Malic Enzyme Activity in Bacteroids from Soybean Nodules

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Soluble extracts of *Bradyrhizobium japonicum* bacteroids from soybean root nodules showed substantial rates of NAD⁺ and NADP⁺ reduction which were malate and MnCl₂ dependent. Pyruvate was formed stoichiometrically and the NAD- and NADP-dependent rates were additive, indicating the presence of two malic enzymes. The NADP-dependent malic enzyme had a high affinity for malate (apparent $K_m = 0.1$ mM) and was stimulated by ammonium. The NAD-dependent malic enzyme had a lower affinity for malate (apparent $K_m = 1.9$ mM) and was stimulated by potassium and ammonium salts. The maximum velocities of the two enzymes were similar and of comparable magnitude to the activities of tricarboxylic acid cycle enzymes in the extracts. Possible roles of the malic enzymes in the metabolism of malate and succinate in bacteroids are discussed.

INTRODUCTION

Transport and metabolism of dicarboxylic C₄-organic acids are essential for the development of nitrogen-fixing bacteroids in legume root nodules (Finan *et al.*, 1981; Ronson *et al.*, 1981; Arwas *et al.*, 1985). These acids support nitrogen fixation by isolated bacteroids (Appleby, 1984) and are present in high concentrations in legume nodules (Stumpf & Burris, 1979). Furthermore, succinate, fumarate and malate are readily transported across the peribacteroid membrane which encloses the bacteroids *in vivo* (Price *et al.*, 1987; Udvardi *et al.*, 1988), and it is generally considered that succinate or malate are the most likely forms of carbon supplied by the plant to support nitrogen fixation (Appleby, 1984; Dilworth & Glenn, 1984).

Although a key role for these organic acids in nitrogen fixation has been established, the details of their metabolism by bacteroids have not been elucidated. The existence of a functional tricarboxylic acid (TCA) cycle in *Rhizobium* and *Bradyrhizobium* species has been demonstrated (Storvall & Cole, 1978; Karr *et al.*, 1984; Appleby, 1984) but these studies have not demonstrated how imported malate and succinate are metabolized. Gluconeogenic enzymes are present in *Rhizobium leguminosarum* bacteroids (McKay *et al.*, 1985) and may be involved in organic acid metabolism. In *Bradyrhizobium japonicum* bacteroids, added malate and succinate are converted to glutamate in long-term incubations (Salminen & Streeter, 1987) but the pathways involved have not been identified.

Malic enzyme (ME), in combination with malate dehydrogenase (MDH), provides a very efficient way of metabolizing added malate (or succinate) since both pyruvate and oxaloacetate (OAA) are provided to the TCA cycle. Such a pathway, involving an NAD-linked ME (EC 1.1.1.38), operates in plant mitochondria (Day & Hanson, 1977). *Escherichia coli* possesses both an NAD- and an NADP-linked ME (EC 1.1.1.40) which are thought to be involved in malate catabolism and generation of reducing power (Murai *et al.*, 1971; Hansen & Juni, 1975). Recently, NAD- and NADP-dependent ME activities have been demonstrated in *R. leguminosarum* (MacKay *et al.*, 1988), but whether these were due to one or two enzymes was not

Abbreviations: MDH, malate dehydrogenase; ME, malic enzyme; OAA, oxaloacetate; TCA, tricarboxylic acid.

determined. There have been no reports to date of ME activity in *Bradyrhizobia*. In this paper we describe an investigation of ME in *B. japonicum* bacteroids isolated from soybean nodules. Evidence is presented which indicates the occurrence of separate NAD- and NADP-dependent enzymes. The possible role of these enzymes is discussed in terms of bacteroid metabolism and nitrogen fixation.

METHODS

Materials. Seeds of soybean [*Glycine max* (L.) Merr.] cv. Bragg were inoculated with *B. japonicum* strain USDA 110 and grown in pots in a glasshouse, as described previously (Day *et al.*, 1987). Nodules were harvested from plants two to three months old.

