FERREDOXIN FROM BACILLUS POLYMYXA*

Y. I. Shethna, N. A. Stombaugh and R. H. Burris
Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706

Received January 29, 1971

SUMMARY

A ferredoxin has been purified from Bacillus polymyxa, strain Hino, a facultative N_2-fixing organism. Optical and electron spin resonance spectra of the ferredoxin have been recorded, and its molecular weight is approximately 9,000. The ferredoxin is reduced reversibly by H_2 in the presence of hydrogenase from Clostridium pasteurianum or B. polymyxa.[2] Purified ferredoxin enhances acetylene reduction, with pyruvate as the substrate, by extracts from which ferredoxin has been partially removed with DEAE-cellulose.

Bacillus polymyxa (strain Hino) fixes N_2 only under anaerobic conditions. The pathway of electron transfer for N_2 reduction by B. polymyxa has not been defined, but data in this paper indicate that ferredoxin, whose properties are described here, can serve as an electron donor in N_2 fixation.

MATERIALS AND METHODS

The cells of B. polymyxa used for isolation of ferredoxin were grown in 150 or 1,500 liter fermentors in a medium free of fixed nitrogen containing 12.2 g K_2HPO_4, 1.35 g KH_2PO_4, 20 g sucrose, 0.1 g $MgSO_4$, 0.015 g $FeCl_3 \cdot 6\ H_2O$, 0.003 g $NaMoO_4 \cdot 2\ H_2O$, 0.09 g $CaCl_2 \cdot 2\ H_2O$ and 0.0001 g biotin per liter of medium. Culture vessels were sparged with 0.03-0.15 liters of high purity N_2/min/liter of culture. After 18 hours, 2-3 g of wet cells/liter were recovered; they were stored at -8°.

Cell-free preparations were made from cells grown for 12-14 hours in 100 liter fermentors; the yield was about 1 g of wet cells/liter. These

*Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. The work was supported in part by grant GB-483 from the National Science Foundation, by research grants AI-00848 and AI-01417 from the Division of Research Grants, National Institutes of Health, U. S. Public Health Service, and by Public Health Service Training Grant no. GM-00236BCH from the National Institute of General Medical Sciences.

cells were stored in liquid N_2.

The ferredoxin was purified essentially as iron-sulfur protein III from Azotobacter vinelandii (1). 1,500 g of wet cell paste, 3,000 ml of 50 mM phosphate buffer (pH 7.3), and 1,500 ml of n-butyl alcohol were stirred vigorously at 4° for 2 hours. The mixture was centrifuged for 2 hours at about 13,000 x g at 4°, and the aqueous and butanol layers were decanted. Approximately 500 ml wet settled volume of DEAE cellulose (Brown Co., DEAE type 40) was added to the aqueous supernatant and stirred for 6-8 hours at 4°. This slurry was washed in a sintered glass funnel with approximately 6 liters of the 50 mM phosphate buffer to remove butanol. The DEAE cellulose was transferred to a 10 cm diameter column and was washed with 0.10 M phosphate buffer (pH 7.3), containing 0.30 M KCl. The dark brown eluate was dialyzed overnight in 0.01 M phosphate buffer, pH 7.3.

The dialyzed eluate was placed on a column (2.4 x 15 cm) of Whatman DE32 cellulose equilibrated with the 0.01 M phosphate buffer, pH 7.3. The column was washed with 10 ml aliquots of phosphate buffer, increasing to 0.02, 0.03 M etc. until a concentration of 0.10 M had been reached. The ionic strength was gradually increased with KCl (0.01, 0.02 M etc.) until a concentration of 0.10 M phosphate (constant) and 0.12 M KCl had been reached; the column was washed with 15-20 bed volumes of this buffer. The molarity was raised to 0.10 M phosphate and 0.16 M KCl, and the brown-green ferredoxin band was displaced almost to the bottom of the column. The top layer of the column together with a tightly bound red protein was removed by scraping. The ferredoxin then was eluted with 0.10 M phosphate and 0.30 M KCl; 2-3 ml fractions were collected.

