Purification and Properties of Nitrogenase from *Rhodospirillum rubrum*, and Evidence for Phosphate, Ribose and an Adenine-Like Unit Covalently Bound to the Iron Protein

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1. The molybdenum–iron (Mo–Fe) protein, iron (Fe) protein and the activating factor of nitrogenase from *Rhodospirillum rubrum* were purified. 2. The Mo–Fe protein has properties similar to those of the Mo–Fe proteins of other nitrogen-fixing organisms. 3. The Fe protein is similar to other Fe proteins with respect to its molecular weight, metal composition and e.p.r. signal. 4. The Fe protein is different from other Fe proteins in that it apparently has two types of subunits rather than one, its u.v. spectrum has an extra peak, and phosphate, ribose and an adenine-like unit are covalently bound to the protein. The presence of these non-protein groups on the protein may explain the requirement for activation of *R. rubrum* Fe protein.

Despite the presence of nitrogenase in diverse groups of bacteria, much of our knowledge about the enzyme has been derived from the study of the nitrogenases from only a few of these bacteria. The nitrogenases from *Clostridium pasteurianum*, *Klebsiella pneumoniae* and *Azotobacter vinelandii* each have been separated into two components, a molybdenum-and-iron-containing protein (Mo–Fe protein) and an iron-containing protein (Fe protein), and each of these has been highly purified and studied in some detail. These nitrogenase components have similar metal contents, molecular weights, e.p.r. and visible spectra and substrate specificity (Emerich, 1977). Mo–Fe proteins from *Chromatium* strain D and *Rhizobium* sp. and both components from *Bacillus polymyxa* have also been purified and shown not to vary from the pattern established for *C. pasteurianum*, *A. vinelandii* and *K. pneumoniae* nitrogenases.

No Fe protein from a photosynthetic bacterium has been characterized before the present report. We have shown that the Fe protein from *Rhodospirillum rubrum* is different from other Fe proteins, as it is isolated in an inactive state and can be activated by a protein-activating factor isolated from membranes of *R. rubrum* (Ludden & Burris, 1976). The activation requires ATP and a bivalent metal ion. The requirement for an ATP-mediated activation explains the non-linear time courses for nitrogenase

Abbreviations used: the proteins of nitrogenase are referred to as the Mo–Fe protein (molybdenum–iron protein) and the Fe protein (iron protein); SDS, sodium dodecyl sulphate.

activity from *R. rubrum* observed by Munson & Burris (1969), and the unusual metal requirements for activation explain the observation of Burns & Bulen (1966) that 25 mM-Mg²⁺ is optimal for nitrogenase activity in the presence of only 5 mM-ATP. The differences between *R. rubrum* Fe protein and those from other more thoroughly studied organisms are discussed in the present paper.

Materials and Methods

Rhodospirillum rubrum (A.T.C.C. 11170) was grown on Ormerod's glutamate medium (Ormerod *et al.*, 1961) in a 20-litre fermenter at 30°C. The fermenter was illuminated by a 600 W Sylvania FCB overhead-projector bulb inside a water-jacketed tube that fits down into the centre of the fermenter. Cells were collected by anaerobic centrifugation at 6000g for 10 min and were stored in liquid N₂ until used. About 2 g of wet cell paste was obtained per litre.

Unless otherwise stated, all operations were anaerobic in the presence of 1 mM-dithionite. Crude extracts were prepared by osmotic shock. Frozen cell paste (50 g) was thawed in 200 ml of glycerol buffer (4 M-glycerol in 100 mM-Tris/acetate, pH 8.1). The thawed cells were centrifuged at 10000g for 15 min, and the glycerol buffer was decanted. Next 10 ml of glycerol buffer mixed with 20 g of 4 mm-diameter glass beads, 10 mg of deoxyribonuclease (Sigma Chemical Co., St. Louis, MO, U.S.A.), 10 mg of ribonuclease (Sigma) and 20 mg of lysozyme was added to the pellet and a thick slurry was made.

After the slurry had stood at 23°C for 15 min, 200 ml of 0°C breaking buffer (100 mM-Tris/acetate, pH 8.1, plus 2 mM-dithiothreitol) was added, and the centrifuge bottle was immediately capped and vigorously shaken for 1 min. The extract was centrifuged twice: 10 min at 10000g to remove unbroken cells, and 90 min at 45000g to remove membrane particles. The second centrifugation was with 40 ml centrifuge tubes (Oak Ridge type) closed with wired-down serum stoppers. The supernatant from the second centrifugation is referred to as the crude extract and the pellet as the chromatophore fraction.