Preparation of extracts. All steps were carried out at 4 °C. Bacteroids were isolated from 10–15 g of nodules and purified by centrifugation in a Percoll density gradient as described previously (Day *et al.*, 1986). Purified bacteroids were resuspended in 6 ml 25 mM-*TES*/NaOH buffer (pH 7.5) which contained 50 mM-KCl, 5 mM-MgSO₄ and 5 mM-DTT and the soluble extract prepared by passing the suspension three times through a Yeda press at 14000 Pa. The broken bacteroids were centrifuged at 100000 *g* for 1 h and the supernatant used for assays. Solid (NH₄)₂SO₄ was used to fractionate crude bacteroid extracts. Pellets were redissolved in the same buffer used to resuspend the bacteroids.

Assay of enzymes. All enzyme assays were carried out at 25 °C. Malic enzyme activity was assayed by measuring the increase in *A*₃₄₀ due to the formation of NADH or NADPH. Standard reaction mixtures contained, in a volume of 1 ml, 50 mM-*HEPES*/NaOH buffer (pH 7), 20 mM-KCl, 0.75 mM-EDTA, 5 mM-L-malate, 1 mM-NAD⁺ or NADP⁺, 6 mM-MnCl₂ and an appropriate volume of bacteroid extract. In experiments where the formation of pyruvate was measured, reactions were stopped by the addition of 0.1 ml of 1 M-HCl and the precipitate removed by centrifugation. Pyruvate in the supernatant was determined spectrophotometrically as described by Wedding *et al.* (1976).

3-Hydroxybutyrate dehydrogenase activity was assayed in reaction mixtures which contained, in a volume of 1 ml, 50 mM-*HEPES*/NaOH buffer (pH 7), 10 mM-acetoacetate, 0.2 mM-NADH and an appropriate volume of bacteroid extract. Isocitrate, malate and pyruvate dehydrogenase activities were measured as described by Karr *et al.* (1984), except that 50 mM-*HEPES*/NaOH buffer (pH 7) was used in the reaction mixtures. Protein was determined by the Lowry method, using bovine serum albumin as standard.

The oxidation of NADH and NADPH by *B. japonicum* extracts was determined after bacteroids had been sonicated for three 1 min periods at 100 W. The sonicate was centrifuged at 14000 *g* for 2 min and the supernatant used to measure O₂ uptake as described by Day *et al.* (1986).

RESULTS

Solubilized extracts of soybean bacteroids displayed substantial rates of Mn²⁺-dependent NAD⁺ and NADP⁺ reduction in the presence of malate (Figs 1 and 2). This reduction was a linear function of the quantity of soluble protein added to the reaction mixtures and was accompanied by the production of stoichiometric quantities of pyruvate (Fig. 1). Addition of NADP⁺ to a reaction mixture containing NAD⁺ caused a rate of increase in *A*₃₄₀ exactly equivalent to the rate of reduction of NADP⁺ alone (Fig. 2), indicating that the rates of NAD⁺ and NADP⁺ reduction were additive.

These results suggest that soybean bacteroids contain both an NADP- and an NAD-linked ME. However, since the bacteroid extract also contains high NAD-dependent MDH activity and can catalyse considerable OAA decarboxylation in the presence of MnCl₂ (Table 1), caution must be exercised when interpreting the data for NAD⁺ reduction. MDH coupled to OAA decarboxylation could also lead to stoichiometric formation of NADH and pyruvate. We think it unlikely that NAD-ME activity was due to the presence of MDH for the following reasons. (1) NAD-ME was stimulated by KCl; MDH and OAA decarboxylation were not (Table 1). (2) Desalting the bacteroid extract had little effect on MDH activity, stimulated OAA decarboxylation, but inhibited NAD-ME (Table 1). (3) Addition to the reaction medium of a quantity of commercial (rabbit muscle) NAD-MDH equivalent to the amount of MDH activity in the bacteroid extract stimulated ME activity by less than 10%. If pyruvate formation were due to MDH in conjunction with OAA decarboxylation, a doubling of the activity should have resulted. (OAA decarboxylation was not rate-limiting.) (4) NAD-ME was very labile after freezing and thawing the bacteroid extract, sometimes losing all activity; yet MDH and OAA

Fig. 1

Fig. 2

Fig. 1. Comparison of pyridine nucleotide reduction and pyruvate formation by (a) NADP- and (b) NAD-dependent malic enzymes of *B. japonicum* bacteroids. Pyridine nucleotide reduction was measured by following the increase in A_{340} . Reactions were stopped after 10 min and the concentration of pyruvate determined as described. ●, NADH or NADPH; ○, pyruvate. The data are from one of duplicate experiments.

