Occurrence of Two Pathways for Malate Oxidation in Bacteroids Isolated from Sesbania rostrata Stem Nodules during C2H2 Reduction

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ABSTRACT
Malate oxidation supported C2H2 reduction by bacteroids isolated from Sesbania rostrata stem nodules. Optimal activity reached 7.5 nanomoles per minute per milligram of dry weight and was in the same order of magnitude as that observed with succinate but always required a lower O2 tension. Malate dehydrogenase (EC 1.1.1.37), purified 66-fold from bacteroids, actively oxidized malate (Km = 0.19 millimolar). Malic enzyme (EC 1.1.1.39) from Sesbania bacteroids had a lower affinity for malate (Km = 2.32 millimolar). Both enzymes exclusively required NAD+ as cofactor and required an alkaline pH for optimal activity. 2-Oxoglutarate and oxalacetate, inhibiting malate dehydrogenase and malic enzyme, respectively, were used to specifically block each malate dehydrogenase activity in bacteroids. The predominance of malate dehydrogenase activity to support bacteroid N2 fixation was demonstrated. The inhibition of O2 consumption by 2-oxoglutarate confirmed the importance of the malate dehydrogenase pathway in malate oxidation. It is proposed that the utilization of malate, with regard to O2, is important in a general strategy of this legume to maintain N2 fixation under O2 limited conditions.

Sesbania rostrata is a legume that grows in West Africa and is characterized by the presence of numerous nodules induced along the stems by the soil bacteria Azorhizobium caulinodans (6, 7). The high capacity of stem nodules to reduce N2 has been demonstrated, but only few studies have been devoted to N2 fixation at the bacteroid level (1, 22). Since the cells of the cortex of stem nodules contain chloroplasts (8), bacteroids could appear privileged with regard to O2 supply and carbon molecule availability, explaining the unusual N2 fixation capacity of this type of nodules. However, by comparison to the free living form of Azorhizobium, bacteroids did not exhibit particular tolerance to oxygen when assayed in steady state experiments where O2 was provided by leghemoglobin or myoglobin (2). In contrast, Sesbania bacteroids have an enhanced capacity for utilizing substrates providing energy and reducing power to nitrogenase. Lactate, which was not utilized by soybean and French bean bacteroids, can act as an energy-yielding substrate to support N2 fixation in Sesbania (22). Moreover, although malate oxidation can be coupled to C2H2 reduction in incubations of soybean bacteroids, it is much more efficient in Sesbania bacteroids. Two enzymatic systems are involved in this oxidation process: malate dehydrogenase (MDH') and malic enzyme (ME), acting as follows:

\[
\text{Malate + NAD}^+(\text{MDH}) \rightarrow \text{oxaloacetate} \quad + \text{NAD}^+(\text{H}) + \text{H}^+(1)
\]

\[
\text{Malate + NAD}^+(\text{ME}) \rightarrow \text{Pyruvate} \quad + \text{CO}_2 + \text{NAD}^+(\text{H}) + \text{H}^+ (2)
\]

Recent work has been focused on ME activity in soybean (3, 13) and pea bacteroids (16) which alone appear able to oxidize malate in the absence of significant oxidative activity of MDH. Some discrepancies exist in these studies concerning the characteristics of soybean enzymes, particularly the nature of the cofactor used. Furthermore, in spite of several suggestions for a possible role of malate to support N2 fixation (3–5, 9), no direct relation between malate oxidation and nitrogenase activity was reported.

In this paper, we have determined the involvement of MDH and ME in oxidizing malate and reducing C2H2 in whole bacteroids of Sesbania. Some characteristics of both enzymes after partial purification are described, and the respective role of each oxidative pathway in functioning nodules is discussed.

MATERIALS AND METHODS

Nodules

Seeds of Sesbania rostrata were surface-sterilized in concentrated H2SO4, washed with sterile distilled water, and germinated for 24 h at 30°C as previously described (22). Seedlings were transferred to a vermiculite-sand mixture (2:1, v/v), and the plants growing in a greenhouse received a nitrogen-free mineral solution (18) supplemented with KNO3 (100 mg·L−1) and Ca(NO3)2 (400 mg·L−1). After 3 weeks, suspensions of Azorhizobium caulinodans, strain ORS 571, were used to inoculate the roots and spray the stems of the plants which were then watered with the nitrogen-free mineral solution alone. Stem inoculation was repeated each week as elongation proceeded and nodules were harvested when they were 4 to 5 weeks old.