Fractions with optical absorbance ratios (A_{280}/A_{400}) less than 3.0 were pooled, and ammonium sulfate was added slowly to 100% saturation. If the ferredoxin was too concentrated it began to precipitate at high concentrations of ammonium sulfate, but this could be prevented by diluting the ferredoxin solution. The A_{280}/A_{400} ratio of the solution after this step usually was 2.0-2.3.

The supernatant remaining after ammonium sulfate precipitation was dialyzed

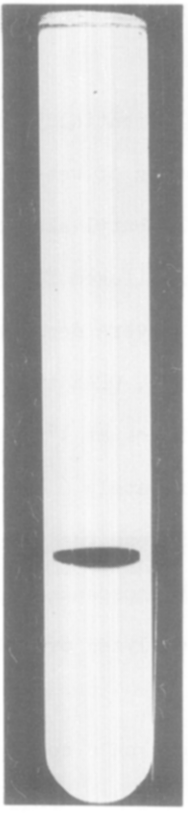

Fig. 1. Pattern of ferredoxin from B. polymyxa after electrophoresis on polyacrylamide gel. The anode was at the bottom; the A_{280}/A_{395} ratio was 1.85.

against 0.025 M Tris (pH 7.3), concentrated in collodion bags (Schleicher and Schuell, Inc.) and placed on a 1.5 x 90 cm column of Sephadex G-100 equilibrated with pH 7.3, 0.025 M Tris-HCl and 0.1 M KCl.

Fractions from the column with A_{280}/A_{400} ratios less than 1.80 were pooled, concentrated, and stored in small vials in liquid N_2. Fractions with A_{280}/A_{400} ratios less than 1.85 were homogeneous in polyacrylamide gel electrophoresis at pH 8.0-9.0 (2) as shown in Fig. 1. Approximately 10 mg of pure ferredoxin was obtained from 1 kg of wet cell paste.

The optical spectrum was recorded on a Cary model 11 spectrophotometer compared to a water blank (Fig. 2). The EPR spectrum was obtained as described in Fig. 3.

The molecular weight was determined by comparison with standard proteins

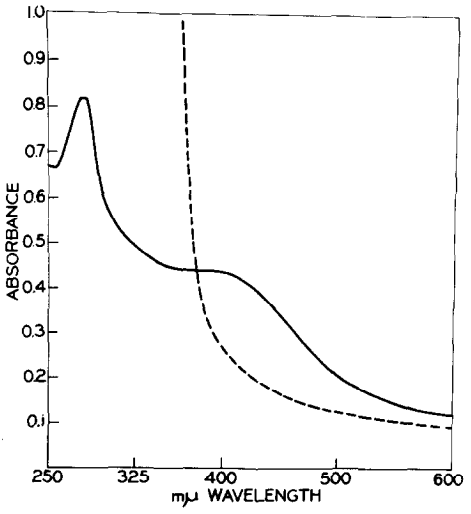

Fig. 2. Optical absorption spectrum of ferredoxin from B. polymyxa; ——— oxidized spectrum was taken with 0.39 mg protein/ml in 25 mM Tris-HCl (pH 7.3), ------reduced spectrum was taken 5 min after the addition of excess $Na_2S_2O_4$. The spectra were recorded with a Cary 11 spectrophotometer.

by column chromatography on Sephadex G-100 equilibrated with 0.10 M KCl and 0.025 M Tris-HCl (pH 7.3).

Cell-free extracts were prepared by washing 15-20 g of cell paste in 400 ml of pH 7.3, 0.010 M Tris-HCl buffer sparged with high purity N_2. The cells were sedimented by centrifugation at 4° and about 8,000 x g for 15 minutes and were resuspended in 10-15 ml of the same buffer. The cells were broken in a French press at 10,000 psi; DNAse was added to the extract to reduce viscosity, and the extract then was centrifuged for 20 minutes at 25,000 rpm in a Spinco model L centrifuge with a no. 30 head. This crude extract had approximately 50 mg protein/ml.

