Alanine Dehydrogenase from Bacteroids and Free Living Cells of *Rhizobium japonicum*

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Alanine dehydrogenase (E.C. 1.4.1.1.) from nitrogenase repressed free living cells of *Rhizobium japonicum 61-A-101* was purified 370 fold to a specific activity of 30.4 µmol pyruvate · min^{-1} · mg $protein^{-1}$. The same enzyme from effective bacteroids from nodules of *Glycine max* var. Mandarin, infected with the same strain was purified 150 fold to a specific activity of 35 units. The enzyme from both preparations was identical in the molecular weight of about 168 kD with four identical subunits of 42 kD. The alanine dehydrogenase is, therefore, different from the same enzyme from *Bacillus subtilis* (molecular weight 228 kD) and from *Anabaena cylindrica* (molecular weight 270 kD). The K_M data for the enzyme from *Rhizobium japonicum* are: 4.7 mmol/l for NH_4^+, 0.68 mmol/l for pyruvate and 44 µmol/l for NADH. Specific activity of the enzyme in total cell extracts from eight other strains of *Rhizobium japonicum* (3 effective strains, 5 ineffective strains) was only 20 to 30% of the activity with strain 61-A-101. No correlation between alanine dehydrogenase activity and nitrogenase activity in these other eight strains was observed. The function of alanine dehydrogenase in *Rhizobium japonicum* in ammonium assimilation and cell wall differentiation is discussed.

Introduction

Amongst all the oxidation levels of nitrogen the reduced form of ammonium (NH_4^+) is the only one which can be incorporated into organic compounds by living cells. Ammonium therefore has a central role within the ecological nitrogen cycle. The primary products of biological ammonium assimilation (gln, glu, asp, asn, ala, ser and carbamyl-phosphate) constitute basic pools for the synthesis of nucleotides, amino acids and proteins.

Since Meers *et al.* (1970) [1] discovered the glutamate cycle, it has been shown in the majority of the organisms examined that primary ammonium assimilation occurs by this pathway [2]. This has also been proven for free-living bacteria of the genus *Rhizobium* [2]. Glutamine synthetase (= GS) and glutamate synthase (= GOGAT) hardly show any activity in symbiotic bacteroids when compared to free-living bacteria [11]. During the development of the bacteroids and concomitant nodule differentiation GS-activity decreases in the *Rhizobium*-cells [3] and glu-DH also shows, like GOGAT, a relatively low activity within the symbionts [4, 5]. Thus in a developed symbiosis almost no NH_4^+ is assimilated into bacteroids. As the cell wall of rhizobia is freely permeable for NH_4^+ [6], nearly all the ammonium accrued by N_2-fixation enters the plant cytoplasm by diffusion and is assimilated by plant cytoplasmic enzymes [2]. The permanent loss of NH_4^+ from the bacteroids is thought to be partly compensated for by the uptake of nitrogenous compounds from the plant, especially via the two amino acids asn and gln, which are the most important forms for the transport of combined nitrogen in the symbiosis of rhizobia and most legumes [7].

Stripf (1978) [8] found high activities of ala-DH which catalyses the reaction: pyruvate + NH_4^+ + NADH ⇄ alanine + H_2O + NAD, in a crude extract of effective bacteroids (designated nod^+/nif^+) of *Rhizobium japonicum*, strain 61-A-101. The activities in the crude extract of ineffective bacteroids (desig-

Abbreviations: A: (purified) alanine dehydrogenase from *Anabaena cylindrica;* ala: alanine; asn: asparagine; asp: aspartic acid; B: (purified) alanine dehydrogenase from *Bacillus subtilis;* BSA: bovine serum albumin; DEAE: diethyl-amino-ethyl; E: (purified) alanine dehydrogenase from effective bacteroids of *Rhizobium japonicum;* F: (purified) alanine dehydrogenase from free living, nitrogenase-repressed cells of *Rhizobium japonicum;* gln: glutamine; glu: glutamic acid; GOGAT: *g*lutamate *o*xoglutarate *a*mino-*t*ransferase (= glutamate synthase); GS: glutamine synthetase; β-ME: β-mercaptoethanol; TES: N-Tris(hydroxymethyl)methyl-2-ethanesulfonic acid; TRIS: Tris(hydroxymethyl)aminomethane; USDA: United States Department of Agriculture.

