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Purification and Some Properties of a Non-Haem Iron Protein from the Bacteroids of Soya-bean (*Glycine max* Merr) Nodules

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A non-haem iron protein was isolated from an extract of soya-bean nodule bacteroids by a procedure including protamine sulphate and heat precipitation followed by chromatography on DEAE-cellulose. The purified protein contains non-haem iron and acid-labile sulphur and exhibits a spectrum with a rather broad absorption shoulder in the region 380–440nm and a more prominent peak at 280nm. From sedimentation-velocity measurements an apparent $s_{20,w}$ value of 1.3S was calculated. The protein functions as an electron carrier between the reducing system of illuminated chloroplast fragments and nitrogenase from nodule bacteroids, but it failed to function as a cofactor for the photochemical reduction of NADP in the presence of spinach chloroplasts. Also, it is inactive as a cofactor in the enzymic degradation of pyruvate to acetyl phosphate and CO_2 in the presence of a ferredoxin-free extract of *Clostridium pasteurianum*. Repeated freezing, storage and thawing of the non-haem iron protein resulted in a marked loss of activity in the photochemical acetylene-reduction assay. A major portion of the activity that was lost was restored as a result of treatment with sodium sulphide, mercaptoethanol and ferrous ammonium sulphate.

Mortenson (1964*a*) and D'Eustachio & Hardy (1964) showed that ferredoxin was required for linking the phosphoroclastic breakdown of pyruvate to nitrogen fixation in reactions containing extracts of *Clostridium pasteurianum*. In the initial experiment (Bulen, Burns & LeCompte, 1964) in which nitrogen fixation by cell-free extracts of *Azotobacter vinelandii* was demonstrated, a crude extract of *C. pasteurianum*, lacking nitrogenase but containing ferredoxin and hydrogenase was utilized to transfer electrons from H_2 gas to the *Azotobacter* nitrogenase. In similar experiments, Dr Robert Klucas (unpublished work) of this laboratory successfully utilized H_2 gas, and ferredoxin and hydrogenase from *C. pasteurianum* as an electron-donor system for soya-bean bacteroid nitrogenase. In addition to reductant, this system like others, required a source of ATP.

Until recently, sodium dithionite had been used almost exclusively as a reductant for the determination of nitrogenase in extracts from *Azotobacter* and nodule bacteroids because natural electron-donor systems for nitrogen fixation in these organisms had not been elucidated. Klucas & Evans (1968) reported that electrons for the nitrogenase system for both *Azotobacter* and soya-bean bacteroids could be supplied by an $NADH_2$-generating system provided that a dye such as benzyl viologen was added as an electron carrier. Some evidence (Evans, 1970) was obtained that the dye in the coupled nitrogenase reaction could be replaced by a crude factor and flavin nucleotide. Koch, Evans & Russell (1967) identified a nodule bacteroid component containing non-haem iron and acid-labile sulphur, but no definite role of this factor in electron transport was established. It would not function in known assays for ferredoxin.

Benneman, Yoch, Valentine & Arnon (1969) have described an electron carrier from *Azotobacter*, referred to as azotoflavin, that functions in the transfer of electrons from photosystem I of spinach chloroplast fragments to nitrogenase from *Azotobacter*. Photochemical reduction of acetylene in this system was dependent on an ATP supply. The azotoflavin appears to be identical with the free-radical flavoprotein isolated and crystallized from *A. vinelandii* by Hinkson & Bulen (1967). By use of the chloroplast-fragment assay, Yoch, Benneman, Valentine & Arnon (1969) also have identified a ferredoxin compound in *A. vinelandii* that also functions in the transfer of electrons from photosystem I to the *Azotobacter* nitrogenase. In connection with this, Mr Sterling Russell from this laboratory has prepared an extract of soya-bean

nodule bacteroids containing active nitrogenase, and Yoch, Benneman, Valentine, Arnon & Russell (1970) submitted a preliminary report indicating that this extract contained a factor that functioned in the transfer of electrons from photosystem I to the *Azotobacter* nitrogenase. The addition of the bacteroid protein to an assay containing chloroplast fragments, an ATP-generating system and a crude bacteroid nitrogenase increased acetylene reduction from 24nmol of ethylene/min without the factor to 45nmol/min with a saturating concentration of it.

The present paper describes the purification and some properties of a compound from soya-bean nodule bacteroids that functions in the transfer of electrons from photosystem I to bacteroid nitrogenase. This non-haem iron component does not function as a cofactor in the phosphoroclastic breakdown of pyruvate or in the photochemical reduction of NADP and therefore classification as a ferredoxin seems unjustified.