Ten ml of this extract was placed on an anaerobic column (1.5 x 12 cm) of DEAE cellulose (Whatman DE32) equilibrated with 0.010 M Tris-HCl (pH 7.3). The column was washed with 0.4 ml aliquots of pH 7.3, 0.010 M, 0.015 M, 0.020 M and 0.025 M Tris followed by 0.025 M Tris plus 0.005 M $MgCl_2$, 0.025 M Tris plus 0.010 M $MgCl_2$, etc. to 0.025 M Tris plus 0.035 M $MgCl_2$. The column then was

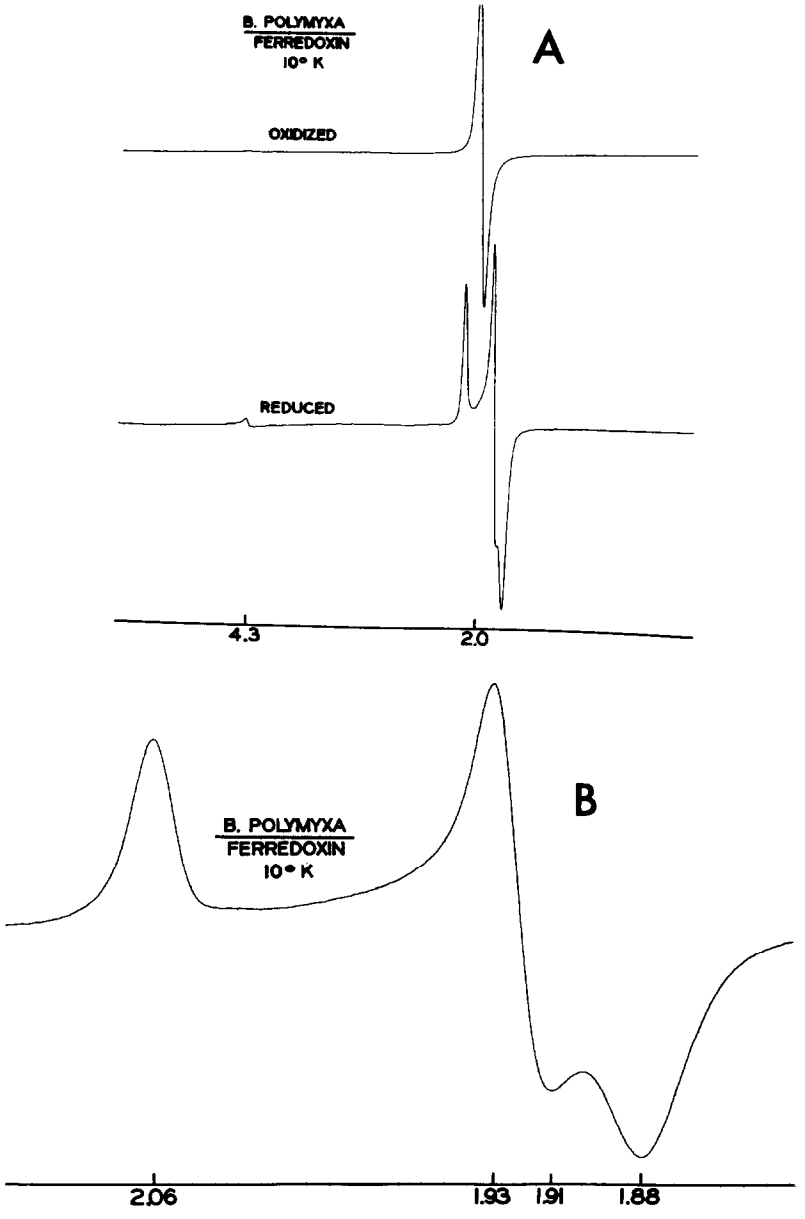

Fig. 3. A. EPR spectra of ferredoxin from B. polymyxa before and after addition of excess $Na_2S_2O_4$ at pH 7. Conditions of EPR spectroscopy were: frequency , 9,150 mHz; modulation (100 KHz) amplitude, 12.5 gauss; incident power, 27 milliwatts; field sweep rate, 1250 gauss/min; time constant, 0.5 sec; temp, 10° K. The spectra are displayed as the first derivative of microwave power absorbed with respect to applied magnetic field, as a function of applied field. The numbers on the abscissa are g-values calculated from the microwave frequency and the magnetic fields at the indicated points. The instrument gain was increased five-fold when the lower spectrum was recorded.
B. EPR spectrum of ferredoxin from B. polymyxa after reduction with excess $Na_2S_2O_4$ at pH 7. The conditions of EPR spectroscopy and spectral display are as in Fig. 3A, except that the field sweep rate was 125 gauss/min, and only the portion of the field between 3,150 gauss and 3,500 gauss is shown.

washed with 2-10 ml of 0.025 M Tris plus 0.035 M $MgCl_2$ until the ferredoxin which had formed a band at the top of the column began to move down the column; as the ferredoxin moved it quickly formed a relatively dark brown band. When this band became obvious, the column was stopped and the top 1-2 cm of the column including the brown band was removed with a Pasteur