimilation in *B. licheniformis*. It has also been studied in several different species where its role is more clearly in the assimilation of NH_3. These include *Anabaena cylindrica* (Rowell & Stewart, 1976), *Rhodopseudomonas capsulata* (Johansson & Gest, 1976), *Streptomyces clavuligerus* (Aharonowitz & Friedrich, 1980) and *Thiobacillus neopolitanus* (Beudeker *et al.*, 1982), although the enzyme amounts in the last organism were very low. Murrell & Dalton (1983) found an alanine dehydrogenase in *Methylococcus capsulatus* (Bath) after growth on methane and ammonia that could utilize both NAD$^+$ and NADP$^+$.

MATERIALS AND METHODS

Growth and maintenance of organism

Pseudomonas MA (A.T.C.C. 23819) was grown in 800 ml batches of minimal salts medium (Bolbot & Anthony, 1980) at 30 °C in baffled shake flasks.

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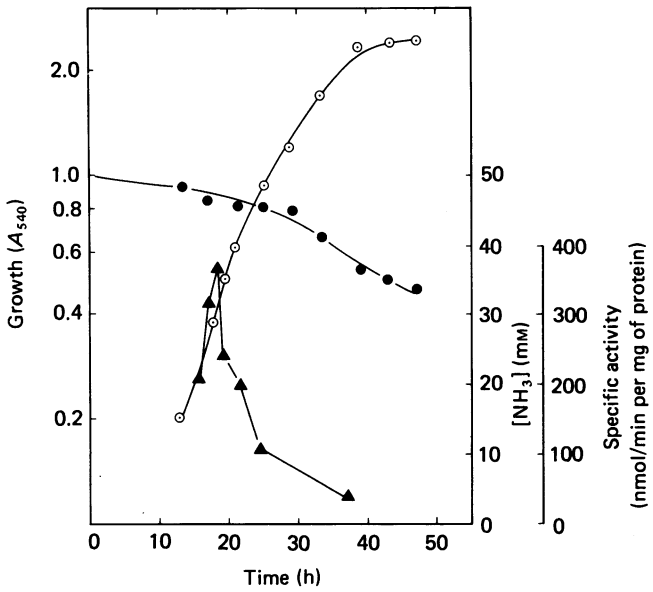

Fig. 1. Alanine dehydrogenase activity and NH_4Cl concentration during growth of *Pseudomonas* sp. strain MA on succinate plus 50 mM-NH_4Cl

⊙, A_{540}; ●, [NH_3]; ▲, specific activity of alanine dehydrogenase.

Succinate or methylamine was used as carbon source, and NH_4Cl or methylamine as nitrogen source. Except as otherwise noted, the concentration of carbon and nitrogen sources used was 50 mM, and growth was initiated with a 1–2% inoculum of cells grown on the same substrates.

Alanine dehydrogenase was purified from cells grown in 10 litre batches of minimal salts medium, containing succinate/NH_4Cl, under forced aeration. Growth was monitored with a Bausch and Lomb Spectronic 20 instrument, at 540 nm with a 1 cm-light-path cuvette.

Cells were harvested by centrifugation at early exponential phase ($A_{540} = 0.4$–0.45), when alanine dehydrogenase activity was highest (Fig. 1), and were washed twice with 20 mM-potassium phosphate buffer (pH 7.2). The cells were then quickly frozen in liquid N_2 and stored at −20 °C until needed.

Preparation of cell extracts

Frozen cells were thawed and resuspended at a ratio of 1 g wet wt. of cells/2 ml of TED buffer [50 mM-Tris/HCl (pH 8.0)/0.1 mM-EDTA/0.5 mM-dithiothreitol]. The cells were disrupted by passage twice through a pre-cooled French pressure cell (American Instrument Co.) at 138 MPa (20000 lb/in²). The exudate was centrifuged at 30000 *g* for 40 min at 0 °C and the pellet discarded.

Protein assay

Protein was determined by the dye-binding method of Bradford (1976). Crystalline bovine serum albumin was used as the reference standard.

Determination of NH_3

NH_3 concentration in the growth medium was determined by the method of McCullough (1967), based on the indophenol reaction of NH_3. At appropriate intervals, 2 ml samples of cells were removed aseptically from an 800 ml culture, the A_{540} was measured and the cells were centrifuged in an Eppendorf 5412 centrifuge for 1.5 min. The supernatant was decanted off and stored at −20 °C for subsequent assay.