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nated nod$^+$/nif$^-$) of strain 61-A-24 were 6–7 times lower than in the effective organisms (40 mU : 280 mU). There was no detectable ala-DH activity in the plant nodule cytoplasm. The appearance of ala-DH and nitrogenase activities seemed to be concatenated.

Although Rowell and Stewart (1976) [9] showed that ala-DH in the cyanobacterium *Anabaena cylindrica* played an additional role in primary ammonium assimilation only at high intracellular NH_4^+ concentrations, this restriction may not be necessarily true for the symbiosis between rhizobia and legumes plants. This may be so because the activities of the enzyme in the crude extracts made from *Anabaena cylindrica* are much lower than in these from *Rhizobium japonicum*, strain 61-A-101: 55 mU : 280 mU. Thus high substrate concentrations may be required in the cyanobacterial system, but not in the rhizobial one.

From these findings the following hypotheses may be postulated:

- Nitrogenase activity in effective bacteroids is correlated with ala-DH activity.
- Ala-DH plays a role in the primay ammonium assimilation, this role becomes particularly important under those conditions where N_2-fixation takes place.

Materials and Methods

Organisms, media and growth

Nine strains of *Rhizobium japonicum* were maintained and grown in a liquid medium designated 20 E [10]. Four strains had the ability to both nodulate host plants and fix N_2, and were therefore characterized as nod$^+$ nif$^+$. These were: 61-A-101, 61-A-124, 110 USDA and 136 USDA; the rest could nodulate host plants, but lacked the ability to fix N_2. These strains 61-A-24, 61-A-164, 61-A-165, RH 31 and 24 USDA were therefore characterized as nod$^+$ nif$^-$. Free-living cells of strain 61-A-101 were obtained in large quantities by culturing in a 5 l fermentation vessel under aerobic conditions. Growth of these cells was stopped after 96 h, at which time the cells were dividing exponentially.

Nodulation and symbiotic nitrogen fixation

Seeds of *Glycine max* var. Mandarin were surface sterilized and germinated as described by Werner and Stripf [11]. The root system of ten days old seedlings was immersed for 10 min in a suspension of medium 20 E with 0.07% agar containing 1×10^7 *Rhizobium* cells · ml^{-1}. Seedlings were grown as described by Werner *et al.* [10]. 28 d to 42 d after infection, plants were uprooted and the root systems only were incubated under an atmosphere of 10% acetylene in air for 1 h in the dark at 28 °C. Finally 0.5 ml gas samples were removed and tested for acetylene reduction activity as described by Werner and Wilcockson [12]. The nodules were then harvested and the size distribution evaluated [13].

Enzyme extraction

Free-living cells of strain 61-A-101 were harvested by centrifugation, washed twice and resuspended in Tris buffer (50 mmol/l Tris/HCl, pH 7.5, 10 mmol/l EDTA and 5 mmol/l β-mercaptoethanol). Other strains were washed and resuspended in TES-buffer (50 mmol/l TES/NaOH, pH 7.5, 10 mmol/l glutathione). Cells were disrupted by passage through a French pressure cell at 2560 MPa (18 000 psi). Cell debris were removed by centrifugation at $35\,000 \times g$ for 20 min and the supernatant used as enzyme source.

Enzyme purification

5 l of batch culture and bacteroids derived from 41 g of fresh nodules were treated as described above at 8 °C, the extracts were diluted 7-fold with buffer and brought to 30% ammonium-sulfate saturation. With the addition of the solid salt precipitated proteins were discarded and $(NH_4)_2SO_4$ was added to the supernatant to a final concentration of 40% saturation. The precipitate was resuspended in Tris-buffer and dialyzed against the same buffer to remove excess $(NH_4)_2SO_4$. Proteins were then applied to a 35×2 cm column of DEAE sephadex A-25, and eluted by a 0.05 to 0.35 mol/l gradient of KCl in Tris buffer. Peak enzyme fractions were pooled and concentrated to 1.0 ml by vacuum dialysis. The concentrated enzyme preparation was subsequently re-chromatographed on a 145×1.6 cm column of sephadex G-200, being eluted with Tris buffer. Fractions which contained ala-DH activity were either directly used for further studies of the kinetic characteristics of the purified enzyme, or were concentrated for structural studies.

Determination of molecular weight

The molecular weight of ala-DH was estimated by gel filtration on sephadex G-200, using the molecular weight standards thyroglobulin (665 000), ferritin (450 000), catalase (240 000), aldolase (158 000) and bovine serum albumin (67 000). v_0 was estimated by "dextran blue 2000".