1 Abbreviations: MDH, malate dehydrogenase; ME, malic enzyme; pO2, oxygen partial pressure.
Bacteroid Preparations

About 25 to 30 g (fresh weight) of stem nodules of *S. rostrata* were crushed anaerobically as described elsewhere (23). The extraction mixture was: 50 mM Na-phosphate buffer (pH 7.4), 5 mM DTT, 2 mM EDTA, 0.2 mM Na-ascorbate, and 10% (v/v) insoluble PVP, and 2 mL of buffer were used per g nodules (fresh weight). After centrifugation of the homogenate at 7000 rpm for 10 min, bacteroids were washed twice in 50 mM Na-phosphate buffer (pH 7.4) containing 2 mM MgSO_4 and 0.3 M sucrose. Chloroplasts sedimented at the upper part of the bacteroid pellet and were scraped off.

Partial Purification of Enzymes

All steps were carried out at 5°C. Bacteroids (3 g fresh weight) were resuspended in 15 mL 50 mM Tris-HCl buffer (pH 8.3) containing 5 mM DTT and 2 mM EDTA and disrupted in a precooled Amino French pressure cell at 110,316 \times 10^6 N m^{-2}. Crude extracts were obtained after centrifugation at 35,000g for 30 min. Solid (NH_4)_2SO_4 was added to the crude extracts to give 40% saturation. After stirring for 30 min, the protein precipitate was sedimented by centrifugation (20,000g, 15 min) and discarded. More (NH_4)_2SO_4 was added to bring the saturation to 60%, and after stirring, the precipitate was collected by centrifugation and dissolved in about 8 mL of 50 mM Tris-HCl buffer (pH 8) containing 5 mM DTT and 2 mM EDTA. Columns (2.5 \times 50 cm) of Sephadex G-200 were prepared and equilibrated with 50 mM Tris-HCl buffer (pH 8) containing 5 mM DTT and 2 mM EDTA (12). The 40 to 60% (NH_4)_2SO_4 fraction was applied to the columns and eluted with 50 mM Tris-HCl buffer at a flow rate of 0.2 mL min^{-1} and 2 mL fractions were collected. Fractions with MDH activity and ME activity were respectively pooled and concentrated in a Diaflo cell fitted with a UM 10 membrane.

Enzyme Assays

For MDH, incubation mixtures received 10 mM Na-malate, 1 mM NAD^+, and enzyme extract in 400 mM hydrazine hydrate/500 mM glycine buffer (pH 9.5) (23).

Standard reaction mixtures for ME contained 15 mM Na-malate, 1 mM NAD^+, 5 mM MgCl_2, 30 mM KCl, and enzyme extract in 50 mM glycylglycine buffer (pH 8.5). In experiments where the formation of pyruvate was measured, reactions were stopped by addition of 0.1 mL of 2 N HClO_4. The mixtures were neutralized with 2 N KOH, and the precipitate was removed by centrifugation (4000g, 10 min). Pyruvate was determined spectrophotometrically in the supernatant with lactate dehydrogenase (20).

All enzymes were assayed spectrophotometrically at 340 nm and 30°C in a final volume of 2 mL with a DU 7 Beckman spectrophotometer. Background rates were established with complete reaction mixture minus the indicated substrate. For both enzymes, 1 unit of activity is defined as the amount of enzyme that reduced 1 \mu mol of NAD^+ min^{-1} at 30°C.

Nitrogen Fixation Assays

Bacteroid incubations (1 mL) were carried out in 7-mL rubber-cap vials in the presence of a gas phase containing 5% (v/v) C_2H_2 and O_2 at different partial pressures in the range of 0.33 to 4.7 kPa in argon. N_2 fixation activity was measured by C_2H_2 reduction (19).

Uptake Experiments

Bacteroids were incubated at 25°C in 2.7 kPa pO_2 in the gas phase. Individual assays (final volume 1 mL) contained Na-malate (0.2 \mu M) enriched with [U-14C]malic acid (5.55 GBq \cdot mmol^{-1}) purchased from CEA (France). The uptake was stopped at regular intervals of time by rapid filtration through Whatman glass microfilter (0.45 \mu m pore size). Filters were then rinsed with 5 mL of the corresponding assay medium, minus labelled substrate, and placed in scintillation vials containing 4 mL Aquamatic. Radioactivity was determined in a Kontron liquid-scintillation counter.

Bacteroid O_2 Consumption

Incubations (4 mL) containing 1 to 2 mg (dry weight) of bacteroids were carried out in the chamber of an O_2 electrode (Rank Bros) without gas phase as described elsewhere (21).

