AN ENDOGENOUS ELECTRON CARRIER FOR THE NITROGENASE SYSTEM OF *RHIZOBIUM* BACTEROIDS[1]

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Received 24, 1969

SUMMARY

A partly purified electron carrier isolated from an extract of *Rhizobium* bacteroids (from soybean root nodules) mediated the transfer of reducing power generated by illuminated spinach chloroplasts to the nitrogenase of *Rhizobium* bacteroids or of *Azotobacter vinelandii*. The electron carrier is reducible by dithionite and, according to preliminary evidence, is similar to the recently reported *Azotobacter* type of ferredoxin.

The reductant required for nitrogen fixation by the nitrogenase system of anaerobes such as *Clostridium pasteurianum* was shown to be ferredoxin (Mortenson, 1964; D'Eustachio *et al.*, 1964). However, *in vitro* nitrogen fixation by the nitrogenase systems of aerobes, e.g. *Azotobacter vinelandii* and *Rhizobium* bacteroids, was achieved only with artificial reductants, such as dithionite (Bulen *et al.*, 1966; Koch *et al.*, 1967). The identity of the endogenous reductants involved in aerobic nitrogen fixation has remained unknown.

Recent experiments with the nitrogenase system of *A. vinelandii* (Benemann *et al.*, 1969; Yoch *et al.*, 1969) have shown that the reductants in nitrogen fixation by that organism can be successfully investigated by the experimental approach of Yoch and Arnon (1969), in which the reducing power for nitrogenase activity is photochemically generated by spinach chloroplasts. One merit of this approach is that illuminated chloroplasts, unlike dithionite, can donate

[1]This investigation was aided by National Science Foundation grants G-13970 (to D. I. Arnon), GB-7036 (to R. C. Valentine), and GB-12116 (to H. J. Evans).

[2]Participated in this investigation by culturing plants and preparing a root nodule extract having nitrogenase activity, as described under Methods.

electrons to nitrogenase only through intermediate electron carriers. The dependence of the coupled chloroplast-nitrogenase system on intermediate electron carriers provides an experimental tool for testing the efficacy of those cellular electron carriers that may be involved in nitrogen fixation.

This communication reports the isolation, by this procedure, of an endogenous electron carrier linked to the nitrogenase system of Rhizobium bacteroids from soybean root nodules. The same factor was also effective as an electron carrier for the Azotobacter vinelandii nitrogenase.

METHODS

The assay for nitrogenase activity (reduction of acetylene to ethylene), the preparation of the A. vinelandii nitrogenase and of spinach chloroplasts were described elsewhere (Benemann et al., 1969; Whatley and Arnon, 1963). An extract of root-nodule bacteroids with an active nitrogenase system was prepared by the method of Klucas et al. (1968) from nodules removed from roots of about 1,000 five-week-old soybean plants.

RESULTS AND DISCUSSION

The nitrogenase activity of extracts of bacteroids from soybean root nodules is inactivated by oxygen (Koch et al., 1967). We found that it is also inactivated by mild heating (60° for 10 min) but that the heat treatment does not destroy an electron carrier activity present in the bacteroid extract - an activity that mediates the transfer of reducing power from illuminated spinach chloroplasts to Azotobacter nitrogenase. Thus, after being heated at 60° for 10 min, the bacteroid extract becomes a source of the electron carrier, free of nitrogenase activity (Table 1). Table 1 also shows that the electron carrier activity of bacteroid extracts, although resistant to heating, is sensitive to oxygen, becoming about 85% inactivated after an exposure of 2 hours to O_2 at 30°.

Heating the crude bacteroid extract at 60° for 10 min under argon gave a precipitate which was removed by centrifugation at 10,000 x g and discarded.

Table 1

EFFECT OF HEATING AND OXYGEN ON THE STABILITY OF ELECTRON CARRIER AND NITROGENASE ACTIVITY OF RHIZOBIUM BACTEROID EXTRACTS

Treatment of bacteroid extract	Electron carrier activity[1]	Dithionite-dependent nitrogenase activity
	(Ethylene produced (mμmoles/min))	
None	34.1	92.5
100% O_2 for 2 hours	4.6	0.5
Heating at 60° for 10 min (under argon)	34.8	2.0

[1]The electron carrier activity of the treated extract was measured by its ability to support acetylene reduction in the coupled chloroplast-nitrogenase system described by Yoch and Arnon (1969).