The crude extract was removed from the centrifuge tube with a syringe and was passed through a DEAE-cellulose column (2.5 cm diameter $\times$ 15 cm). The negatively charged nitrogenase proteins were bound tightly to the top of the column, and the cytochromes and other proteins were washed through. The column was washed sequentially with 100 ml portions of buffers (50 mM-Tris/acetate, pH 7.6, plus 2 mM dithiothreitol) containing 100, 200 or 400 mM-NaCl. The 100 mM-NaCl fraction was discarded. The 200 mM-NaCl wash eluted a dark-brown band containing Mo-Fe protein, and this was frozen and stored at -20°C for later use. The Fe protein was in a fraction including everything from the end of the Mo-Fe-protein fraction to the end of the dark-brown band eluted with 400 mM-NaCl. Immediately after elution, the Fe-protein fraction was diluted with 1.5 vol. of buffer and put on a DEAE-cellulose column (1 cm $\times$ 6 cm) to concentrate it. The Fe protein was eluted from this column with 400 mM-NaCl as a tight band in 5-10 ml volume.

The concentrated Fe protein was desalted on a Sephadex G-25 column (3 cm $\times$ 10 cm) before being electrophoresed on a preparative polyacrylamide-gel column (3 cm $\times$ 7 cm). Solid sucrose was added to the concentrated, desalted Fe protein to give a 10% (w/w) solution, and this was layered on the gel column under the upper reservoir buffer. The 3 cm-diameter gel had a 5 cm-long section of 8.3%-acrylamide separating gel and a 2 cm-long section of stacking gel. The acrylamide/bisacrylamide ratio was 40:1. Both the gel buffer and the reservoir buffer were 100 mM-Tris/borate, pH 8.3. Before application of the protein, the gel was pre-run for 3 h in the presence of dithionite at 60 V (150 pulses/s). The protein was run into the gel slowly (overnight) at 40 V (150 pulses/s, 6 mA) and then was run through the separating gel at 150 V (150 pulses/s, 20 mA). The temperature was kept low by putting the gel in a water jacket or by running the gel column in the cold-room. Two ferredoxins preceded the Fe protein through the gel and were collected and kept. The Fe protein was eluted with 100 mM-Tris/acetate (pH 8.0) on to a DEAE-cellulose column (0.6 cm $\times$ 4 cm); this column served as an on-line concentrator from which the pure Fe protein was eluted with 400 mM-NaCl in

Tris/acetate, pH 7.7. The Fe protein was stored in liquid N₂ until used. By this means 20 mg of pure Fe protein can be obtained in about 30 h after thawing the cells.

Further purification of the Mo-Fe protein

The Mo-Fe protein (in 200 mM-NaCl/Tris/acetate, pH 7.7, from DEAE-cellulose) was purified by applying it to a DEAE-cellulose column (2.5 $\times$ 10 cm) after diluting it with buffer; it was eluted with a 200 ml linear gradient from 100 to 300 mM-NaCl. Further purification was achieved by precipitation with poly(ethylene glycol) 4000; the Mo-Fe protein remained soluble at 15% (w/w) poly(ethylene glycol), but was precipitated at 30% poly(ethylene glycol). This preparation was not consistently free from all other protein bands as observed on analytical gels, but it was free from Fe protein, activating factor and any contaminating compounds giving e.p.r. signals.

Purification of activating factor

This was eluted from chromatophores of either glutamate- or NH₃-grown cells with 50 mM-Tris/acetate containing 0.5 M-NaCl and 2 mM-dithiothreitol. After centrifugation at 45000g for 1 h, the activating-factor-containing supernatant was made 30% (w/w) in poly(ethylene glycol); this precipitated the activating factor. The factor was collected by centrifugation at 27000g for 20 min and then was resuspended in 50 mM-Tris/acetate containing 2 mM-dithiothreitol. Activating factor at this stage contained contaminating pigments, and, if glutamate-grown cells were used, Mo-Fe protein. The contaminants could be separated from activating factor on an anaerobic DEAE-cellulose column (2.5 $\times$ 10 cm) by eluting with a gradient from 0 to 150 mM-NaCl in Tris/acetate, pH 7.7, total volume 200 ml. A Blue Sepharose affinity column was also used in purification of activating factor, but yields were low; activating factor was eluted from the column with NAD⁺ (10 mM).