Enzyme assays

Alanine dehydrogenase was assayed by monitoring initial rates of NADH oxidation at 340 nm in a Gilford 222 spectrophotometer. The reaction mixture contained (μmol, in a final volume of 1.0 ml): pyruvate, 20; NH_4Cl, 100; NADH, 0.25; Tris/HCl buffer (pH 9.0), 100. For monitoring the deaminating reaction, the assay mixture contained (μmol, final vol. 1 ml): alanine, 50; NAD^+, 1; Tris/HCl bufffer (pH 9.0), 100.

Glutamate dehydrogenase ($NADP^+$-dependent) was assayed as previously described (Bellion & Tan, 1984). Amino acids used in enzyme assays were all of the L-series.

Electrophoresis

Polyacrylamide gels are described by the nomenclature of Hjerten (1962); T denotes the total amount of acrylamide and *NN'*-methylenebisacrylamide ('bis') in 100 ml of solvent; C denotes the amount of 'bis' expressed as a percentage of the total amount of monomer. Routine electrophoresis was performed on gels of the following compositions: stacking gel, 50 mM-Tris/HCl (pH 6.7), 0.05% (v/v) *NNN'N'*-tetramethylethylenediamine ('TEMED'), 0.1% (w/v) ammonium persulphate, 3.08% T and 2.50% C; separating gel, 750 mM-Tris/HCl (pH 8.9), 0.06% 'TEMED', 0.05% ammonium persulphate, 6.0% T and 3.23% C. The running buffer was 10 mM-Tris/glycine (pH 8.3). Protein was applied in samples of 10–50 μg, and was detected after electrophoresis by a modification of the method of Chrambach *et al.* (1967). Gels were fixed in 10 vol. of 12% (w/v) trichloroacetic acid for 15 min, and stained with a freshly prepared solution of Coomassie Brilliant Blue R-250 (0.1% in 12% trichloroacetic acid) at 60 °C for 1 h. The gels were destained overnight in 7% (v/v) acetic acid.

Electrophoresis in the presence of SDS was performed by a modification of the procedure described by Weber & Osborn (1969). Protein samples were denatured by incubation in solution containing 1% (w/v) SDS, 1% 2-mercaptoethanol, 4 M-urea and 10 mM-phosphate buffer (pH 7.0) at 37 °C for 2 h. The gels used were slightly modified in that gels composed of 9.24% T and 3.51% C were used. Proteins used for M_r standards were obtained from Bio-Rad.

Electrophoretic mobilities and protein content of stained bands were calculated from densitometric scans at 554 nm by using a Gilford 2410-S linear transport device.

Activity staining

Alanine dehydrogenase activity was detected after electrophoresis on polyacrylamide gels by immersing the gels in a solution containing alanine (500 μmol), NAD^+ (5 μmol), Tris/HCl (pH 9.0) (500 μmol), phenazine methosulphate (0.5 mg) and Nitro Blue Tetrazoleum (1.0 mg) in a final volume of 5 ml. The reaction was terminated after 15 min by washing and immersing the gels in 7% acetic acid. Alanine dehydrogenase appeared as a single purple band of formazan precipitate. When

Table 1. Summary of purification procedure for alanine dehydrogenase

1 unit = 1 μmol of NAD^+ formed/min at 30 °C.

Step	Vol. (ml)	Activity (units)	Protein (mg/ml)	Recovery (%)	Specific activity (units/mg)
Crude extract	25.0	201	25.5	100	0.322
Streptomycin sulphate treatment	26.0	178	21.5	89	0.321
$(NH_4)_2SO_4$ precipitation	11.5	148	21.5	74	0.600
Affinity chromatography	15.0	97	0.12	48	53.6
DEAE-cellulose chromatography and concentration	2.5	45	0.14	22	129

alanine was omitted from the reaction mixture, no bands appeared.

Enzyme purification

All procedures were carried out at 2 °C, and centrifugations at 30000 *g* for 10 min at 0 °C.

(1) Streptomycin sulphate treatment. A neutralized 10% (w/v) streptomycin sulphate solution was added slowly to crude extract with stirring (0.1 ml of streptomycin sulphate solution/1.0 ml of extract). The solution was stirred for an additional 5 min and centrifuged to remove precipitated nucleic acids.