Fig. 2. Change in A_{340} due to the reduction of (a) NAD^+ and (b) NADP^+ by malic enzymes of *B. japonicum* bacteroids. MnCl_2 and NADP^+ were added to standard reaction mixtures (which contained NAD^+) as indicated. The numbers on the traces give the $\text{nmol NAD(P)H formed min}^{-1} (\text{mg protein})^{-1}$. The data are from one of four replicate experiments.

Table 1. *NAD-ME, MDH and OAA decarboxylation activities in B. japonicum bacteroid extracts*

KCl (10 mM) and MnCl_2 (5 mM) were added to the standard reaction medium as indicated. ME activity was measured as NAD^+ reduction in the presence of malate, MDH as NADH oxidation in the presence of OAA, and OAA decarboxylation as pyruvate formation in the absence of malate and NAD^+ . The values are the means $\pm$ standard errors of four determinations.

Assay conditions	Activity [$\text{nmol min}^{-1} (\text{mg protein})^{-1}$]		
	NAD-ME	OAA decarboxylation	MDH
Crude extract			
+ KCl, - MnCl_2	0	0	ND
+ KCl, + MnCl_2	11.9 ± 0.6	18.1	633 ± 55
- KCl, + MnCl_2	4.0 ± 0.7	16.5	633 ± 55
Filtered extract*			
- KCl, + MnCl_2	4.2 ± 0.6	38.7	566 ± 16

ND, Not determined.

* Extract was passed through a Sephadex G-50 column prior to assay.

decarboxylation were not affected (results not shown). (5) Finally, we used $(\text{NH}_4)_2\text{SO}_4$ precipitation to partially separate the enzymes. The MDH activity of the extract was confined to the 50–70% $(\text{NH}_4)_2\text{SO}_4$ fraction, whereas greater than 85% of the NAD- and NADP-ME activities were in the 30–50% fraction and less than 10% in the 50–70% fraction. These two

Fig. 3

Fig. 4

Fig. 3. Effect of pH on NAD- and NADP-dependent malic enzymes and NAD-dependent MDH of *B. japonicum* bacteroids. Activity of the 30–50% $(\text{NH}_4)_2\text{SO}_4$ fraction (ME) or 50–70% $(\text{NH}_4)_2\text{SO}_4$ fraction (MDH) was measured using the standard reaction mixtures, except that a combination of 25 mM-MES and 25 mM-Tris buffers was used. MDH activity was assayed in the direction of malate oxidation. Each point is the mean of four determinations; the standard errors were 10% or less of the values. ■, NAD-dependent ME; ○, NADP-dependent ME; ●, NAD-dependent MDH.

Fig. 4. Effect of L-malate concentration on NAD- and NADP-dependent malic enzymes of *B. japonicum* bacteroids. Reaction mixtures were as described for the standard assay except that the concentration of L-malate was varied. ●, NAD-dependent ME; ○, NADP-dependent ME. The data are from one of duplicate experiments.

partially purified fractions were used to examine the effect of pH on ME and MDH activities. NAD-ME activity peaked at pH 7, whereas NADP-ME was maximally active near pH 7.5 (Fig. 3). The pH profile of NAD-dependent MDH activity (measured as malate oxidation) was quite distinct from that of NAD-ME, reaching optimum activity at pH 8–8.5 (Fig. 3). Clearly, at pH 7, there is no correlation between MDH and ME activities, and we therefore conclude that soybean bacteroids possess both an NAD- and an NADP-linked ME.