U.v. spectra

U.v. spectra of the Fe proteins from three organisms were recorded with a Cary 14 spectrophotometer. The proteins were precipitated twice in 1% HClO₄ to remove iron-sulphur centres. After the second precipitation, the proteins were resuspended in 1 ml of 100 mM-Tris base; the final pH was about 9.0.

Polyacrylamide gels

Gels for native proteins were run as described by Ornstein (1964) and Davis (1964) under anaerobic conditions. The anaerobic gels were pre-run for 30 min at 100 V in the presence of 0.5 mM-dithionite in the upper (cathode) buffer reservoir.

SDS/polyacrylamide gels were run aerobically as described by Laemmli (1970). Native gels were

stained as described by Reisner *et al.* (1975). SDS/polyacrylamide gels were stained with Coomassie Brilliant Blue R-250 in water/acetic acid/methanol (5:1:5, by vol.) and destained in the same medium minus stain.

Molecular weights

Molecular weights of Fe proteins were determined by SDS/polyacrylamide-gel electrophoresis, whereas molecular weights of the Mo-Fe protein were determined by SDS/polyacrylamide-gel electrophoresis and gel-filtration chromatography.

Metal analysis

Iron analyses were carried out as described by Van De Bogart & Beinert (1967), and molybdenum assays by the method of Clark & Axley (1955).

Amino acid composition

Amino acid analysis of the Fe protein hydrolysed with 6M-HCl was carried out by D. Omilianowski on a Durrum D-500 amino acid analyser. Cysteine was determined as cysteic acid after oxidation with performic acid. Tryptophan was not determined.

E.p.r. spectra

E.p.r. spectra were obtained at 9.2GHz with a Varian E-9 instrument at 13 K.

Digestion of proteins for phosphate content, and assay of phosphate

Proteins were precipitated twice in 5ml of 5% (w/v) trichloroacetic acid before digestion. All added ATP or phosphate could be removed from a protein sample by this method of protein precipitation. To the precipitated samples 2ml of 2M-HCl was added and the mixtures were boiled to dryness. Then 1ml of 2M-HCl and 20 μ l of H₂O₂ (30%, w/v) were added, and again the solution was boiled to dryness. Five sequential additions of 100 μ l of H₂O₂ with heating to dryness between additions were used to complete the hydrolysis; theoretical yields of phosphate from ATP, AMP, pyrophosphate or glucose 6-phosphate were obtained. Test tubes used in these assays were soaked in deionized water for 1h and then rinsed again in deionized water before use. A modification of the molybdate assay for phosphate was used (Chen *et al.*, 1956).

Analysis for ribose and deoxyribose

The orcinol (Dische, 1962, p. 484) and diphenylamine (Dische, 1962, p. 505) assays were used for determination of ribose and deoxyribose respectively. Proteins were precipitated in 5% trichloroacetic acid, before being analysed for ribose and deoxyribose.

Analysis for adenine and nicotinamide

The fluorescence assays of Yuki *et al.* (1972) for adenine and of Udenfriend (1964, p. 304) for nicotinamide were used. Proteins for adenine assays were precipitated twice in 5% trichloroacetic acid followed by a single wash with 100mM-acetic acid to remove the interfering trichloroacetic acid. Proteins used for nicotinamide assays were precipitated with 1% HClO₄.

Preparation of ³²P-labelled Fe protein

A 500ml culture of *R. rubrum* was grown on a medium (Ormerod *et al.*, 1961) containing NH₃ (0.5g of NH₄Cl/l) as a nitrogen source and one-tenth the normal phosphate buffer concentration; 10mM-Tris/HCl buffer, pH7.0, was added to these low-phosphate growth media. The NH₃-grown cells were collected and resuspended in 500ml of medium lacking nitrogen and phosphorus (Ormerod *et al.*, 1961), and 3mCi of potassium [³²P]phosphate was added. The cells were incubated in the light under an atmosphere of N₂ overnight. In the absence of fixed nitrogen, nitrogenase was synthesized; this was confirmed by assaying the whole cells for acetylene reduction.