Protein Determination

Protein content of the different extracts was determined by the method of Lowry et al. (15) with BSA as standard.

RESULTS

Malate Oxidation and Acetylene Reduction by Stem Nodule Bacteroids

Addition of malate to bacteroid incubations strongly enhanced C_2H_2 reduction, which was very low when supported by the endogenous reserves alone (Fig. 1). Malate efficiency

![Figure 1. Effect of pO_2 in the gas phase upon bacteroid C_2H_2 reduction. Incubation mixtures (1 mL) contained bacteroids (10 mg dry weight) and 10 mM malate (○), succinate (●), or no exogenous substrate (△) in 25 mM Na-phosphate buffer (pH 7.4). Assays were at 25°C with shaking (140 rpm). DW, dry weight.](image-url)
increased with increasing pO$_2$ up to 2.7 kPa and then declined. When succinate was used as an energy-yielding substrate, about the same optimal C$_2$H$_2$ reduction activity was reached (7.5 nmol·min$^{-1}$·mg$^{-1}$) but required higher O$_2$ tension in the gas phase (4 kPa).

Since two different enzymatic pathways can be involved in malate oxidation (MDH and ME), 2-oxoglutarate and oxalate, commonly described in the literature as competitive inhibitors of MDH and ME, respectively (11, 17, 26), were added to incubation mixture to test their ability to inhibit malate oxidation. Increasing concentrations of 2-oxoglutarate or oxalate were added to incubations under conditions of optimal bacteroid C$_2$H$_2$ reduction as in Figure 1. In both cases, activity of Sesbania bacteroids was inhibited (Fig. 2), indicating that malate oxidation coupled to C$_2$H$_2$ reduction involved both MDH and ME activity. However, the 2-oxoglutarate effect was more pronounced, since 50% inhibition occurred at a concentration of 2.4 mm, whereas the same inhibition level required 7.5 mm oxalate. These two inhibitor concentrations were used to study the effects of increasing pO$_2$ on malate oxidation under these conditions.

C$_2$H$_2$ reduction was lowered by addition of each inhibitor over the range of O$_2$ tensions used, but the effect of 2-oxoglutarate was always greater (Fig. 3). Optimal activity was depressed 22% by oxalate, whereas 2-oxoglutarate, in inhibiting MDH, caused a 53% inhibition rate of bacteroid C$_2$H$_2$ reduction. The effects of both inhibitors increased with increasing pO$_2$ in the gas phase. In the absence of malate in incubations, each inhibitor was unable to act as carbon source for supporting bacteroid N$_2$ fixation.

![Figure 2](image-url)  
**Figure 2.** Inhibitory effect of oxalate and 2-oxoglutarate upon C$_2$H$_2$ reduction activity of Sesbania bacteroids. Incubations were performed with 2.7 kPa pO$_2$ in the gas phase and contained bacteroids (10 mg dry weight [DW]), 10 mm malate and increasing concentrations of oxalate (●) or 2-oxoglutarate (○) in 25 mm Na-phosphate buffer (pH 7.4). Assays were at 25°C with shaking (140 rpm).

![Figure 3](image-url)  
**Figure 3.** Effect of oxalate and 2-oxoglutarate upon C$_2$H$_2$ reduction by Sesbania bacteroids in relation to pO$_2$ in the gas phase. Incubation mixtures (1 mL) contained bacteroids (10 mg dry weight [DW]), 10 mm malate and 7.5 mm oxalate (●), 2.4 mm 2-oxoglutarate (○), or no inhibitor (△) in 25 mm Na-phosphate buffer (pH 7.4). Assays were at 25°C with shaking (140 rpm).

To aid in determining whether the effects observed under our experimental conditions were due to direct effects of inhibitors at the enzymatic level or some other phenomenon, malate transport kinetics studies were conducted with isolated bacteroids. Under a pO$_2$ allowing optimal C$_2$H$_2$ reduction (2.7 kPa), 14C-malate uptake by Sesbania bacteroids was linear for 15 min with an initial velocity of 1.2 nmol·min$^{-1}$·mg$^{-1}$ dry weight. This rate of transport was not modified when assays were conducted either under lower O$_2$ tensions (0.7 or 1.3 kPa) or in air. With the knowledge of concentrations of inhibitors responsible for 50% inhibition of C$_2$H$_2$ reduction, corresponding to a ratio of substrate versus inhibitor (S/I) of 4 and 1.3 (Fig. 2), malate uptake was followed in the presence of 2-oxoglutarate or oxalate concentrations with ratios S/I ranging from 20 to 0.5. A very slight decrease in malate uptake, which did not exceed 2 to 3%, was observed only for the highest concentrations of both inhibitors. Transport kinetics were also determined with [U-14C]2-oxoglutarate and [U-14C]oxalate alone, and, in both cases, inhibitor uptake rates were linear during 15 min with an initial rate of 0.3 nmol·min$^{-1}$·mg$^{-1}$ dry weight.