MATERIALS AND METHODS

Chemicals

Reagent-grade chemicals or the highest grade available were obtained from commercial sources. Creatine phosphate, 2,6-dichlorophenol-indophenol, 2-mercaptoethanol, ATP (sodium salt), and tris were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. TES [*N*-tris-(hydroxymethyl)methyl-2-aminomethanesulphonic acid] was obtained from Calbiochem, Los Angeles, Calif., U.S.A. Na_2S, ferrous ammonium sulphate, $MgCl_2$ and CaC_2 (for acetylene generation) were obtained from J. T. Baker, Phillipsburg, N.J., U.S.A. The protamine sulphate was obtained from Eli Lilly Corp., Indianapolis, Ind., U.S.A., and sodium ascorbate and polypropylene glycol (P-400) from Matheson, Coleman and Bell, Cincinnati, Ohio, U.S.A. Polyvinylpolypyrrolidone was obtained from the General Aniline and Film Corp., San Francisco, Calif., U.S.A. The pre-purified N_2 and argon were obtained from National Cylinder Gas, Portland, Oreg., U.S.A. Traces of O_2 were removed from gases by the method of Lux (1959).

Preparation and assay of bacteroid non-haem iron protein

Crude bacteroid extract. Soya-bean plants (*Glycine max* Merr. var. Chippewa) inoculated with a commercial preparation of *Rhizobium japonicum* were cultured and the nodules were harvested as described by Klucas, Koch, Russell & Evans (1968). The crude bacteroid extract was prepared from 300g batches of nodules by a method in which polyvinylpolypyrrolidone and sodium ascorbate were added to the preparation medium to prevent enzyme inactivation by phenolic compounds. The procedure as described by Klucas *et al.* (1968) was used with the following exceptions. Anaerobic conditions were not maintained after the separation of the bacteroids from the brei by centrifugation. The bacteroids were washed in 0.02M-TES buffer, pH7.5, and subsequently suspended in 0.1M-TES buffer at pH8.5 before they were ruptured in a French pressure cell at 16000lb/in^2. A typical preparation in which 300g of nodules was processed gave 45ml of crude extract with a protein content of about 50mg/ml.

Protamine sulphate and heat treatments. Sufficient 2% protamine sulphate was added with stirring to 45ml of crude bacteroid extract (2.3g of protein) to obtain a concentration of 80mg of protamine sulphate/g of protein. After stirring at 4°C for 5min the protamine sulphate precipitate was removed by centrifugation at 48000g for 20min with an Ivan Sorvall RC2B refrigerated centrifuge. The supernatant liquid was placed in stainless-steel centrifuge tubes, gassed with purified N_2, sealed and then heated at 55°C for 3min. The tubes were cooled immediately in an ice bath and centrifuged at 48000g for 30min. The supernatant liquid was collected and the precipitate was discarded.

Chromatography. The bacteroid non-haem iron protein was further purified on columns of DEAE-cellulose (Whatman DE 32). DEAE-cellulose for use in preparation of the columns was precycled with 0.5M-NaOH and then equilibrated with 0.02M-TES buffer, pH7.5. The columns were maintained at 4°C by operation in a cold-room or by use of an external water jacket connected to a temperature-controlled water bath. The flow rates of the columns were about 1ml/min.

In the first DEAE-cellulose chromatography (Table 1), a packed column (2.5cm × 11cm) was used. The extract (48ml containing 1196mg of protein) from the protamine sulphate and heat treatments was placed on the column. After the non-haem iron protein had been adsorbed on the surface of the DEAE-cellulose, the column was eluted with a stepwise gradient consisting of 140ml of 0.08M-$MgCl_2$ followed by 50ml of 0.2M-$MgCl_2$. Both salt solutions were dissolved in 0.02M-TES buffer, pH7.5. The crude greenish-brown non-haem iron protein was collected in 14ml of the buffered 0.2M-$MgCl_2$ solution.

Salts were removed from the non-haem iron protein by use of 100–200 mesh Bio-Gel P-2 (Bio-Rad Laboratories, Los Angeles, Calif., U.S.A.). The column (2.5cm × 9cm) was equilibrated and eluted with 0.02M-TES buffer, pH7.5.

In the second DEAE-cellulose chromatography a column (1.7cm × 12cm) was used. The desalted bacteroid non-haem iron protein (14ml containing 11.7mg of protein) was layered on to the column and eluted with a linear gradient of $MgCl_2$ as described in the legend of Fig. 2. The effluent from the column was collected in 5ml or 2ml fractions with a Gilson Medical Electronics fraction collector and was continuously monitored for protein by measurement of E_{280}. The non-haem iron protein from this column was desalted with Bio-Gel P-2 by the procedure described for removing salts from the eluate from the first DEAE-cellulose column.