Gel electrophoresis

SDS-Polyacrylamide disc gel electrophoresis was carried out according to Neville [14] on 10% gels, 3 mm thick, using Tris/borate buffer at pH 8.5, containing 0.1% SDS for the cathode and Tris/HCl buffer at pH 9.3 for the anode. Protein solutions were supplied with Tris/borate buffer containing 5% SDS (30 µl/50 µg protein) and 5 µl of 1 mol/l β-mercaptoethanol and lyophilized at 20 °C. The dried protein was dissolved in water (0.5 µl per 1 µg protein) and denatured by incubation at 50 °C for 30 min. After centrifugation the supernatants were added to a mixture of bromphenol blue (tracking dye) and sucrose. The electrophoresis was performed at a voltage of 150 V ant at a current of 44 mA at the beginning. Gels were stained for protein using 0.05% Coomassie Brilliant Blue in TCA/methanol/glacial acetic acid/water (5.5/14.6/6.7/73.2 (w/w)) over night at room temperature. Destaining was carried out by washing in glacial acetic acid/methanol/water (4.8/28.6/66.7) for several days until the gel was transparent and showed clear protein patterns. Polyacrylamide gel electrophoresis was carried out according to the system explained above, with some modifications: there was no stacking gel provided and SDS was generally missing, protein solutions were directly taken into the gel chambers without any further treatment.

The molecular weight standards used included: α-gelatine (95 000), bovine serum albumin (67 000), chicken ovalbumin (45 000), chymotrypsinogen A (25 000) and cytochrome C (12 500), and without SDS: ferritin (450 000), catalase (248 000), aldolase (158 000), α-gelatine (95 000) and bovine serum albumin (67 000) respectively.

Protein determinations

The method of Bradford [15] was used with bovine serum albumin as standard.

Alanine dehydrogenase (deaminating)

Activity was estimated by following the reduction of NAD^+ at 340 nm. The reaction mixture contained, in addition to the enzyme, 200 µmol sodium carbonate/bicarbonate, pH 10.0; 1.4 µmol NAD^+, and 20 µmol l-alanine, in a total volume of 2 ml.

Alanine dehydrogenase (E.C. 1.4.1.1.) (aminating)

Activity was estimated by following the oxidation of NADH at 340 nm. The reaction mixture contained, in addition to the enzyme, 200 µmol Tris/HCl, pH 8.6; 0.2 µmol NADH; 2.0 µmol sodium pyruvate and 16.5 mmol NH_4Cl in a total volume of 2 ml.

Glutamate dehydrogenase (E.C. 1.4.1.2.)

Activity was estimated as above, using 2 µmol 2-oxoglutaric acid instead of pyruvate. The assay was carried out at pH 8.4 which is the pH optimum [8].

Results

Enzyme purification and molecular structure

Alanine dehydrogenase was purified from both N_2-fixing bacteroids living in root nodules of *Glycine*

Table I. Purification scheme for alanine dehydrogenase derived from *Rhizobium japonicum* strain 61-A-101, free-living nitrogenase repressed cells.

Purification step	Total protein [mg/ml]	Specific activity [µmol NADH oxidized $min^{-1} \cdot mg\ protein^{-1}$]	Total activity [µmol NADH oxidized $\cdot min^{-1}$]	Yield [%]
crude extract	1500	0.082	122.9	100
30–40% ammonium sulfate precipitate	84.37	1.93	97.2	79.1
DEAE-sephadex	3.51	9.24	32.9	26.8
Sephadex G-200	0.417	30.4	12.7	10.3

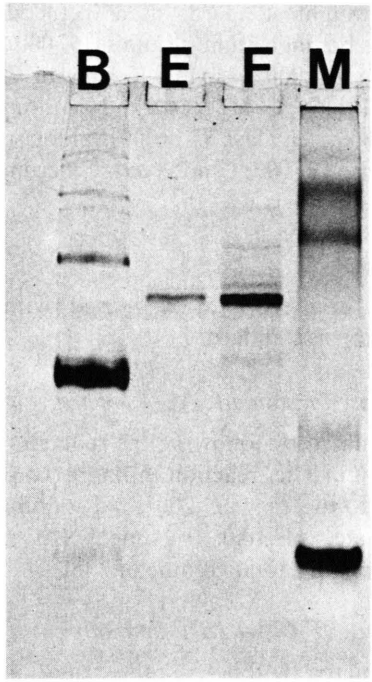

Fig. 1. Polyacrylamide gel electrophoresis of purified alanine dehydrogenase from *Rhizobium japonicum* 61-A-101. That from effective bacteroids ("E") and that from nitrogenase repressed bacteria ("F") in comparison with the purified enzyme from *Bacillus subtilis* ("B", obtained from Boehringer, Mannheim). "M" shows marker proteins.