The reaction mixture used to measure dithionite-dependent nitrogenase activity of the Rhizobium bacteroid extract contained, in addition to bacteroid extract (3.75 mg protein), treated as indicated, the following in μmoles: HEPES buffer, pH 7.4, 50; Mg^{++}, 5; creatine phosphate, 40; ATP, 4; creatine phosphokinase, 0.05 mg; and sodium dithionite, 20. To measure electron carrier activity, the above reaction mixture was modified by adding a washed A. vinelandii nitrogenase (4.6 mg protein) and replacing the sodium dithionite by spinach chloroplasts (300 μg chlorophyll); ascorbate, 10 μmoles; and 2,6-dichlorophenol indophenol, 0.05 μmoles. Final volume, 1.5 ml. Light intensity, 9,000 foot-candles. Gas phase, 73% argon and 27% acetylene. The reaction was carried out at 30° for 30 min.

The supernatant solution was used as the source of the electron carrier. Its effectiveness in coupling the reducing power generated by illuminated spinach chloroplasts to Azotobacter nitrogenase and to the native bacteroid nitrogenase is shown in Fig. 1. Activity of the washed Azotobacter nitrogenase had an absolute requirement for the bacteroid electron carrier. The crude bacteroid extract which contained both nitrogenase and electron carrier showed significant activity without added carrier. Nevertheless, the addition of carrier to the crude bacteroid extract gave a marked increase in nitrogenase activity, at a rate parallel to the carrier-dependent nitrogenase activity of Azotobacter.

A further purification of the bacteroid electron carrier is summarized in Table 2. The supernatant solution (after the heat treatment) was passed through a DEAE-cellulose column previously equilibrated with 0.02 M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer, pH 7.2. Most of the electron

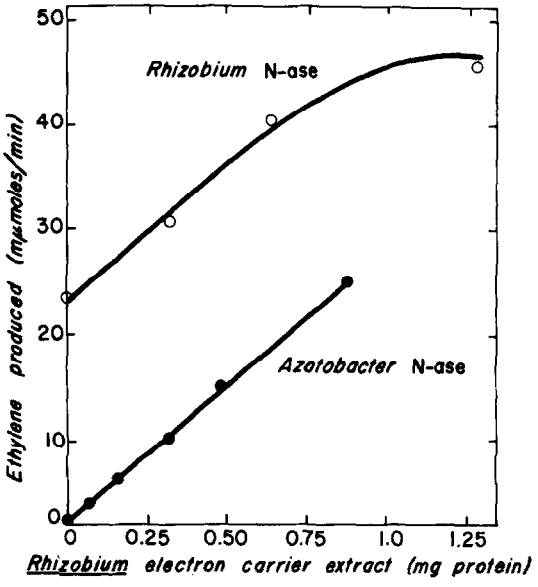

Figure 1. Effect of an electron carrier fraction isolated from a Rhizobium bacteroid extract on the coupling of the reducing power generated by illuminated chloroplasts to nitrogenase activity. The complete reaction mixture contained, in addition to the Rhizobium bacteroid electron carrier fraction (see text), spinach chloroplasts (300 μg chlorophyll), and the following in μmoles: HEPES buffer, pH 7.4, 50; ascorbate, 10; 2,6-dichlorophenol indophenol, 0.05; Mg^{++}, 5; creatine phosphate, 40; ATP, 4; and creatine phosphokinase, 0.05 mg. The source of nitrogenase was either a crude Rhizobium bacteroid extract, 2.5 mg protein, or a washed A. vinelandii nitrogenase preparation, 4.6 mg protein. Other conditions were as described in Table 1.

Table 2

PURIFICATION OF ELECTRON CARRIER FROM A RHIZOBIUM BACTEROID EXTRACT

	Protein (mg/ml)	Total protein	Total activity (units)[1]	Specific activity (units/mg)
Crude extract	12.5	150	1800	12.0
Heated extract[2]	3.1	52.6	1770	33.7
DEAE chromatography	1.35	1.75	510	292

[1] 1 unit = 1 mμmole ethylene reduced/min. Electron carrier activity was measured by its ability to couple the reducing power generated by illuminated spinach chloroplasts to acetylene reduction by A. vinelandii nitrogenase.

[2] Crude extract heated at 60° for 10 min under argon.

carrier activity was localized on the column in a dark brown band. The column was washed with the same buffer which contained, successively, 0.15 M and 0.3 M NaCl. The brown band was then eluted with the same buffer containing 0.8 M chloride and contained 80% of the total electron carrier activity. Apart from the brown band, low but significant electron-carrier activity was observed in several fractions from the DEAE-cellulose column preceding the main fraction. This activity could have come from contamination by the main electron carrier fraction or it might have resulted from the presence of other electron carriers yet to be identified.

Preliminary results indicate that the partially purified, brown electron-carrier fraction is not a flavoprotein; it is reducible by dithionite and its spectrum, showing a broad shoulder between 360 and 415 mμ, appears to be similar to that of the Azotobacter type of ferredoxin (Yoch et al., 1969). However, the similarity of properties of these two factors needs further investigation. An iron-sulfur protein, without "consistent enzyme activity," was earlier isolated from Rhizobium bacteroid extracts by Koch et al. (1967).

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