The cells were collected and broken by osmotic shock (see the Materials and Methods section). After centrifuging at 10000g for 10min, the extract was put on a DEAE-cellulose column (0.5cm $\times$ 5cm); the column was washed with 5ml of 150mM-NaCl and then with 5ml of 400mM-NaCl. The 400mM-NaCl eluted a brown band of protein that was placed on anaerobic slab acrylamide gel (4% stacking gel, 10% separating gel) and run at 200V for 2h. The section of gel containing the Fe protein was cut out and mashed by passing it through a 5ml syringe. The protein was eluted from this mash with 2ml of buffer (50mM-Tris/acetate, pH7.7) containing 0.1% SDS and 0.1% 2-mercaptoethanol. The buffer was removed with a Pasteur pipette after 12h and the acrylamide mash was washed once with another 2ml of the same buffer. The protein was precipitated from the combined buffer fractions with 20% trichloroacetic acid and then was resuspended in 0.5ml of the SDS buffer containing 10% (v/v) glycerol. This protein preparation was separated on 12% acrylamide gels containing 0.1% SDS, and the gels were stained for protein, scanned and then sliced into 2mm sections for scintillation counting of radioactivity. The identity of the ³²P-labelled Fe protein was confirmed by co-electrophoresis with purified Fe protein. Each slice of gel was added to a scintillation vial containing 15ml of Aquasol counting solution (New England Nuclear, Boston, MA, U.S.A.), and its radioactivity was measured on the ³²P channel of a Beckman LS-100 scintillation counter.

Nitrogenase activity

Nitrogenase was assayed by acetylene reduction (Ludden & Burris, 1976). High concentrations of Mg^{2+} (25 mM) and Mn^{2+} (0.5 mM) were used in the assay of *R. rubrum* nitrogenase. Ethylene was determined on a Varian 600D flame-ionization gas chromatograph after separation from acetylene on a column (180 cm $\times$ 1.8 mm internal diam.) of Porapak R at 50°C.

Protein analysis

Protein was measured by the micro biuret method (Goa, 1953) with bovine serum albumin as a standard. Before they were analysed, all protein samples were precipitated with trichloroacetic acid to remove interfering Tris.

Reagents

ATP and creatine kinase (EC 2.7.3.2) were obtained from Sigma, phosphocreatine was from Pierce Chemical Co., Rockford, IL., U.S.A., and dithionite from J. T. Baker Co., Phillipsburg, NJ, U.S.A. Enzyme-grade Tris from Grand Island Biochemicals, Grand Island, NY, U.S.A., was used in all buffers, and dithiothreitol was from Calbiochem, La Jolla, CA, U.S.A. Gases used were from Matheson, Joliet, IL, U.S.A., and were purified by passing them over BASF catalyst R3-11 from Chemical Dynamics Corp., South Plainfield, NJ, U.S.A. Acrylamide and bisacrylamide were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. Whatman DE-52 DEAE-cellulose (Reeve Angel, Clifton, NJ, U.S.A.) was used in all DEAE-cellulose columns, and Blue Sepharose CL 6B was obtained from Pharmacia, Piscataway, NJ, U.S.A. Acetylene was generated from CaC_2 .

Other proteins

Azotobacter nitrogenase components were provided by R. V. Hageman of our laboratory. *Bacillus polymyxa* and *C. pasteurianum* components used for ribose and phosphate analyses were provided by D. W. Emerich of our laboratory. *C. pasteurianum* Fe protein for the spectrum was obtained from M. Henzl, Department of Biochemistry, University of Wisconsin-Madison.

Results and Discussion

The purification schemes for *R. rubrum* Mo-Fe and Fe proteins presented in Table 1 are comparable with those for other nitrogenases. A DEAE-cellulose column holds both highly acidic nitrogenase components. The preparative gel system was essentially the same as that used by Shah & Brill (1973) for the purification of *A. vinelandii* nitrogenase. The lower yield of *R. rubrum* Fe protein from the preparative gel compared with that reported by Shah & Brill (1973) for *A. vinelandii* Fe protein appears to result from an inherent low stability of the *R. rubrum* Fe protein, because we have reproduced their high yield of *A. vinelandii* Fe protein on our preparative gels.

In contrast with previous reports (Burns & Bulen, 1966), we have found no evidence for cold-lability of either the active or inactive form of *R. rubrum* Fe protein, either in crude extracts or as a pure protein.

The degrees of purification for *R. rubrum* Mo-Fe and Fe proteins from the crude extract were 12.5- and 9.6-fold respectively. These values are somewhat low compared with other nitrogenase systems, but analysis of the crude extract on a gel (Fig. 1) shows that *R. rubrum* Mo-Fe and Fe proteins each represent about 10% of the soluble protein in the crude extract. This high percentage of nitrogenase reflects

Table 1. Purification and yield of nitrogenase components of *R. rubrum*
For further details see the Results and Discussion section.