Standard assay procedure. The activity of bacteroid non-haem iron protein was measured by its capacity to function as a carrier between photosystem I and the nitrogenase system. A complete reaction mixture in a final volume of 1.5ml contained the following (μmol): TES buffer, pH7.5, 30; ATP, 7.5; creatine phosphate, 50; $MgCl_2$, 10; sodium ascorbate, pH7.2, 20; 2,6-dichlorophenol-indophenol, 0.05. In addition each reaction contained 0.2mg of creatine phosphokinase, tris-washed

chloroplast fragments (0.6mg of chlorophyll), bacteroid nitrogenase (3.9mg of protein with a specific activity of 40nmol of ethylene/min per mg of protein) and the amount of bacteroid non-haem iron protein indicated in the various legends of tables or figures. The quantity added was sufficient to produce no more than 550nmol of ethylene in a period of 20min. The reaction vessels were flushed five times with high-purity argon. The final gas mixture contained 0.9atm of argon and 0.1atm of acetylene. After incubation for 20min in a 25°C water bath with light-intensity of 1500 ft-candles, the reactions were terminated by injection of 0.5ml of 15% (w/v) trichloroacetic acid. Ethylene production was measured by gas chromatography (Kelly, Klucas, & Burris, 1967). One unit of bacteroid non-haem iron protein activity is defined as the amount catalysing the reduction of 1nmol of acetylene/min in the standard assay.

Other preparations and assays

Nitrogenase. A crude extract containing active nitrogenase was prepared from soya-bean nodule bacteroids as described by Klucas *et al.* (1968). Strictly anaerobic conditions were maintained throughout the purification procedure. To 100ml of crude extract (45–50mg of protein/ml) in 0.1M-TES buffer, pH7.5, was added with stirring 25ml of polypropylene glycol. After being stirred in an ice bath for 5min the precipitated protein was removed by centrifugation at 48000g for 15min and an additional 25ml of polypropylene glycol was added to the supernatant liquid. After stirring for 5min, the precipitate was collected by centrifugation at 15000g for 15min and the supernatant liquid was discarded. The precipitate was dissolved in 35ml of 0.02M-TES buffer, pH7.5, and stored in liquid N_2 until it was used.

The nitrogenase assay procedure, as described by Klucas *et al.* (1968) was followed except that acetylene reduction rather than nitrogen fixation was measured. The gas mixture in each reaction was composed of 0.9atm of argon and 0.1atm of acetylene. Reactions were terminated by the addition of 0.5ml of 15% (w/v) trichloroacetic acid. Ethylene production was measured by gas chromatography by using the method of Kelly *et al.* (1967).

Ferredoxin–NADP reductase. A homogeneous preparation of this enzyme was kindly supplied by Dr Norman I. Bishop. It was obtained from *Scenedesmus obliquus* and purified initially by the method described by Powls, Wong & Bishop (1969). Further purification was accomplished by use of preparative gel electrophoresis. Photochemical reduction of NADP was assayed spectrophotometrically by the method of McSwain & Arnon (1968).

Scenedesmus *ferredoxin.* An electrophoretically homogeneous preparation isolated by the method of Powls *et al.* (1969) was kindly supplied by Dr Norman I. Bishop. The concentration was determined by the extinction coefficient of 9.8×10^3 at 421nm (Matsubara, 1968).

Clostridium *ferredoxin.* This preparation was prepared from dried *C. pasteurianum* cells (kindly supplied by Dr Robert Klucas) by the method of Mortenson, Valentine & Carnahan (1962). The concentration of the purified material was determined by use of the extinction coefficient of 17.3×10^3 at 390nm (Mortenson, 1964*b*).

Phosphoroclastic enzyme. The phosphoroclastic enzyme was prepared from dried *C. pasteurianum* cells and assayed by the methods of Mortenson *et al.* (1962) except that the assay mixture contained 11 rather than 110μmol of pyruvate/ml.

Chloroplast fragments. Chloroplasts were prepared from spinach leaves by the method of Schwartz (1966). Photosystem II was inactivated by washing the chloroplasts with 0.8M-tris chloride for 10min as described by Yamashita & Butler (1969). The chlorophyll content of the fragments was determined by the method of Arnon (1949) and the chloroplast fragment suspension was diluted to a final concentration of 2mg of chlorophyll/ml with the suspending medium 0.4M-sucrose–0.01M-NaCl–0.05M-tris chloride buffer, pH7.5.