The two forms of ME could be distinguished by the marked difference in their response to increasing malate concentration (Fig. 4). The NAD-ME displayed typical hyperbolic kinetics, whereas activity of the NADP-dependent enzyme was inhibited at concentrations of malate greater than 2 mM. Kinetic constants of the enzymes were determined by fitting the data of Fig. 4, together with those from a duplicate experiment with a different bacteroid extract, to the Michaelis–Menten equation as described by Duggleby (1981). The K_m for L-malate and the maximum velocity of NAD-ME were 1.9 ± 0.4 mM and 8.4 ± 0.1 nmol min⁻¹ (mg protein)⁻¹, respectively (means $\pm$ standard error). The values of the corresponding parameters for NADP-ME (calculated from non-inhibitory concentrations of malate) were 0.10 ± 0.01 mM and 10.1 ± 1.1 nmol min⁻¹ (mg protein)⁻¹. Thus, the maximum velocity was similar for the two enzymes, but NADP-ME had an affinity for malate which was approximately 20-fold greater than that of NAD-ME.

NAD- and NADP-ME were activated by ammonium ions. Activity of NAD-ME in extracts that had been extensively dialysed was stimulated approximately threefold when 10 mM- NH_4Cl was added to the reaction mixtures, and fourfold when the concentration of NH_4Cl was increased to 50 mM (Table 2). NAD-ME was activated to some extent by 50 mM-KCl but not by NaCl. NADP-dependent ME activity was stimulated 1.7- and 2.7-fold by 10 and 20 mM- NH_4Cl , respectively, but KCl and NaCl had no effect (Table 2). Coenzyme A, which is an activator of ME of plants (Macrae, 1971), did not stimulate either of the bacterial enzymes.

The activity of ME was compared to activities of certain TCA cycle enzymes and 3-hydroxybutyrate dehydrogenase in the bacteroid extracts (Table 3). The NAD- and NADP-ME rates were somewhat less than isocitrate dehydrogenase and fumarase activities, but greater

Table 2. *Effect of salts on NAD- and NADP-dependent malic enzymes of B. japonicum bacteroids*

The 30–50% $\text{NH}_4(\text{SO}_4)_2$ fraction of the bacteroid extracts was dialysed for 2 h against 1000 vols 10 mM-Tris/HCl buffer (pH 7). Activity was measured using the standard reaction mixture described, except that KCl was omitted. The values are the means $\pm$ standard errors of four determinations.

Addition (mM)	Activity [nmol min ⁻¹ (mg protein) ⁻¹]	
	NAD-ME	NADP-ME
None	10.4 $\pm$ 2.0	8.6 $\pm$ 2.0
KCl (10)	10.9 $\pm$ 1.7	9.3 $\pm$ 1.0
KCl (50)	19.3 $\pm$ 0.4	10.1 $\pm$ 0.8
NaCl (10)	9.6 $\pm$ 0.1	9.5 $\pm$ 0.5
NaCl (50)	9.1 $\pm$ 1.3	8.0 $\pm$ 0.1
NH ₄ Cl (10)	33.2 $\pm$ 3.5	15.0 $\pm$ 3.9
NH ₄ Cl (50)	44.3 $\pm$ 4.6	23.8 $\pm$ 3.9

Table 3. *Activity of enzymes in the soluble extract of B. japonicum bacteroids*

Enzyme	Activity* [nmol min ⁻¹ (mg protein) ⁻¹]
Malic enzyme (NAD) (EC 1.1.1.38)	8.0 $\pm$ 1.7 (3)
Malic enzyme (NADP) (EC 1.1.1.40)	8.1 $\pm$ 1.5 (3)
Pyruvate dehydrogenase (EC 1.2.4.1)	1.3 $\pm$ 0.5 (4)
Isocitrate dehydrogenase (NADP) (EC 1.1.1.42)	22 $\pm$ 2 (3)
Fumarase (EC 4.2.1.2)	21 (1)
Malate dehydrogenase (NAD) (EC 1.1.1.37)†	940 $\pm$ 233 (4)
Malate dehydrogenase (NADP) (EC 1.1.1.82)†	52 (2)
3-Hydroxybutyrate dehydrogenase (EC 1.1.1.30)	128 $\pm$ 41 (7)

* The number of replicate extractions used to determine the activity $\pm$ standard error is indicated in parenthesis.