(2) $(NH_4)_2SO_4$ precipitation. The supernatant from the previous step was adjusted to 40% saturation by the slow addition of recrystallized $(NH_4)_2SO_4$, and the precipitate was removed by centrifugation. The supernatant was then adjusted to 55% saturation in similar fashion. The precipitate was collected by centrifugation and dissolved in a minimum amount of TED buffer, then desalted by passage through a column of Sephadex G-25 (1.5 cm × 15 cm).

(3) Affinity chromatography. The pooled fractions from the desalting column were applied on to a 1.5 cm × 12 cm column of Affi-gel Blue (Bio-Rad) previously equilibrated with TED buffer, and the column was washed until no more protein was detected in the effluent [Bradford (1976) method]. Alanine dehydrogenase was eluted with TED buffer containing 0.25 mM-NADH.

(4) DEAE-cellulose chromatography. The active fractions from the previous step were pooled and adsorbed on to a column of DEAE-cellulose (2.5 cm × 10 cm) previously equilibrated with TED buffer. The column was washed to remove unbound protein, and a linear gradient of 50–500 mM-TED buffer was applied. The enzyme was eluted as single peak. The fractions with the highest activity were pooled and concentrated in a Aminco ultrafiltration cell with a YM-5 membrane.

The purified enzyme was stored at 0 °C, where it retained 43% of its initial activity after 15 days.

Estimation of M_r

The M_r of alanine dehydrogenase was estimated by the gel-filtration method of Andrews (1965), by using a Sephadex G-200 column (2.5 cm × 92 cm) previously equilibrated with TED buffer. Cytochrome *c* (M_r 13370) and myoglobin (16900) from horse heart, ovalbumin (45000), bovine serum albumin (66000), yeast alcohol dehydrogenase (141000), bovine liver catalase (250000), jack-bean urease (483000) and thyroglobulin (670000) were used as standards.

Materials

Affi-gel blue (100-200 mesh) was purchased from Bio-Rad. DEAE-cellulose, dithiothreitol, standard proteins for calibration of gel-filtration columns, phenazine methosulphate, Nitro Blue Tetrazolium, Coomassie Brilliant Blue R-250 and most substrates used for enzyme assays were purchased from Sigma. Electrophoresis chemicals were obtained from Eastman Kodak. NH_4Cl (reagent grade) was recrystallized from 0.5 mM-EDTA.

RESULTS

Enzyme purification

The results of a typical enzyme purification are depicted in Table 1. A 400-fold purification was achieved, with a 22% recovery and a final specific activity of 129 units/mg. Polyacrylamide-gel electrophoresis revealed the presence of only one major band and three very faint minor bands (Fig. 2). From the integrated peaks of densitometer scans, the purity was estimated to be > 98%.

Estimation of M_r

From gel-filtration studies, the M_r of alanine dehydrogenase was estimated to be 217000. Electrophoresis in the presence of SDS revealed a minimum M_r of 53000.

Kinetic properties

The enzyme showed a pH optimum of 9.0 and exhibited typical Michaelis–Menten kinetics. K_m values determined with non-variable substrates under saturating conditions were (mM): NH_3, 26; pyruvate, 4.3; NADH, 0.059. *In vitro*, the rate of the deaminating reaction was only 2.2% of the aminating reaction, also with a pH optimum of 9.0. Attempts to increase the rate of the deaminating reaction by varying the reaction conditions were unsuccessful.

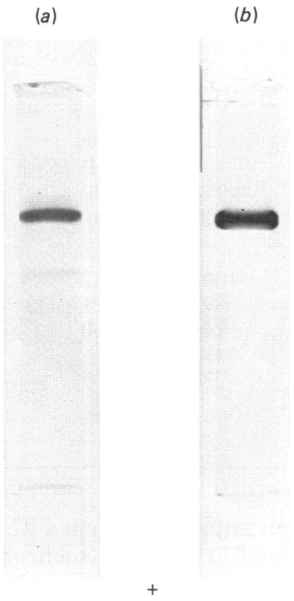

Fig. 2. Polyacrylamide-gel electrophoresis of purified alanine dehydrogenase from *Pseudomonas* sp. strain MA grown on succinate plus 50 mM-NH_4Cl

(*a*) Gel stained for protein by Coomassie Blue; (*b*) gel stained for enzyme activity. Electrophoresis under non-denaturing conditions and staining were performed as described in the Materials and methods section.