Creatine phosphokinase. A crystalline preparation of the enzyme was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. A solution containing 2mg/ml was prepared in 0.2M-TES buffer, pH7.5, and stored at −20°C.

Protein. Protein was measured by a modification of the Folin method (Lowry, Rosebrough, Farr & Randall 1951) with crystalline bovine serum albumin as standard. Proteins in samples containing $MgCl_2$ were precipitated by adding 1ml of 15% (w/v) trichloroacetic acid for each 0.5ml of sample. The precipitated proteins were collected by centrifugation at 10000g for 10min and the protein content was determined by the modified Folin method. Protein measurements were not corrected for any differences in behaviour of non-haem iron proteins and serum albumin in the modified Folin procedure.

Iron and acid-labile sulphur. Acid-labile sulphur was determined by the method of Fogo & Popowsky (1949). The concentration of the standard solution of sodium sulphide used in the procedure was determined by iodometric titration.

Iron contents of samples were determined colorimetrically by an *o*-phenanthroline method (Ballentine & Burford, 1957).

Sedimentation velocity. A Spinco model E analytical ultracentrifuge, equipped with the RTIC temperature-control unit and electronic speed control was used for sedimentation experiments. Measurements on photographic plates were made with a Nikon model 6C microcomparator. Calculations were made as described by Schachman (1957).

RESULTS AND DISCUSSION

Purification

A summary of the results of purification of bacteroid non-haem iron protein is presented in Table 1. The protamine sulphate and heat treatments produced 117% increase in yield of activity units. The most probable explanation for this unexpected but reproducible result is that an inhibitor was removed in the initial purification step. Application of the supernatant from the protamine sulphate and heat treatments (fraction II, Table 1) to the first DEAE-cellulose column resulted in the adsorption of a dark band of material on the surface of the DEAE-cellulose. A large quantity of protein failed to adhere to the column and was eluted with 0.02M-TES buffer, pH7.5.

Table 1. *Summary of the results of the purification*

Procedures for the preparation of the crude extract, the protamine sulphate and heat treatments and DEAE-cellulose chromatography are described in the Materials and Methods section. Results of the second DEAE-cellulose chromatography are presented in Fig. 1.

Fraction no.	Preparation	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Yield (%)
I	Crude extract	45	2262.0	2036	0.9	100
II	Supernatant from protamine sulphate and heat treatments	48	1196.0	4425	3.7	217
III	First DEAE-cellulose chromatography (0.2M-$MgCl_2$ eluate)	14	11.7	2012	172.0	99
IV	Second DEAE-cellulose chromatography (120–152 ml fractions from $MgCl_2$ gradient elution)	32	2.7	1080	400.0	53

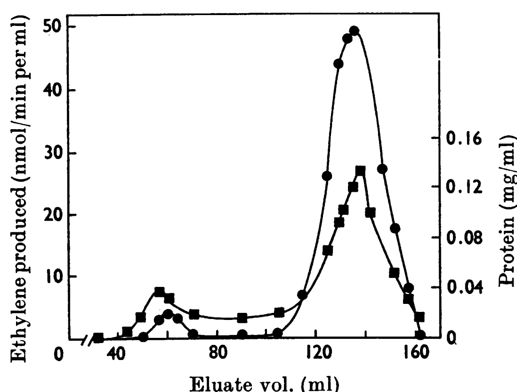

Fig. 1. Second DEAE-cellulose chromatography of bacteroid non-haem iron protein. The eluate from the first DEAE-cellulose chromatography was desalted and rechromatographed on DEAE-cellulose (see the Materials and Methods section) by using a linear gradient consisting of 150 ml each of 0.05M- and 0.25M-$MgCl_2$ in 0.02M-TES buffer, pH 7.5. The initial 15 ml of effluent represents the void volume of the column. The linear elution gradient initiated at a concentration of 0.05M-$MgCl_2$ extends from 15 ml of eluate to 160 ml of eluate, where the concentration of $MgCl_2$ was 0.15M. ●, Activity; ■, protein concentration.

The addition of 0.08M-$MgCl_2$ in 0.02M-TES buffer, pH 7.5, resulted in the elution of considerable quantities of other proteins including two yellow components with spectral characteristics of flavoproteins and a haemoprotein component with properties similar to those of cytochrome P-450 (Appleby, 1969). Finally, the addition of 0.2M-$MgCl_2$ in 0.02M-TES buffer, pH 7.5, eluted the partially purified band of bacteroid non-haem iron protein. This component represents less than 1% of the protein in the original crude extract.