Fraction	Volume (ml)	Protein concentration (mg/ml)	Specific activity (nmol of acetylene reduced/min per mg of protein)	Purification (fold)	Yield (%)
<i>R. rubrum</i> Mo-Fe protein					
Crude extract	160	7.0	138	—	100
1st DEAE-cellulose column	25	10.4	617	4.5	103
DEAE-cellulose gradient column	40	2.2	1540	11.2	88
Poly(ethylene glycol) precipitation	8	5.8	1725	12.5	52
<i>R. rubrum</i> Fe protein					
Crude extract	160	7.0	88	—	100
1st DEAE-cellulose column	47	3.8	645	7.3	115
2nd DEAE-cellulose column	11	12.6	535	6.1	74
Preparative gel electrophoresis	1.5	14.4	845	9.6	18

the fact that most of the *R. rubrum* protein is membrane-bound and is centrifuged out before the specific activity of the crude extract is measured.

The analytical gels in Fig. 1 show that the Fe protein is greatly enriched on the first DEAE-cellulose column (compare gels 7 and 8). The resolving power of the preparative gel also is illustrated; the fraction shown in gel 8 was concentrated and added to the preparative gel, and gel 1 shows the purity of the material collected from the preparative gel. The two bands at the bottom of gel 8 are ferredoxins, and these can be collected as pure proteins from preparative gels.

The specific activity for the Fe protein is recorded in Table 1 as 845 nmol of C_2H_2 reduced/min per mg of Fe protein. More highly active preparations have been obtained (the highest activity obtained was

1215), but normal preparations have specific activities of 800–1000.

The Mo–Fe protein is routinely purified by the method shown. This preparation, although it has a high specific activity, is not consistently free from all other bands on the gels. Separation on a preparative gel removes almost all of these bands so that molecular-weight, subunit and metal analyses can be made, but, because the yield is low, this step is usually omitted. The Mo–Fe protein did not crystallize under the conditions described for crystallization of *A. vinelandii* Mo–Fe protein by Shah & Brill (1973); instead it was precipitated and lost much of its activity.

Molecular weights and subunits

The molecular weights of *R. rubrum* Mo–Fe protein as determined by Sephadex gel filtration and by SDS/polyacrylamide-gel electrophoresis were 230000 and 234000 respectively; these agree with each other and are comparable with values obtained for other Mo–Fe proteins. Only one major band appears for *R. rubrum* Mo–Fe protein on SDS/polyacrylamide gels, but this is insufficient evidence to claim that there is only one type of subunit; Kennedy *et al.* (1976) have reported the problems involved in determining the subunit structure of Mo–Fe proteins by SDS/polyacrylamide-gel electrophoresis.

The Fe protein from *R. rubrum* shows two bands on SDS/polyacrylamide gels (Fig. 2). The molecular weights of the subunits are 30000 and 31500. The appearance of two bands was affected neither by the SDS concentration used (0.1 or 1.0%) nor by heating the SDS-treated protein at 100°C for 5 min before applying it to the gel. We have observed no change in the intensity of either band during activation of *R. rubrum* Fe protein. A two-directional gel (isoelectric focusing and SDS/polyacrylamide) run by Gary Roberts by O'Farrell's (1975) method showed that the two bands differ in molecular weight and in isoelectric point. When *A. vinelandii* Fe protein is run simultaneously with *R. rubrum* Fe protein in a slab gel, only one band is formed for *A. vinelandii* Fe protein.

Fig. 1 shows that active and inactive Fe proteins behave the same on native gels. Both active and inactive forms of the Fe protein are labile to O_2 and polymerize when oxidized by air.

Metal analysis

Table 2 summarizes the molecular weights, subunit compositions and metal contents of the *R. rubrum* proteins. Metal contents are similar to those found in other nitrogenases. The iron analysis was performed on the inactive Fe protein, and this indicates that a change in iron composition is not the basis for activation; other Fe proteins also have

Fig. 1. Separation of the *R. rubrum* Fe protein of nitrogenase from other proteins on native polyacrylamide gels. Gels: 1, 2, *R. rubrum* Fe protein, inactive; 3, 4, *R. rubrum* Fe protein, active; 5, 6, *R. rubrum* Fe protein, active and inactive; 7, crude extract; 8, *R. rubrum* Fe protein from first DEAE-cellulose column; 9, 10, crude activating factor; 11, O_2 -treated active *R. rubrum* Fe protein; 12, O_2 -treated inactive *R. rubrum* Fe protein. Identical amounts (about 100 μ g) of protein are on gels 1–4 and 11 and 12. Gels are 10% acrylamide, acrylamide/bisacrylamide ratio 20:1. The gels were pre-run for 20 min in the presence of dithionite. O_2 -treated samples were made anaerobic before being placed on the gels. For O_2 treatment, 100 μ l of *R. rubrum* Fe protein was mixed with 100 μ l of 30% (w/w) sucrose and was left in a 5 ml vial exposed to air for 5 min at 23°C with occasional shaking. It was then made anaerobic again by evacuating and flushing three times with H_2 , after which 10 μ l of 100 mM- $Na_2S_2O_4$ was added. Abbreviations: Rr1, *R. rubrum* Mo–Fe protein; Rr2, *R. rubrum* Fe protein.

approximately four iron atoms per molecule of Fe protein.