Table 2. Substrate specificity of alanine dehydrogenase from *Pseudomonas* MA grown on succinate plus 50 mM-NH_4Cl

Compound tested	Concn. used (mM)	Relative rate of reaction
Pyruvate	20	100
Hydroxypyruvate	20	35
3-Bromopyruvate	20	23
α-Oxoglutarate	20	0
NH_4Cl	100	100
Hydroxylamine	100	0
Methylamine	100	0
NADH	0.25	100
NADPH	0.25	0

Substrate specificity and inhibitors

The relative rates of reaction with amines, α-oxo acids and NADPH are shown in Table 2. Reactions with hydroxypyruvate and 3-bromopyruvate were catalysed at only 35% and 23% of the rate of that with pyruvate. The rate obtained with oxaloacetate as substrate was 28% of that with pyruvate, although this activity was most probably a result of the spontaneous decarboxylation of oxaloacetate to pyruvate. α-Oxoglutarate and glyoxylate could not replace pyruvate, hydroxylamine and methylamine were unable to replace NH_4Cl, and NADPH could not replace NADH. The result observed with 3-bromopyruvate was intriguing, because this substance is used as an affinity label and usually inactivates enzymes by alkylation (Meloche & Golusker). When the reaction was done with 10 times the usual NADH concentration and the products were subjected to t.l.c. on cellulose (Brinkman NM300), with a mobile phase of butanol/acetic acid/water (4:1:1, by vol.), dried and sprayed with ninhydrin, a product (presumably 3-bromoalanine), whose mobility was significantly less than that of authentic alanine, was detected.

In order to determine some general properties of the enzyme and of its possible regulation, the effects of various compounds on the amination reaction were determined by their addition to the assay mixture. The enzyme was very sensitive to inhibition by $HgCl_2$ (0.01 mM) and dithiobisnitrobenzoic acid (0.1 mM). These inhibitions were reversed on addition of 2-mercaptoethanol (final concn. 7 mM), with 72% and 56% of the initial activity restored respectively. Metal chelators had no effect on activity. Of L-amino acids tested, only alanine and serine were significant inhibitors, inhibiting the reaction at 59% and 43% when used at 2 mM and 1 mM respectively. Surprisingly, glutamate, which causes a powerful repression of the enzyme (see below), was ineffective as an inhibitor, suggesting that it does not regulate the activity of the enzyme within the cell. Hydroxypyruvate was the strongest inhibitor of all the oxo acids tested, inhibiting the reaction by 60% at a concentration of 4 mM. Since this compound was also a weak substrate for the enzyme, the inhibition was likely due to direct competition. Although hydroxylamine was unable to act as an NH_3 analogue in the reaction (see above), it was inhibitory (50%) at a concentration (40 mM) above the K_m for NH_3. The exact type of observed inhibition was not determined. NAD^+ had a moderately strong effect, inhibiting the reaction by 35% at a concentration of 0.1 mM.

Amounts of alanine dehydrogenase under various growth conditions

We have previously shown that alanine dehydrogenase is not synthesized when cells grow on methylamine as

Table 3. Specific activities of alanine dehydrogenase found under different conditions of growth

Abbreviation: nd, none detected. Values in parentheses are percentages of that in the first line.

Growth conditions		Specific activity (nmol/min per mg of protein)	
Carbon source	Nitrogen source		
Succinate	NH_4Cl (50 mM)	340	(100)
Succinate	NH_4Cl (10 mM)	105	(31)
Succinate	Methylamine (50 mM)	nd	–
Methylamine (50 mM)	(Methylamine)	nd	–
Succinate	NH_4Cl (50 mM)+ alanine (5 mM)	235	(69)
Succinate	NH_4Cl (50 mM)+ glutamate (5 mM)	41	(12)
Succinate	Alanine (20 mM)	226	(66)
Succinate	Glutamate (20 mM)	32	(9)
Alanine (20 mM)	(Alanine)	539	(159)
Glutamate (20 mM)	(Glutamate)	47	(14)