Details of the second DEAE-cellulose chromatography are shown in Fig. 1. The desalted bacteroid non-haem iron protein from the first DEAE-cellulose chromatography adhered to the second DEAE-cellulose column and was eluted by a buffered $MgCl_2$ gradient. The first peak (Fig. 1) emerged at a concentration of $MgCl_2$ of 74–89 mM. It contained a relatively small proportion of both the total protein and activity units that were originally applied to the column. The spectrum of this (50–70 ml of eluate, Fig. 1) and analogous fractions in other experiments suggested a flavoprotein as a major constituent. It seems highly probable that the major portion of the original component of this type was discarded in the earlier steps of the purification procedure. This fraction (50–70 ml of eluate) appeared to be analogous to the azotoflavin isolated from *A. vinelandii* by Benneman *et al.* (1969). The properties of this fraction have not been studied in detail and further experimentation is essential for its characterization.

The bacteroid non-haem iron protein was eluted from the second DEAE-cellulose column by the $MgCl_2$ gradient at concentrations ranging from 0.123 to 0.148M (Fig. 1). This peak contained most of the protein and the activity units that were applied to the column (Fig. 1 and Table 1).

Some physical properties

A solution of bacteroid non-haem iron protein containing 2 mg of protein/ml is brown to dark amber in appearance. The spectrum (Fig. 2) of the purified material reveals a broad absorption band in the range 380–440 nm and a more prominent absorption peak at 280 nm. There is evidence of a slight shoulder near 320 nm. The extinction of the bacteroid non-haem iron protein at 390 nm is 56% of that at 280 nm. The comparable value for the

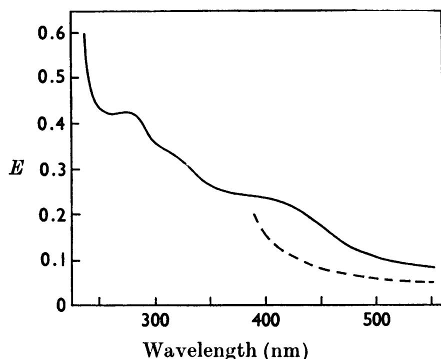

Fig. 2. Spectra of purified bacteroid non-haem iron protein. A 1 ml sample of the peak fraction (130–132 ml of eluate of Fig. 1) containing 0.11 mg of protein/ml was placed in a quartz cuvette of 1 cm light-path and spectra were recorded in air with and without the addition of 0.1 mg of sodium dithionite. A Cary model 11 spectrophotometer was used. ——, in air; ----, dithionite-reduced.

ferredoxin from *C. pasteurianum* is 78% (Buchanan, Lovenberg & Rabinowitz, 1963) and that from *S. obliquus* is 55% (Matsubara, 1968).

The bacteroid non-haem iron protein is bleached as a result of the addition of sodium dithionite (Fig. 2). The spectrum of the reduced protein exhibits no apparent absorption peaks in the visible region and in this respect is similar to that of ferredoxins. When a sample of the non-haem iron protein was shaken in air, the spectra recorded at four 30-min intervals afterwards indicated that slightly over 50% of the reduced non-haem iron protein was oxidized in 2h.

The purified bacteroid non-haem iron protein was examined by the isoelectric-focusing technique (Electrofocusing Equipment Instruction manual, LKB Produkter Stockholm-Bromma 1, Sweden). A single band of protein was detected at an isoelectric point of 6.0 but the eluted protein showed no activity in the standard assay for the non-haem iron protein. Treatment of the inactive protein with sodium sulphide, ferrous ammonium sulphate and mercaptoethanol (Malkin & Rabinowitz, 1966) resulted in restoration of less than 1% of the original activity applied to the isoelectric-focusing column.

The schlieren patterns obtained from the analytical ultracentrifugation experiment (Fig. 3) indicate that the purified preparation of bacteroid non-haem iron protein is essentially homogeneous. The only evidence of impurities as shown by the schlieren patterns is some accumulated material in the bottom of the cell used for centrifugation. This accumulation could have resulted from