E.p.r. spectra

The peaks in the e.p.r. spectrum of the Mo-Fe protein from *R. rubrum* have been recorded previously (Burris & Orme-Johnson, 1976). This spectrum is not notably different from that of other Mo-Fe proteins. The e.p.r. spectra of the active and inactive Fe proteins show no differences, and they are like those of other active Fe proteins. The spectrum for the inactive protein suggests that the inactivity cannot

be attributed to absence of or gross distortion of the Fe-S centre. The change in e.p.r. signal observed when MgATP is added to other Fe proteins was not observed when MgATP was added to either active or inactive *R. rubrum* Fe protein.

U.v. spectra

The u.v. spectra of nitrogenase proteins have been little investigated. Only Yates & Planque (1975) have reported the spectrum of the Fe protein in the region below 330 nm. The intense absorbance by dithionite may have discouraged examination in this region. We report the u.v. spectra of *R. rubrum* Fe protein, *A. vinelandii* Fe protein and *C. pasteurianum* Fe protein. The contributions to the absorbance by dithionite and the Fe-S centres were eliminated by precipitating the proteins as described in the Materials and Methods section. Both *C. pasteurianum* and *A. vinelandii* Fe proteins have absorption peaks near 275 nm (Fig. 3) and the magnitude of the absorbance reflects the calculated amount of tyrosine present. *R. rubrum* Fe protein, on the other hand, shows peaks at both 275 and 268 nm. Because the tyrosine and phenylalanine compositions of the proteins are quite similar (*A. vinelandii* Fe protein contains 15 residues each of tyrosine and phenylalanine, *C. pasteurianum* Fe protein has 18 tyrosine and 11 phenylalanine residues, *R. rubrum* Fe protein has 19 tyrosine and 11 phenylalanine residues per protein molecule), the 268 nm peak from the *R. rubrum* Fe protein can be accounted for only by some non-amino acid group attached to the protein. The spectrum presented from *R. rubrum* Fe protein is that of the inactive protein.

Amino acid composition

The amino acid composition of *R. rubrum* Fe protein was (in terms of amino acid residues/molecule of Fe protein): aspartate+asparagine, 60; threonine, 31; serine, 27; glutamine+glutamate, 71; proline, 17; glycine, 66; alanine, 67; cysteine, 12; valine, 32; methionine, 16; isoleucine, 32; leucine, 56; tyrosine, 19; phenylalanine, 11; histidine, 10; lysine, 30; and arginine, 26. The composition is normal for Fe

Fig. 2. Separations of purified *R. rubrum* Fe protein into two types of subunits on SDS/polyacrylamide gels. Slot 1, bovine serum albumin and cytochrome *c*; slots 2, 3 and 4, partially activated *R. rubrum* Fe protein; slot 5, *A. vinelandii* Fe protein, not heated before electrophoresis; slot 6, *A. vinelandii* Fe protein, heated before electrophoresis; slot 7, another preparation of *R. rubrum* Fe protein; slots 8 and 10, inactive *R. rubrum* Fe protein, heated; slots 9 and 11, inactive *R. rubrum* Fe protein, not heated; slot 12, creatine kinase and ovalbumin. Acrylamide gels (10%) with 0.1% SDS (acrylamide/bisacrylamide ratio 3.7:0.3) were used. Gels were run for 15 min at 100 V to get the protein into the stacking gel and then at 250 V for 1.5 h. The resolution of the gel is demonstrated by the separation (slot 12) of creatine kinase and ovalbumin, which differ in molecular weight by 3000.

Table 2. Properties of nitrogenase components from *R. rubrum*

Molecular weights were determined on Sephadex G-200 columns or by polyacrylamide-gel electrophoresis in the presence of SDS.