**O$_2$ Consumption by Sesbania Bacteroids**

Malate stimulated bacteroid respiration, reaching a rate of 14.6 nmol O$_2$·min$^{-1}$·mg$^{-1}$, as compared to 3.7 nmol·min$^{-1}$·mg$^{-1}$ with only endogenous reserves (Fig. 4). Addition of oxalate or 2-oxoglutarate to the incubation mixtures caused a decline in the O$_2$ consumption capacities of bacteroids which appeared more sensitive to 2-oxoglutarate than to oxalate. A concentration of 4.6 mm of 2-oxoglutarate was sufficient to
inhibit O₂ uptake 50%, whereas more than 10 mM oxalate was necessary to reach the same level of inhibition. It should be noted that endogenous rates of bacteroid respiration were not affected by either 2-oxoglutarate or oxalate, confirming that these two substances cannot act as energy-yielding substrates.

Partial Purification of MDH and ME from Bacteroids

Distinguishing between MDH and ME activities in crude extracts of bacteroids was not possible by measuring NAD⁺ reduction. The generation of pyruvate was specifically used to determine ME activity. This method appeared accurate since pyruvate and NADH were stoichiometrically produced during the decarboxylation of malate by partially purified ME (results not given). To further confirm the validity of this procedure, we determined also that there was no oxaloacetate decarboxylation by ME.

Most of activity of both enzymes was in the 40 to 60% (NH₄)₂SO₄ saturation fraction with a purification of 5.5- and 4-fold for MDH and ME, respectively (Table Ⅰ). Separation of ME and MDH was achieved by chromatography on Sephadex G-200 (Fig. 5), the two enzymes emerging as single peaks behind the main peak of protein, as previously observed by Johnson and Hatch (12). For a typical purification, the recovery was about 30% for the two proteins with 66- and 41-fold purification for MDH and ME, respectively (Table Ⅰ).

Specific Characteristics of Bacteroid MDH and ME

The kinetic parameters for the two enzymes were determined in standard assays containing increasing malate concentrations in the range 0.4 to 25 mM. By the use of double-reciprocal plots, the Kₘ values for L-malate were determined to be 0.19 mM and 2.32 mM for MDH and ME, respectively, with corresponding Vₘₐₓ values of 223 and 44 µmol NAD⁺ reduced·min⁻¹·mg⁻¹. The pH-dependent rates of malate oxidation catalyzed by both enzymes were determined between pH 7 and 10 (Fig. 6). The two enzymes exhibited low activity at pH 7, but malate oxidation rapidly increased with increasing pH values. Optimal rates were found at pH 8.5 and 9.5 for ME and MDH, respectively. About 50% of MDH activity (20.4 µmol·min⁻¹·mg⁻¹) remained at pH 8.5 where ME activity was optimal (5.1 µmol NADH·min⁻¹·mg⁻¹). In contrast, when MDH activity was the highest (40.8 µmol·min⁻¹·mg⁻¹ at pH 9.5), ME activity was present in the extracts, but only at very low level (1.6 µmol·min⁻¹·mg⁻¹).

To discriminate better between MDH and ME, the effects of other parameters, such as divalent cations or inhibitors, were tested under our experimental conditions. ME exhibited an absolute requirement for Mg²⁺ to decarboxylate malate, Mn²⁺ being much less efficient (data not shown). In contrast, the presence of Mg²⁺ in incubations did not affect the activity of MDH. In experiments with the inhibitors, oxalate and 2-oxoglutarate, ME appeared more sensitive than MDH, since 50% inhibition was reached with about 0.1 mM oxalate, whereas the same MDH inhibition level required 0.3 mM 2-oxoglutarate (Fig. 7). Moreover, oxalate did not affect MDH.

Table I. Purification of MDH and ME from Sesbania Bacteroids

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activity and, reciprocally, ME was not sensitive to 2-oxoglutarate, confirming the strict specificity of these inhibitors.