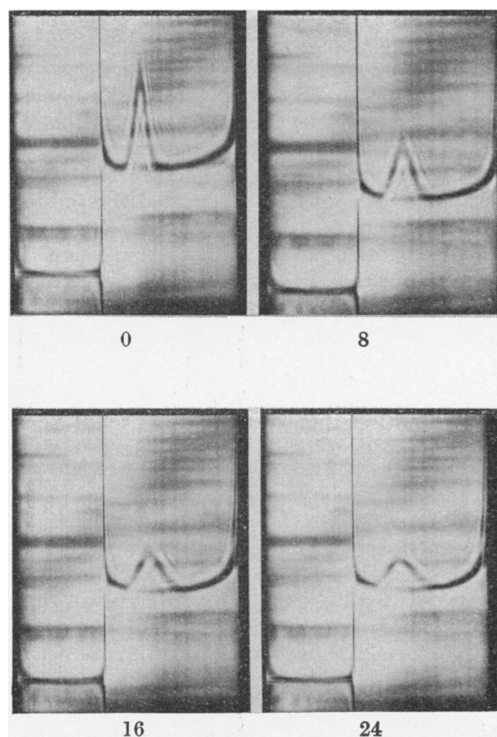

Fig. 3. Schlieren patterns of the sedimentation of bacteroid non-haem iron protein. Sedimentation velocity was determined at 9°C with a Spinco model E analytical ultracentrifuge by using a synthetic-boundary cell and an AnD rotor at 52000 rev./min. The purified non-haem iron protein (concentrated preparation of fraction IV, Table 1) contained 2 mg of protein/ml in 0.02M-TES buffer, pH 7.5. Schlieren patterns were photographed at 8 min intervals.

denaturation of the bacteroid non-haem iron protein during preparation and centrifugation. This conclusion is supported by results of experiments, to be discussed below, showing the instability of the bacteroid non-haem iron protein. From the sedimentation-velocity measurements obtained from the schlieren patterns (Fig. 3), an $s_{20,w}$ value of 1.3 S was calculated by using the methods of Schachman (1957). Although insufficient purified non-haem iron protein was available for measurement of all the parameters necessary for an accurate molecular-weight determination, some reasonable estimations can be made.

The iron contents of bacteroid non-haem iron protein and adrenodoxin (Kimura, Suzuki, Padmanabhan & Samejima, 1969) are comparable. One might assume therefore that the partial specific volume of 0.70 ml/g at 20°C and the diffusion

coefficient of 11.2×10^{-7} cm^2/s reported for adrenodoxin (Kimura *et al.* 1969) could be applied in estimating the molecular weight of bacteroid non-haem iron protein. By using these values and the sedimentation-velocity measurements, a tentative molecular weight of 9400 has been calculated. This tentative estimate may be contrasted with a molecular weight of 20000 reported for the ferredoxin isolated from *A. vinelandii*.

The subjection of bacteroid non-haem iron protein to a series of storage periods after freezing resulted in a deterioration in activity (Table 2). In other experiments, the brown-to-amber appearance of samples progressively disappeared and acid-labile sulphur content decreased with increasing number of storage periods after freezing. After the specific activity of a sample had deteriorated to 23 units/mg of protein (Table 2) the addition of mercaptoethanol, sodium sulphide and ferrous ammonium sulphate, followed by the removal of excess of these reagents (Malkin & Rabinowitz, 1966), caused the specific activity of the sample to be restored to 122. This response appears similar to the reported reconstitution (Malkin & Rabinowitz, 1966) of clostridial ferredoxin after loss of activity from mersalyl and Chelex resin treatments.

An experiment was conducted in which three samples of bacteroid non-haem iron protein were incubated in an ice bath for 2h under three different gas compositions; namely, 100% argon, 100% O_2 and air. A parallel series of three samples was incubated under similar conditions except that the temperature was maintained at 30°C. Assays of samples after the incubation periods indicated that all retained 92% or more of the activity except that the sample incubated under 100% O_2 at 30°C retained only 25% of its activity and the sample incubated under air at 30°C retained 82% of its original activity. Yoch *et al.* (1969) have found that storage of crude bacteroid non-haem iron protein under O_2 results in loss of activity.

From analyses of three freshly prepared bacteroid non-haem iron protein samples mean iron and acid-labile sulphur contents of 0.31 and 0.35 μg-atoms respectively/mg of protein were obtained. These results are similar to those reported for the *Azotobacter* ferredoxin (Yoch *et al.* 1969).

Biological properties

Role in the photochemical reduction of acetylene. It is obvious (Table 3) that the photochemical reduction of acetylene in the coupled assay system is dependent on bacteroid nitrogenase, bacteroid non-haem iron protein, an ATP-generating system and a photochemical electron-donor system consisting of chloroplast fragments, ascorbate and 2,6-dichlorophenol-indophenol. When the non-haem iron protein was omitted from the complete reaction mixture, 2.9 nmol of ethylene was produced in 20 min (Table 3). This activity is undoubtedly due to endogenous ferredoxin in the spinach chloroplast-fragment preparation. The magnitude of the endogenous activity of the system appeared to be related to the quantity of chloroplast fragments added to assays. The endogenous ferredoxin in chloroplast fragments prepared by the tris-extraction method (Yamishita & Butler, 1969) was consistently less than that of chloroplast fragments prepared by a method involving a heat treatment (Benneman *et al.* 1969).