	Molecular weight	Subunits (molecular weight)	Fe*	Mo*
<i>R. rubrum</i> Mo-Fe protein	234 000 (SDS) 230 000 (G-200)	4 (58 500)	20	1.7
<i>R. rubrum</i> Fe protein	61 500 (SDS)†	2 (30 000 and 31 500)	3.5	—

* Atoms of Fe or Mo per molecule of protein of molecular weight shown in the Table.

† It is assumed that the Fe protein has two subunits, as is the case for all other Fe proteins examined to date; on 'native' gels without SDS the Fe proteins from *R. rubrum* and *A. vinelandii* moved almost together.

Fig. 3. Ultraviolet spectra of the Fe proteins from *A. vinelandii* (Av2), *C. pasteurianum* (Cp2) and the inactive form from *R. rubrum* (Rr2)

Fig. 4. Separation of ^{32}P -labelled Fe protein from *R. rubrum* on SDS/polyacrylamide gel
—, Protein measured by A_{545} ; ----, radioactivity.

proteins. Cysteine was determined as cysteic acid after performic acid oxidation. Tryptophan was not determined, but judged on the u.v. spectrum it probably is absent from *R. rubrum* Fe protein; there

is no tryptophan in *A. vinelandii* Fe protein or *C. pasteurianum* Fe protein. No unusual amino acids were evident, although one or two modified amino acids out of 600 residues could easily be missed. The amino acid composition of *R. rubrum* Mo-Fe protein was not determined.

Presence of phosphate on the Fe protein

Fig. 4 shows that Fe protein, purified from cells de-repressed for nitrogenase in the presence of [^{32}P]orthophosphate, co-electrophoreses with a ^{32}P peak. Furthermore, the phosphate group is tightly bound, as it is not possible to remove it from purified *R. rubrum* Fe protein by precipitation with acetone, trichloroacetic acid, HClO_4 or acetic acid, pH4.0. The phosphate group remains with the protein during chromatography on Sephadex G-75, as well as when the protein is eluted from a DEAE-cellulose column with a salt gradient. Thus *R. rubrum* Fe protein is the first nitrogenase protein to be shown to be a phosphoprotein. The phosphate contents of some other Fe proteins are shown in Table 3; the data indicate that the Fe proteins from *C. pasteurianum*, *A. vinelandii* and *B. polymyxa* contain no covalently bound phosphate. Alternatively, these Fe proteins may have bound phosphate at times, but occur

Table 3. Phosphate and ribose content of Fe proteins
Results are based on the following molecular weights: *C. pasteurianum*, 58000; *B. polymyxa*, 60000; *A. vinelandii*, 64000; *K. pneumoniae*, 66000; *R. rubrum*, 61500.

Protein	Ribose (nmol/nmol of protein)	Phosphate (nmol/nmol of protein)
<i>R. rubrum</i> Fe protein	2-3	2-3
<i>A. vinelandii</i> Fe protein	0.60	0.54
<i>C. pasteurianum</i> Fe protein	0.40	0.39
<i>B. polymyxa</i> Fe protein	0.33	0.22
<i>K. pneumoniae</i> Fe protein	0.41	—

Table 4. Measurement of the relative 'adenine' contents of *R. rubrum* and *A. vinelandii* Fe proteins

The exciting wavelength was 328 nm. Fluorescence was read at 382 nm.

	Relative fluorescence (%)
Blank	20
5 nmol of AMP	37
10 nmol of AMP	53
0.37 mg of <i>R. rubrum</i> Fe protein	62
0.52 mg of <i>A. vinelandii</i> Fe protein	25
Hydrolysate of <i>R. rubrum</i> Fe protein	37

mainly in the dephosphorylated form. There is no experimental support for this alternative; it does not fit the model for activation-deactivation of *R. rubrum* Fe protein and there is no physiological evidence for an activation system for the other nitrogenases.

Ribose on the Fe protein

The presence of ribose (or some other pentose) on the Fe protein has not been demonstrated as rigorously as the presence of phosphate; however, Fe protein from *R. rubrum* always carries 2-3 ribose molecules per molecule of Fe protein. Once again, nitrogenases from other organisms have low contents of ribose relative to *R. rubrum* Fe protein (see Table 3). The group on the Fe protein gives the positive orcinol test for ribose, but this test does not exclude the possibility that some pentose or hexose other than ribose may be the actual sugar present. The diphenylamine test is negative, indicating that deoxyribose is not present.