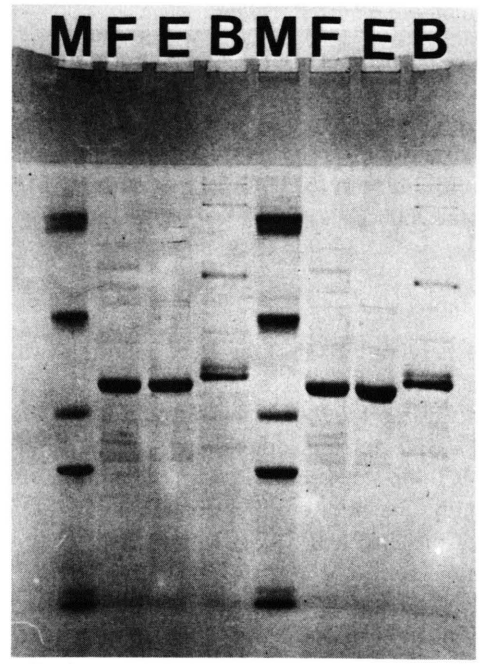

Fig. 2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of alanine dehydrogenase from "E", "F" and "B". 20–30 µg of protein was applied per lane. The origin is at the upper end of the gel. M = marker proteins; from top: α-gelatine; bovine serum albumin; chicken egg albumin; chymotrypsinogene A; cytochrome C.

max and from free-living, nitrogenase repressed cells. Results of a typical enzyme purification from a nitrogenase repressed batch culture are presented in Table I. The enzyme was purified 370-fold with a maximum specific activity of 30.4 µmol NADH oxidized · min^{-1} · mg $protein^{-1}$. From effective bacteroids a specific activity of 35 units was obtained. As shown in Fig. 1, only one major protein band was observed upon polyacrylamide gel electrophoresis of the purified enzymes. The R_m of the protein band was constant and independent of the enzyme source.

Data on the molecular weight of ala-DH

A comparison of the major protein bands in Fig. 1 indicates that there is no difference between "E" and "F". Therefore molecular weight estimation of native ala-DH was carried out only with "F". These data, presented in Fig. 3, are based on the elution pattern from a sephadex G-200 column and is given relative to the elution of several known standard proteins. Thus estimated, the molecular mass of ala-DH is 168000 daltons which is distinctly lower than the molecular weights of similar enzymes purified from *Anabaena cylindrica* [9] with 270000 and *Bacillus subtilis* [16] with 228000. As Figs. 2 and 4 show, the purified enzyme dissociates upon treatment with SDS to give a single protein band of molecular weight of 42000. From these data it appears likely that the native enzyme consists of four indistinguishable sub-units.

pH optima

Fig. 4 shows that the optimum pH for ala-DH activities in the aminating and deaminating reactions are pH 8.6 and 10.0–10.5 respectively. Independently of the enzyme source the activity of purified ala-DH in the aminating reaction at pH 8.6 is thirteen times higher than the activity in the reverse direction at pH 10.0.

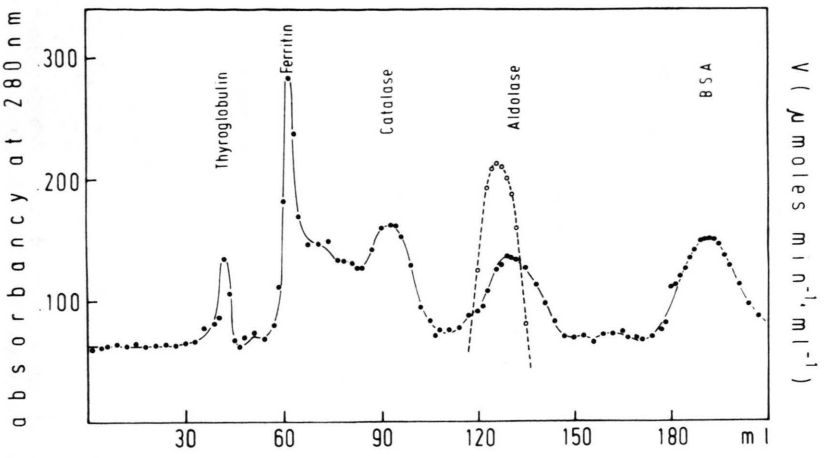

Fig. 3. Estimation, by gel filtration on Sephadex G-200, of molecular weight of alanine-dehydrogenase derived from *Rhizobium japonicum* 61-A-101 from nitrogenase repressed cells (dashed line). Marker proteins as indicated (solid line).