† Assayed in the direction of OAA reduction: the oxidative activity of NAD-MDH at pH 7 was 68 $\pm$ 5 nmol min⁻¹ (mg protein)⁻¹.

Table 4. *Oxidation of NADPH by sonicated B. japonicum bacteroids*

Purified bacteroids were sonicated for three 1 min periods at 100 W. The sonicate was centrifuged for 2 min at 14000 g and the supernatant assayed for O₂ uptake as described by Day *et al.* (1986). The final concentrations of succinate and NAD(P)H in the reaction mixtures were 10 mM and 1 mM, respectively.

Substrate	O ₂ uptake (nmol min ⁻¹ ml ⁻¹)
Experiment 1	
Succinate	179
NADH	495
NADPH	14
NADH + NADPH	495
Experiment 2	
Succinate	165
NADH	481
NADPH	12

than pyruvate dehydrogenase activity. Hydroxybutyrate dehydrogenase activity was substantially faster than ME and the TCA cycle enzyme activities. It is interesting to note that some TCA cycle dehydrogenases were NADP-linked and others NAD-linked (Table 3), yet only NADH was oxidized by sonic particles from bacteroids (Table 4). The rate of O₂ uptake with NADH was similar to that with succinate, whereas with NADPH it was much slower. NADPH

and NADH together gave the same rate as NADH alone (Table 4), suggesting that the oxidation of NADPH was catalysed by the NADH dehydrogenase.

DISCUSSION

The results demonstrate that *B. japonicum* bacteroids from soybean nodules possess both an NAD-linked and an NADP-linked ME. As malate or succinate are likely to be supplied to the bacteroid *in vivo* it seems reasonable to suggest that at least one of these malic enzymes is involved in organic acid oxidation during N_2 fixation. The reported O_2 :malate ratios of approximately 3 during malate-supported respiration of soybean bacteroids (Price *et al.*, 1987), as well as the results of McKay *et al.* (1988), are indicative of ME involvement during aerobic metabolism. However, careful analysis of the products of malate oxidation under microaerobic conditions is required before a role in N_2 fixation can be ascribed definitively to ME.

It could be argued that NAD-ME is more likely to function in N_2 fixation, as it has a relatively low affinity for malate and would consequently only be active when ample substrate was available (e.g. from the plant cytosol). The higher affinity enzyme (i.e. NADP-ME) could be involved in 'maintenance' activities in the bacteroid. The results of Table 4 support these proposals; NADH was readily oxidized by sonicated bacteroids and therefore could contribute to ATP production *in vivo*. In contrast, NADPH was oxidized poorly by the electron transport chain of the bacteroids and hence is unlikely to be quantitatively important in energy production (assuming that a soluble transhydrogenase, which could transfer reducing equivalents from NADPH to NADH, is not active in *B. japonicum*). However, NADPH could be used to reduce nitrogenase (Dilworth & Glenn, 1984).

Preliminary experiments with free-living *B. japonicum* strain USDA 110 indicate that NAD-ME expression is stimulated by dicarboxylic acids (malate and succinate), whereas expression of NADP-ME is not (Quinnell & Day, unpublished results). Stimulation of ME expression in *R. leguminosarum* by succinate has also been reported (McKay *et al.*, 1988). This fits well with a role for NAD-ME in support of nitrogenase, since dicarboxylates are probably supplied to bacteroids during N_2 fixation (Appleby, 1984; Dilworth & Glenn, 1984). The stimulation of ME activity by NH_4Cl would ensure maximum activity when ammonia concentrations are high, as occurs during nitrogen fixation. However, further work involving purification of the enzymes and identification of inducer metabolites in cultures of free-living *B. japonicum* is required to confirm this hypothesis.

ME may also be important in other aspects of the carbon metabolism of bacteroids and free-living bacteria. In addition to supplying acetyl-CoA for the TCA cycle, ME could provide NADPH and pyruvate for various biosynthetic pathways. Our results suggest that NADP-ME is more likely to have such a role.

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