DISCUSSION

MDH and ME were separated and partially purified from bacteroids isolated from S. rostrata stem nodules. An interesting feature of Sesbania MDH is its high affinity for malate. This is in contrast to soybean bacteroids where MDH has low activity with malate as substrate, but large amounts of oxaloacetate are reduced (14, 25). Another apparently unique characteristic of Sesbania bacteroids is that they contain only one ME which catalyzes the oxidative decarboxylation of malate but not of oxaloacetate. This property allowed an accurate measurement of its activity by the determination of pyruvate generated. In addition to these characteristics, Sesbania bacteroid ME exhibited an absolute requirement for NAD* and Mg**. These characteristics led us to define this ME as a malate:NAD* oxidoreductase decarboxylating (EC 1.1.1.39). In soybean bacteroids, two types of ME were detected: one functioning with NAD* as cofactor exhibited a $K_m$ for malate of 1.9 mM (3), a value not far from those determined here with Sesbania bacteroids (2.3 mM); and another, requiring NADP*, had a higher affinity for malate with a $K_m$ of 0.1 mM (3, 13). However, it should be noted that the specific activities of ME reported by these authors for crude extracts of soybean bacteroids were generally low.

Malate exerted a fourfold stimulation of optimal Sesbania bacteroid $C_2H_2$ reduction with only endogenous reserves. This points out the existence of an efficient coupling between malate oxidation and nitrogenase activity (Fig. 1). Since two oxidative pathways, ME and MDH, were possible, it was of interest to define the respective role of each. The stronger alkaline pH requirement (9.5) for optimal ME activity, which corresponded to a very low ME activity (Fig. 6), could be used to separate malate oxidation pathways, but whole bacteroids did not tolerate high alkaline conditions. On the other hand, the large differences in MDH and ME $K_m$ values for malate could also be a criterion to differentiate their role in malate oxidation. However, the presence of substantial quantities (3.5 mM) of malate in the host cell cytosol due to the activity of a MDH reducing oxaloacetate (23) allows for the simultaneous participation of MDH and ME in Sesbania bacteroids for $C_2H_2$ reduction. Any restriction in the malate availability for bacteroids could limit ME activity due to its higher $K_m$ for malate. In this way, the role of the peribacteroid membrane in controlling exchanges between the cytosol and bacteroid could be important. In studies with soybean (24) and French bean (10) where malate was actively transported to bacteroids, any modification of the integrity of the vesicle membrane could cause a drop in the supply of malate which would then be oxidized only by MDH. The use of specific inhibitors, added to bacteroid incubations, gives more accurate and discriminating information on each oxidation pathway of malate (Fig. 3). The low level of $C_2H_2$ reduction remaining after addition of 2-oxoglutarate to inhibit MDH indicated the predominant role of this enzyme in malate oxidation. This was confirmed by the only slight depressive effect of oxalate on $C_2H_2$ reduction, in spite of the high sensitivity of ME to this inhibitor (Fig. 7). The similarity in transport kinetics of 2-oxoglutarate and oxalate was in favor of a direct effect of these inhibitors at the enzyme level instead of differences in their uptake by bacteroids.

These inhibitors, in decreasing the catalytic activity of Sesbania bacteroid MDH and ME, which both require NAD*, probably limited NAD* reduction and availability. Therefore, the pool of NADH reoxidized by the electron transport chain of bacteroids was restricted, explaining the decline observed in $O_2$ consumption (Fig. 4). In this way, the greater effect of...
both inhibitors on $C_2H_2$ reduction, when $pO_2$ was increased, could be related to nitrogenase inactivation by excess oxygen. It should be also noted that bacteroid respiration was always less sensitive to both inhibitors than nitrogenase activity (see Figs. 2 and 4), confirming the tight coupling between malate oxidation and $C_2H_2$ reduction. The fact that both enzymes provide NADH instead of NADPH is of interest in terms of energy for bacteroid nitrogenase, since NADPH, in general, is poorly reoxidized by the respiratory chain of bacteroids (3).

Thus, in addition to the capacity to use lactate as an energy-yielding substrate (22), Sesbania bacteroids are able to actively oxidize malate for reducing $N_2$. These two compounds require lower $O_2$ supply than those of other organic acids such as succinate. The ability of these bacteroids to use a wide range of substrates can be interpreted as a novel strategy developed by this tropical legume in response to variations in the $O_2$ availability. Such a situation occurs during the rainy season in West Africa when the lower part of Sesbania stems are immersed in $O_2$ restricted conditions. By using successively, or simultaneously, oxidation of malate and lactate associated with alcoholic fermentation, Sesbania is able to maintain a significant $N_2$ fixation activity (22).

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