No appreciable reduction of acetylene occurred in reaction mixtures that were incubated in the dark (Table 3). The exposure of complete reaction mixtures to 3200 rather than 1500 ft-candles of light-intensity did not appreciably affect the rate of acetylene reduction in the range where most acetylene reduction measurements were made.

Table 2. *Reconstitution of bacteroid non-haem iron protein after deterioration of activity*

Bacteroid non-haem iron protein (0.84 mg of protein/ml) was assayed by the standard procedure (see the Materials and Methods section). In each treatment the non-haem iron protein was frozen and stored in liquid N_2 and then thawed and maintained in an ice bath for about 1 h before refreezing. In treatment no. 5, bacteroid non-haem iron protein (0.63 mg of protein) was treated with 0.6 μmol each of Na_2S and ferrous ammonium sulphate and 30 μmol of mercaptoethanol. The mixture (final vol. 2.7 ml) was incubated for 10 min at 37°C and then passed through a column (1.5 cm × 11 cm) of Bio-Gel P-2. The eluate was assayed by the standard method.

Treatment no.	Conditions	Specific activity (units/mg of protein)
1	Initial assay	177.0
2	Assayed after freezing and storage for 7 days	84.5
3	Assayed after second freezing and storage for additional 2 days	32.4
4	Assayed after third freezing and storage for an additional 15 days	23.1
5	Sample from treatment no. 4 assayed after the addition of mercaptoethanol, Na_2S and ferrous ammonium sulphate	122.7

Table 3. *Essential components for the photochemical reduction of acetylene*

The complete reaction mixture (final vol. 1.5ml) contained: a photochemical reducing system [consisting of chloroplast fragments, 2,6-dichlorophenol-indophenol (DCIP) and ascorbate]; bacteroid nitrogenase; an ATP-generating system, and buffer (see the standard assay procedure for the bacteroid non-haem iron protein in the Materials and Methods section). The specific activity of the purified bacteroid non-haem iron protein (0.13mg of protein) was 130 units/mg of protein.

Reaction system	Ethylene produced (nmol/20min)
Complete	336.0
Complete, chloroplasts, DCIP and ascorbate omitted	0.4
Complete, nitrogenase omitted	0.3
Complete, ATP-generating system omitted	0.4
Complete, bacteroid non-haem iron protein omitted	2.9
Complete, incubated in the dark	1.0

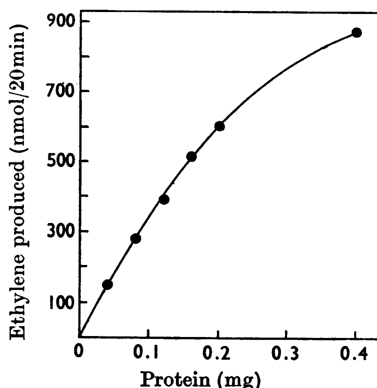

Fig. 4. Relationship between rate of photochemical reduction of acetylene and the concentration of bacteroid non-haem iron protein. The standard assay procedure was followed. Results were corrected for the low rate of acetylene reduction (6nmol/20min) that occurred in the absence of added bacteroid non-haem iron protein. The concentration of purified bacteroid non-haem iron protein was varied as indicated.

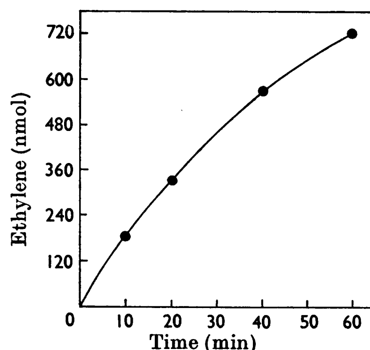

Fig. 5. Time-course of the photochemical reduction of acetylene. The standard assay procedure for bacteroid non-haem iron protein was followed except that the time of incubation was varied as indicated. Each reaction mixture contained 0.13 mg of protein (fraction III, Table 1).

After establishing the optimum concentration of components necessary for photochemical reduction of acetylene, the relationship between activity in the coupled assay and the quantity of bacteroid non-haem iron protein added was determined (Fig. 4). These results illustrate an approximately linear relationship between bacteroid non-haem iron protein concentration and rate of ethylene production in the range up to 0.2mg of protein.