pipette connected to an aspirator. This removed most of the ferredoxin from the crude extract. The column containing hydrogenase, the Mo-Fe protein, the Fe protein and trace amounts of ferredoxin was washed with 0.025 M Tris-HCl plus 0.10 M $MgCl_2$. The anaerobically collected "extract minus ferredoxin" contained approximately 15-20 mg of protein/ml.

The ability of hydrogenase fractions from both C. pasteurianum and B. polymyxa plus H_2 to reduce B. polymyxa ferredoxin was tested by flushing a 10 mm light path quartz cuvette with H_2 several times and injecting 0.2 ml of a freshly prepared hydrogenase fraction from either organism. The reaction was followed with a Cary model 11 spectrophotometer over a range of 325-600 nm.

RESULTS AND DISCUSSION

The optical spectrum is recorded in Fig. 2. The oxidized spectrum shows the characteristic protein peak at 280 nm plus a peak near 400 nm. The 400 peak disappeared after reduction. The spectrum resembles the spectrum of iron-sulfur protein III from A. vinelandii. The $Na_2S_2O_4$ accounts for the end absorption below 400 nm.

The EPR spectra of the ferredoxin, before and after reduction with dithionite, are recorded in Figures 3A and 3B. With C. pasteurianum ferredoxin (3), no signals in the vicinity of $g = 2$ were observable at 77° K, so lower temperatures had to be employed. Also, as with clostridial ferredoxin, a narrow signal near $g = 2$ was given by the protein prior to reduction (4). In contrast to the complex signals given by clostridial ferredoxin on reduction, the ferredoxin from B. polymyxa gives a signal resembling chloroplast ferredoxins, which perform one-electron transfers (5). Ferredoxin from B. polymyxa may represent a form intermediate between the bacterial and plant classes of iron-sulfur proteins.