Substrate specificity and effect of substrate concentrations

It was found that the enzyme was strictly NADH/NAD$^+$ specific, activity being undetectable with NADPH/NADP$^+$. Pyruvate could be substituted by oxalacetate without any loss of activity. There are no other accepted substrates that could replace ammonium.

The kinetic behaviour of the enzymes from N_2-fixing bacteroids and nitrogenase repressed free-living bacteria with various primary substrates was studied. Determinations of K_M were carried out at the optimum pH for each reaction. The data in Fig. 5 show the concentration effects for the primary substrates of the aminating reaction. At pyruvate concentrations above 2.5 mmol/l some substrate inhibition occurs, whereas with the enzyme derived from *Bacillus subtilis* a straight line response is obtained (Fig. 5b). Lineweaver-Burk plots of the effect of NH_4Cl concentration on ala-DH activity yield straight lines. The slopes of these lines vary with pH (Fig. 5c) and the calculated K_M^{app} values range from 4.65 mmol/l at pH 8.6 or above to 50.0 mmol/l at pH 7.4. When the pK_M for NH_4Cl is plotted against pH a straight line with unit slope occurs in the range over which the K_M^{app} varies, at pH 8.6 (free-living cells) and 8.8 respectively (effective bacteroids) there is an inflexion with no change in pK_M above this value (Fig. 5d). The same phenomenon has been observed for ala-DH of *Anabaena cylindrica* [9]. The authors suggest from these data that it is NH_3 rather than NH_4^+ which may be the actual substrate for ala-DH, as the low K_M values for NH_4Cl at high pH values coincide with the pK_a of the dissociation of NH_4^+ to $NH_3 + H^+$ (pK_a 9.2). The K_M values of the different substrates are summarized in Table II.

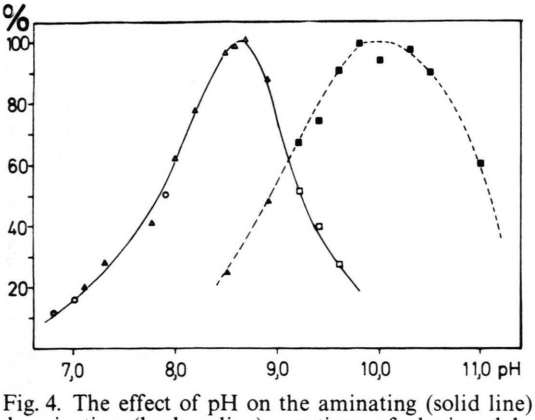

Fig. 4. The effect of pH on the aminating (solid line) and deaminating (broken line) reactions of alanine dehydrogenase derived from effective bacteroids of *Rhizobium japonicum* 61-A-101. The buffer systems used were potassium phosphate (○), Tris/HCl (△), sodium carbonate/bicarbonate (□); all 0.1 M ionic strength. % of maximum activity.

Various compounds were tested for their inhibitory effect on ala-DH activity. The results are presented in Table III. Of the amino acids tested, l-alanine, D-alanine, l-serine and l-threonine markedly inhibited the aminating activity of the enzyme. The difference between the enzyme from effective bacteroids ("E") and free-living bacteria ("F") were insignificant, except for the effects of l-threonine and glycine. Table III compares the inhibitory effect of glycine on the ala-DH activity from different sources. Since it is known that the glycine content of