sole carbon and nitrogen source (Bellion & Bolbot, 1983). Thus its synthesis is clearly a function of the nature of the carbon and nitrogen sources present in the growth medium. To investigate this phenomenon further, we measured specific activities of the enzyme in cells grown with different carbon and nitrogen sources, and the results are depicted in Table 3. For comparative purposes the enzyme-activity measurements were done at cell densities equivalent to those of succinate/NH_4Cl-grown cells that showed the highest amounts of enzyme, that is at mid-exponential phase. Cells grown on media other than succinate/NH_4Cl grew a little more slowly than those on succinate/NH_4Cl, and grew to lower final cell densities, e.g. for alanine-grown cells, doubling time 8 h, final cell density corresponding to 0.52 mg dry wt./ml of culture medium, and for succinate-grown cells, doubling time $4\frac{1}{2}$ h, final cell density corresponding to 1.18 mg dry wt./ml of culture medium. The production of the enzyme in cells grown on NH_4Cl (50 mM) was severely repressed by 5 mM-glutamate (an 8-fold decrease), but only slightly by 5 mM-alanine. When alanine was the sole carbon and nitrogen source, the enzyme was maximally induced, but surprisingly the enzyme was still induced to a relatively high extent when alanine was the sole nitrogen source. Replacement of alanine by glutamate in the last two growth conditions resulted in minimal synthesis of alanine dehydrogenase but a slight induction of an $NADP^+$-dependent glutamate dehydrogenase (14 nmol/min per mg of protein) was observed. This activity was not detected in appreciable amounts under any of the other growth conditions used.

Measurements of ammonia uptake or excretion

The observation that the specific activity of alanine dehydrogenase was apparently dependent on NH_3 concentration in the growth medium prompted us to determine the concentration of NH_3 throughout the growth periods for different growth conditions. Cells grown on succinate/50 mM-NH_4Cl utilized only about 32% of the NH_3 supplied (Fig. 1). Cells grown solely on 20 mM-alanine excreted NH_3 into the culture medium to a final concentration of 9.4 mM, showing that the amino acid was deaminated, thereby indicating that the induced alanine dehydrogenase was in fact operating in the oxidative direction under this growth condition. Glutamate-grown (20 mM) cells also released NH_3 into the medium, to a final concentration of 5.6 mM.

DISCUSSION

Alanine dehydrogenases have been observed in several different organisms and in different roles. When operating in the aminating direction, the enzyme can be used as a means of nitrogen assimilation, and it is so used in *Streptomyces clavuligerus* (Aharonowitz & Friedrich, 1980), *Anabaena cylindrica* (Rowell & Stewart, 1976) and *Rhodopseudomonas capsulata* (Johansson & Gest, 1976). The findings reported here indicate that this is the case in *Pseudomonas* sp. strain MA, but only when NH_4Cl is available in high concentration as the nitrogen source. Several lines of evidence lead to this conclusion. (1) The purified enzyme has a K_m for NH_3 of 26 mM. Although not a true indicator of the ability of an enzyme to function at low substrate concentrations, a K_m value of this magnitude certainly is not conducive to efficient functioning under such conditions. This point was originally made by Meers *et al.* (1970) to rationalize the absence of glutamate dehydrogenase and the presence of glutamine:2-oxoglutarate aminotransferase when *Aerobacter aerogenes* (*sic*) was grown in low NH_3 concentration (see also Dalton, 1979, for further discussion of this topic). Therefore, this fact, taken with the relatively low catalytic-centre activity of this enzyme ($1.17 \times 10^2\ s^{-1}$), would render the enzyme somewhat inefficient for NH_3 assimilation when the concentration is significantly lower than this value and when the enzyme amount is also low. Similarly high K_m values were also found in *S. clavuligerus* and *A. cylindrica*, although in *R. capsulatus* a value of 8.3 mM was reported. Accordingly the enzyme was found in high quantities in *Pseudomonas* MA when 50 mM-NH_3 was present as the nitrogen source. Lowering the NH_3 concentration to 10 mM caused a 3-fold decrease in the enzyme amount, whereas growth on methylamine (i.e. no free NH_3) resulted in complete repression of alanine dehydrogenase. (2) The enzyme operated *in vitro* almost exclusively in the aminating direction. (This finding is at variance with the results from *S. clavuligerus* and *A. cylindrica*, where the enzyme was essentially freely reversible.) (3) The enzyme was highly specific for NH_3 and for its coenzyme. No reaction was observed with methylamine, hydroxylamine or NADPH. It is less specific towards the oxo acid, since hydroxypyruvate and 3-bromopyruvate were able to act (slowly) as substrates in the reaction. (4) Glutamate (5 mM) severely repressed synthesis of the enzyme in succinate/NH_4Cl-grown cells, again reflecting the anabolic role of the enzyme under these growth conditions. We were surprised to find that 5 mM-alanine did not likewise repress synthesis of the enzyme, as might be expected for an enzyme that synthesizes alanine. Additionally, the enzyme was also not repressed substantially in cultures of cells growing on alanine as the sole nitrogen source, and in fact it was maximally induced when alanine was the sole source of carbon and nitrogen. Thus the enzyme is induced also by the presence of alanine in the growth medium, and this fact could explain the lack of repression of alanine dehydrogenase in succinate/NH_4Cl-grown cells by 5 mM-alanine.