Fig. 5 shows that the rate of the photochemical reduction of acetylene in the coupled nitrogenase assay decreased to some extent with increasing duration of the incubation period. This might have been associated with a denaturation of bacteroid non-haem iron protein, bacteroid nitrogenase, or both, since both are known to be labile.

Relative effectiveness in ferredoxin assays. The bacteroid non-haem iron protein was examined in a series of different assays for ferredoxin. It failed to function as a cofactor in the photochemical reduction of NADP (Table 4). Four experiments in which different samples of bacteroid non-haem iron protein were tested consistently produced negative results. In contrast, ferredoxin from *S. obliquus* catalysed the photochemical reduction of NADP at a rapid rate. Ferredoxin from *C. pasteurianum* was not tested in the photochemical reduction of NADP, but Tagawa & Arnon (1962) found it to be an effective electron carrier in this assay. It is noteworthy that Yoch *et al.* (1969) have reported that ferredoxin from *A. vinelandii* effectively catalyses the transfer of electrons from photosystem I to NADP whereas our experiments show that the bacteroid non-haem iron protein lacks this capacity.

Ferredoxins from *C. pasteurianum* and *S. obliquus* and the bacteroid non-haem iron protein are all active electron carriers in the photochemical

Table 4. *Comparison of ferredoxins from* S. obliquus *and* C. pasteurianum *with non-haem iron protein from bacteroids as cofactors in the photochemical reduction of NADP and of acetylene and in the phosphoroclastic breakdown of pyruvate*

The reaction mixture for NADP reduction (final vol. 3ml) contained the following (μmol): *N*-tris(hydroxymethyl)methylglycine buffer, pH 7.5, 50; sodium ascorbate, 20; dichlorophenol-indophenol, 0.2; NADP, 0.5; sucrose, 120; tris chloride, pH 7.5, 15; NaCl, 0.05. In addition each reaction contained tris-washed chloroplast fragments (20mg of chlorophyll) and 0.24mg (6 units) of ferredoxin–NADP reductase. Ferredoxin from *S. obliquus* (0.081 mg) and non-haem iron protein from bacteroids (0.064 mg of fraction IV, Table 1) were added as indicated. The standard assay procedure for bacteroid non-haem iron protein (acetylene reduction) was followed. Reactions (1.5ml) contained ferredoxin from *S. obliquus* (0.081 mg), and *C. pasteurianum* (0.13 mg) and non-haem iron protein from bacteroids (0.12mg). The complete reaction mixture for phosphoroclastic enzyme activity (final vol. 1 ml) contained the following (μmol): potassium phosphate, pH 6.5, 50; sodium pyruvate, 11. The phosphoroclastic enzyme preparation contained 1.5mg of protein and sufficient endogenous coenzyme A for maximum activity. Reactions as indicated contained *C. pasteurianum* ferredoxin (0.13mg) or bacteroid non-haem iron protein (0.11 mg).

Source of ferredoxin	NADP reduction (nmol/min per mg of ferredoxin)	Acetylene reduction (nmol/min per mg of non-haem iron protein	Acetyl phosphate formed* (nmol/min per mg of ferredoxin)
S. obliquus	950	70	—
Soya-bean nodule bacteroid	0	203	0
C. pasteurianum	—	325	1300

* Product of the phosphoroclastic reaction (Mortenson *et al.* 1962).

reduction of acetylene in the presence of bacteroid nitrogenase. The ferredoxin from *S. obliquus*, however, is less effective than the electron carriers from the other sources. Bacteroid non-haem iron protein consistently failed to function as a cofactor in the phosphoroclastic breakdown of pyruvate. As expected, ferredoxin from *C. pasteurianum* catalysed this reaction at a rapid rate. Ferredoxin from *S. obliquus* was not tested in this assay, but on the basis of properties reported by others (Matsubara, 1968) it would be expected to be active.

These results are consistent with the reports of Bulen *et al.* (1964) and with unpublished work by Dr Robert Klucas indicating that nitrogenases from *Azotobacter* and nodule bacteroids respectively may be coupled to a hydrogenase system from *C. pasteurianum* provided that *Clostridium* ferredoxin is supplied. The incapacity of bacteroid non-haem iron protein to function in either the photochemical reduction of NADP or in the phosphoroclastic breakdown of pyruvate provides an explanation for the failure of previous efforts to discover this compound in nodule bacteroids. All efforts to substitute bacteroid non-haem iron protein for benzyl viologen in the assay (Klucas & Evans, 1968) in which electrons from an $NADH_2$-generating system are transferred to benzyl viologen and then to bacteroid nitrogenase have so far failed.

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