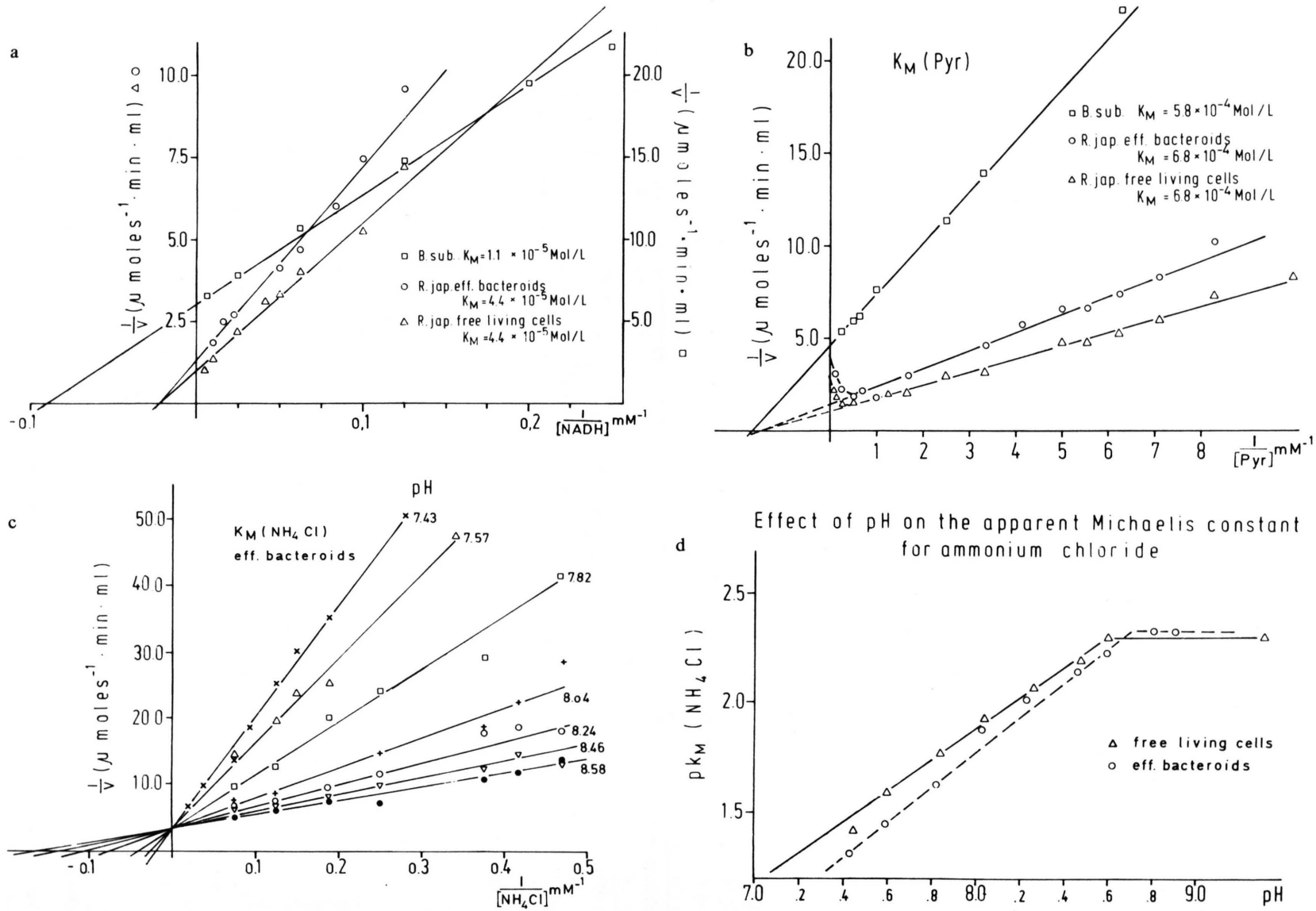

Fig. 5. Double reciprocal plot showing the effect of variation in the concentration of the primary substrates on the aminating activity of alanine dehydrogenase derived from *Rhizobium japonicum*, effective bacteroids (○), nitrogenase repressed bacteria (△) and enzyme from *Bacillus subtilis* (□): a) NADH, b) pyruvate, c) NH_4Cl (effective bacteroids), d) effect of pH on the apparent Michaelis constant for ammonium chloride.

Table II. Comparison of structural and kinetic properties of alanine dehydrogenase derived from *Anabaena cylindrica* (A), *Bacillus subtilis* (B) and *Rhizobium japonicum* (E) and (F).

	Origin			
	A[b]	B[a]	E	F
molecular weight	270	228	168	168
number of sub-units	6	4	4	4
molecular weight of sub-units	43	53–56	42	42
specific activity of the crude extract	55	240	240	82
maximum specific activity	38	30[c]	35	30.4
aminating activity: desaminating activity	1:12	1:13	1:13	1:13
pH-optimum				
– aminating	8.0	8.9–9.0	8.6	8.6
– desaminating	9.6	10 –10.5	10–10.5	10–10.5
K_M [mmol/l]				
NADH	0.015	0.023	0.044	0.044
pyruvate	0.11	0.54	0.68	0.68
NH_4Cl	8–133	38	4.7–50	4.7–35.7
NAD^+	0.014/0.060	0.18		
l-alanine	0.4	1.73		

[a] From Yoshida and Freese, [b] from Rowell and Stewart, [c] from Boehringer, Mannheim.