The elaboration of alanine dehydrogenase in large quantity when alanine is used as the sole source of carbon, nitrogen and energy indicates that the oxidative cleavage reaction to pyruvate and NH_3 is probably an important step in the utilization of alanine by this organism. This suggests that, in this organism, the enzyme may also play a significant catabolic role, as has been found in several *Bacillus* species (Freese & Oosterwyk, 1963; McCormick & Halvorson, 1964; Yoshida & Freese, 1965). This was unexpected, since the enzyme purified from NH_3-grown cells operated *in vitro* essentially only in the direction of amination, and suggested that possibly a different form of alanine dehydrogenase was present in alanine-grown cells. However, polyacrylamide-gel electrophoresis under non-denaturing conditions of extract from alanine-grown cells, followed by staining for alanine dehydrogenase as described in the Materials and methods section, revealed a single band of activity with the same mobility as that from NH_3-grown cells (results not shown), indicating that the two activities were probably catalysed by the same enzyme. It is possible that equilibrium conditions *in vivo* may be such as to allow for the deamination

reaction at a rate that enables cell growth, but the enzyme was not purified from alanine-grown cells to test this hypothesis.

Thus we propose that alanine dehydrogenase has two roles in *Pseudomonas* sp. strain MA. These are in the assimilation of NH_3 and in the utilization of alanine. The former role is similar to the role of $NADP^+$-dependent glutamate dehydrogenase in *Pseudomonas* AM1, but, since *Pseudomonas* AM1 was unable to grow on glutamate as either carbon or nitrogen source, glutamate dehydrogenase has no similar catabolic role in that organism (Bellion & Tan, 1984). Additionally, *Pseudomonas* AM1 is devoid of alanine dehydrogenase after growth on succinate/NH_4Cl (50 mM) medium (Bellion & Bolbot, 1983). However, the alanine dehydrogenase of *Pseudomonas* MA differs from those found in similar roles in other organisms in chemical and physical properties. The M_r of the enzyme as determined by gel filtration, 217000, is somewhat smaller than those reported from the other organisms. The M_r obtained by electrophoresis under denaturing conditions, 53000, suggests a four-subunit structure for the enzyme. A four-subunit structure was also suggested for *S. clavuligerus*, but in that case the subunit M_r was 92000, as determined by density-gradient centrifugation, whereas in *A. cylindrica* a structure composed of six subunits each of M_r 43000 was proposed. Thiol groups seem to be essential for the activity of alanine dehydrogenase, since it was severely inhibited by Hg^{2+} ions and by dithiobisnitrobenzoate, but this inhibition was relieved by 2-mercaptoethanol, as has been found previously (Rowell & Stewart, 1976). Chelating agents were without effect, indicating no metal-ion requirement for activity, and some L-amino acids were inhibitory to the reaction.

The role of the alanine dehydrogenase in the assimilation of NH_3 in *Pseudomonas* sp. strain MA is important only under certain growth conditions and, as Fig. 1 shows, only for a short period of time during the initial growth phase of the cells, after which the activity of the enzyme rapidly declines. We do not know if this decline in activity is due to repression or inactivation, or a combination of both. One possibility is that at this point the intracellular concentration of glutamate (which brings about severe repression when included in the growth medium) becomes sufficiently high to repress the enzyme, even though the NH_3 concentration in the medium is still sufficient for enzyme induction. After that time, the task of further NH_3 assimilation, when and if needed, must be taken over by the 'GS/GOGAT' pathway, thereby explaining our previous finding of the presence of both enzyme systems during growth under these conditions (Bellion & Bolbot, 1983).

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