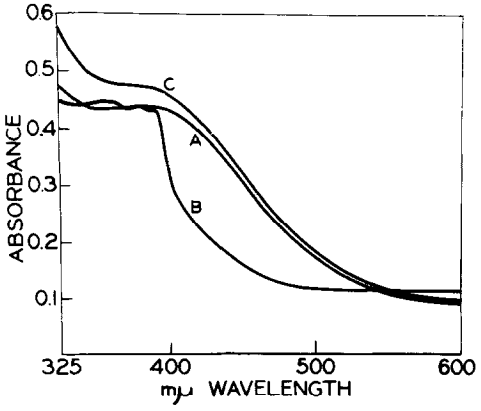

Fig. 4. Spectral changes indicating the reaction between B. polymyxa ferredoxin and hydrogenase from C. pasteurianum in the presence of H_2. A sample of the purified ferredoxin, containing 0.39 mg protein/ml, was flushed several times with H_2 in a quartz cuvette capped with a serum stopper. Spectrum A was taken, and 10 min after the addition of 0.2 ml of a freshly prepared hydrogenase fraction, spectrum B was taken. Spectrum C was taken after shaking the sample exposed to air. Similar spectra were obtained with hydrogenase from B. polymyxa.

The molecular weight, as determined by comparison with movement of known proteins on Sephadex G-100, was near 9,000. This value is intermediate between those found for the ferredoxins from C. pasteurianum and spinach.

It is evident from Fig. 4 that hydrogenase from C. pasteurianum can activate H_2 for the reduction of ferredoxin from B. polymyxa. Line C of Fig. 4 also illustrates that the process is reversible. As the same result was obtained with hydrogenase from B. polymyxa, it may be concluded that ferredoxin has the potential to serve as an electron transfer agent in B. polymyxa. By analogy with other N_2-fixing agents, it is logical that it functions as an electron donor in N_2 fixation.

This conclusion is supported by the demonstration that extracts from B. polymyxa depleted in ferredoxin have a decreased capacity for reduction of acetylene, and that the activity for acetylene reduction is restored by added ferredoxin (Table I). Under the conditions described, activity was increased 4.3 fold by added ferredoxin. Enhancements of 10-20 fold have been obtained but have not been consistent. Although ferredoxin was reduced by H_2 via hydrogenase,

Table 1

RESTORATION BY FERREDOXIN OF ACTIVITY FOR ACETYLENE REDUCTION BY EXTRACTS OF B. POLYMYXA DEPLETED IN FERREDOXIN

	Nanomoles C_2H_2 reduced/min/mg protein	
	A	B
Crude extract	1.71	1.39
Extract minus Fd	0.262	0.0745
Extract minus Fd plus purified Fd		
110 µg added	0.722	0.288
220 µg added	1.13	-
330 µg added	-	0.212
Dithionite control	3.55	2.73

Samples in the two experiments were run in duplicate at 30° for 60 min under 0.1 atm C_2H_2. All samples except the dithionite control contained 11 mg pyruvate per ml, 1.0 mg ATP per ml, and less than 0.1 mg coenzyme A per ml. The dithionite control contained per ml 2.5 mg ATP, 13 mg phosphocreatine, 0.5 mg creatine kinase and 2.6 mg $Na_2S_2O_4$. The total reaction volume always was 1.0 ml.

electron donors other than H_2 also should effect reduction of acetylene, N_2 and other substrates with the aid of ferredoxin.

Ferredoxins generally are separated with ease from the Mo-Fe protein and the Fe protein responsible for N_2 fixation, but the separation is difficult in extracts from B. polymyxa. Hence, it was necessary to scrape DEAE cellulose from the top of the column to aid in the separation. Further work will reveal how much the ferredoxin from B. polymyxa differs from other ferredoxins in isoelectric point, O-R potential, composition and other properties.

Acknowledgment: The authors thank Dr. W. Orme-Johnson for the EPR measurements and Dr. J. Garver for his aid in culturing the organisms in quantity.

REFERENCES

1. Shethna, Y. I., Biochem. Biophys. Acta, 205, 58 (1970).
2. Davis, B. J., Ann. N. Y. Acad. Sci., 121, 404 (1964).
3. Palmer, G., Sands, R. H., and Mortenson, L. E., Biochem. Biophys. Res. Comm., 23, 357, (1966).
4. Orme-Johnson, W. H., and Beinert, H., Biochem. Biophys. Res. Comm., 36, 337 (1969).
5. Hall, D. O., and Evans, M. C. W., Nature, 223, 1342 (1969).