Table III. The effect of various amino acids on the activity of alanine dehydrogenase from *Rhizobium japonicum:* effective bacteroids ("E") and nitrogenase repressed bacteria ("F") compared with the enzymic activity as derived from *Bacillus subtilis* and *Anabaena cylindrica*[a].

Compound	% inhibition			
	Anabaena cylindrica	*Bacillus subtilis*	*Rhizobium japonicum*	
			E	F
l-alanine	69	66	79	78
D-alanine		42	56	54
l-serine	40	79	72	72
l-threonine		56	50	41
glycine	65	16	30	24
l-asparagine		2	7	8

[a] Data taken from Rowell and Stewart

rhizobia is above the average for gram-negative bacteria [17] and that the intracellular amino acid concentration in gram-positive *Bacillus*-species is 20 times higher than it is in gram-negative bacteria [18], the data presented may indicate a greater tolerance for glycine by ala-DH in rhizobia.

Bacteroids of several strains of *Rhizobium japonicum* (both nif^+ and nif^-), grown in symbiosis with *Glycine max* var. Mandarin, were isolated from the nodules either four or six weeks after infection. The crude bacteroid extracts were tested for ala-DH and glu-DH. The results are presented in Table IV. Table IV clearly shows that only the ala-DH specific activity of strain 61-A-101 is significantly higher than the other strains examined. The differences between other effective strains and the

Table IV. Specific activities of enzymes from bacteroids of various *Rhizobium japonicum* strains, 28 d and 42 d after infecting *Glycine max* roots.

Age	28 d after infection					42 d after infection				
Strain	Nodule size [μm]	Protein [mg/ml]	Nitrogenase[a]	ala-DH [munits/mg protein]	glu-DH [munits/mg protein]	Nodule size[b] [μm]	Protein [mg/ml]	Nitrogenase[a]	ala-DH [munits/mg protein]	glu-DH [munits/mg protein]
61-A-101		3.00	23.8	138	27	1600–3300 **2600**	1.11	8.7	110	18
61-A-101		0.67	45.0	220	36					
61-A-124		3.50	31.0	44		1600–4000 **2000**	5.16	5.1	113	18
110 USDA		1.00	81.0	33	40	1600–4000 **2600**	5.10	8.7	55	20
136 USDA		4.40	22.4	26	13	1600–4000 **2600**	4.52	18.2	54	24
61-A-24	1600–2600 **2000**	0.08	0.0	43	81	1600–3000 **1600**	0.49	0.0	19	26
61-A-164	1600–3300 **1800**	0.58	0.0	3	14	1600–2800 **2000**	3.45	0.0	66	19
61-A-165		0.58	0.0	0.0	18	1600–2600 **1600**	4.28	0.0	72	36
24 USDA	1600–2000 **1800**	0.25	0.0	0.0	9	1600–2000 **1600**	0.31	0.0	35	165
RH 31		1.37	0.0	40	31	1600–2800 **2000**	2.23	0.0	45	21

[a] Nitrogenase: nmol $C_2H_4 \cdot h^{-1} \cdot$ mg nodule fresh weight^{-1}, [b] size range: medium size fraction bold print.

ineffective ones are negligable. The specific activity of glu-DH in bacteroids derived from four weeks old nodules was approximately as high as the ala-DH activity. In strain 61-A-101, however, glu-DH activity was not higher than in other strains. The ineffective strain 61-A-24 shows a glu-DH activity which is twice that of the ala-DH activity. Six weeks after infection the glu-DH activity of the effective strains was diminished, whilst in the ineffective strains it was increased, this was particularly so with strain 24 USDA: 165 mU in which the glu-DH activity was 7-fold higher than the ala-DH activity.

Discussion

The most striking result from these experiments is that effective strains of *Rhizobium japonicum* cannot be consistently characterized by high specific ala-DH activities. Strain 61-A-101, which had been tested earlier by Stripf [8], was the only one out of nine strains, from which a significantly higher specific ala-DH activity was obtained. It appears therefore that it is a peculiar characteristic of strain 61-A-101 and not generally applicable. The gene regions for ala-DH probably may be at least doubled within the genome of strain 61-A-101, a higher constitutive level of m-RNA may be formed. This would result in increased enzyme protein synthesis. Table IV shows that ala-DH activity of strain RH 31 (which is nod$^+$/nif$^-$, and was originally derived from strain 61-A-101 by UV-treatment [19]) is comparable with all the other strains tested. This indicates that the additional gene regions in strain RH 31 have been affected by the nif mutation, since only the usual (obligate) amount of ala-DH activity is present. The data presented in Table IV show furthermore that there are neither negative nor positive correlations between the enzyme activities of the two oxido-reductases ala-DH and glu-DH. The latter in particular shows great deviations and no trend can be discerned. In its structural properties the enzyme from *Rhizobium japonicum* differs markedly from that isolated from other organisms. Yoshida and Freese (1964) [16] specify 228 kd as the molecular mass of the ala-DH purified from *Bacillus subtilis*. Upon dilution their enzyme disintegrated into four almost identical sub-units (53 to 56 kd). These units exhibited a diminished activity and they

themselves were probably comprised of two similar parts, each of 27 kd. Rowell and Stewart [9] found that the ala-DH of *Anabaena cylindrica* was composed of 6 identical or almost identical subunits, each 43 kd, resulting in a total molecular mass of 270 kd for the native enzyme. The ala-DH of *Rhizobium japonicum*, however, exists dissociated as 4 subunits, each 42 kd. The complete, catalytically active enzyme thus weighs 168 kd. Table II clearly shows that the maximal ala-DH specific activities of the purified enzymes from all the sources tested are approximately equal. The ratio between the activities of the aminating and deaminating reactions was found to be constant in all cases (12:1 and 13:1 respectively). All enzymes proved to be strictly NAD/NADH-dependent. One peculiar property of the ala-DH from *Rhizobium japonicum* was non-specificity towards oxalacetate as substrate which could substitute for pyruvate to 100%.

The calculated values of the apparent Michaelis constants for NH_4^+ are lower (4.7 mmol/l – 5.9 mmol/l) than those previously published by Dunn and Klucas (1973): 7.4 mmol/l [20]. These data support previous suggestions [11, 8] that ala-DH plays a part in primary ammonium assimilation within N_2-fixing bacteroids in those cases where there is an excess of NH_4^+ available in the cells.

Besides its role in the primary ammonium assimilation, ala-DH is also known in a completely different context: both at the sporulation and germination periods of the *Bacillus subtilis* cell cycle, ala-DH appears in early stages in large quantities.

During the complex development from free-living rhizobia to symbiotic bacteroids within legumes root nodules some changes in the composition of the microorganisms cell wall also occur [21]. Doubtless the cell wall plays an important role both in the differentiation of bacteria to bacteroids and also in maintaining the balance between the plant tissue and the rhizobia. The cell wall of bacteroids is generally characterized by an increased permeability [21]. One possible reason for this could be a lack of l- and/or D-alanine, as both forms are components of the "rigid layer" of gram-negative bacteria cell walls: l-ala connects oligopeptides via lactosyl-groups to heteropolymeric chains consisting of N-acetylglucosamine and N-acetylmuraminic acid. D-ala is situated at the carboxy terminal of oligopeptide chains and is linked with the diaminoacids of adjacent oligopeptides (*e.g.* meso-diaminopimelinic acid or lysine). Thus a lack of ala may impair the cross-linkages within the mureinsacculus resulting in the cell wall loosing some stability and becoming more permeable. As the differentiation from free-living rhizobia to symbiotic bacteroids proceeds, ala-DH could possibly drastically diminish the intracellular content of ala by mechanisms involving deamination. A possible analogy may be drawn between these processes and the events described [22] on the induced germination of *Bacillus* spores by l-ala. The specific activities of the ala-DH derived from effective and ineffective bacteroids of *Rhizobium japonicum* are largely variable and no characteristic trend can be discerned. However the maximal values for the specific activities from bacteroids and free-living bacteria of *Rhizobium japonicum* and also vegetative cells of *Bacillus subtilis* are approximately equal, as is the relationship between the activities in the aminating and deaminating reactions. We may therefore draw the conclusion that the functions of ala-DH in *Bacillus subtilis* and *Rhizobium japonicum* are similar, both in metabolism (primary ammonium assimilation and the rapid formation of key metabolites pyruvate, and NH_3 from l-ala) and also in cell differentiation (transformation to, or from, normal vegetative cells to specialized cell types). These roles appear to be independent of nitrogenase activity or cell type.

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