Adenine and nicotinamide

Table 4 shows that *R. rubrum* Fe protein gives a positive test for adenine, whereas *A. vinelandii* Fe protein gives a response just above the background value. Acetone interfered with the assay, and trichloroacetic acid quenched the fluorescence. Therefore

Fig. 5. Fluorescence spectra of AMP and the *R. rubrum* Fe protein (Rr2) that had been treated with glyoxal hydrate. The exciting wavelength was 325 nm.

the trichloroacetic acid precipitated proteins were washed with 100mm-acetic acid before assay. Yuki *et al.* (1972) report that none of the other major purines or pyrimidines give a positive test for adenine and that methylation of the 6-amino group of adenine destroys its reactivity. Tris or *p*-amino-benzoic acid contributed little to the fluorescence of a blank assay; *p*-aminobenzoic acid is an organic amine with $\lambda_{\max.}$ at 266 nm, and Tris was the buffer for *R. rubrum* Fe protein before precipitation. Neither phosphate nor ribose interfered with the assay. The fluorescence spectrum of the treated protein differs from that of AMP (Fig. 5), indicating that the molecule attached to the Fe protein may be a modified form of adenine or a closely related compound. If all the reagents are added to the Fe protein, but the mixture is not heated (Yuki *et al.*, 1972), there is no fluorescence. Therefore tyrosine or phenyl-alanine fluorescence is not a factor in this assay. Material obtained after hydrolysis of *R. rubrum* Fe protein for 1 h in 1 M-HCl at 100°C shows a positive test for adenine. After hydrolysis, the material was separated from large peptides on a Sephadex G-25 column (1 cm $\times$ 10 cm), freeze-dried and then purified on a Dowex 1 column in a Pasteur pipette. Material from the Dowex column contained bound phosphate and absorbed u.v. light near 268 nm. Adler *et al.* (1974) investigated the covalent binding of a fluorescent adenine analogue to glutamine synthetase, and, when the 'adenine' compound bound, there was a blue shift of its $\lambda_{\max.}$ Because NAD^+ is an inhibitor of activation (P. W. Ludden & R. H. Burris, unpublished work), it seemed possible that part or all of the NAD^+ molecule might be involved in activation, so nicotinamide fluorescence was measured. Both the fluorescence assay for nicotinamide and the assay for a cyanide adduct of nicotinamide were negative.

Quantities of phosphate, ribose and adenine on the Fe protein

Phosphate, ribose and adenine were all determined on a single batch of protein. Table 5 shows the quantity of each group on the Fe protein. The ribose assay is the most susceptible to overestimation, because turbidity from protein may interfere.

Table 5. Concentrations of phosphate, ribose and adenine present in *R. rubrum* Fe protein

The data are based on a mol.wt. of 61500 for the Fe protein of *R. rubrum*.

	nmol/16 nmol of protein	nmol/nmol of protein
Phosphate	33	2.06
Ribose	47	2.94
Adenine	30	1.87

Assays for ribose on other batches of *R. rubrum* Fe protein gave values closer to two ribose molecules per molecule of Fe protein. We surmise that the correct composition of protein/phosphate/ribose/adenine is 1:2:2:2. Our working hypothesis is that *R. rubrum* Fe protein has two subunits and that each subunit has one molecule each of phosphate, ribose and adenine attached.

We have described a method for rapid purification of *R. rubrum* Mo-Fe and Fe proteins from amounts of cell paste normally obtained from one or two 18-litre cultures of cells. The nitrogenase system is unique because the Fe protein as isolated is inactive, and we suggest that covalently bound phosphate, ribose and adenine (or an adenine-like molecule) render the Fe protein inactive. Apparently this unique control system functions only in the regulation of *R. rubrum* nitrogenase and not in its catalytic activity, because once activated the Fe protein can function with the Mo-Fe protein to reduce substrates (Munson & Burris, 1969) and hydrolyse ATP (Burns & Bulen, 1966) and it can cross-react with nitrogenase components from other organisms (Biggins *et al.*, 1971; D. W. Emerich & R. H. Burris, unpublished work).

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References

- Adler, S. P., Mangum, J. H., Magni, G. & Stadtman, E. R. (1974) in *Metabolic Interconversion of Enzymes* (Fisher, E. H., ed.), pp. 221–233, Springer-Verlag, New York
- Biggins, D. R., Kelly, M. & Postgate, J. R. (1971) *Eur. J. Biochem.* **20**, 140–143
- Burns, R. C. & Bulen, W. A. (1966) *Arch. Biochem. Biophys.* **113**, 461–463
- Burris, R. H. & Orme-Johnson, W. H. (1976) in *Proceedings of the First International Symposium on Nitrogen Fixation* (Newton, W. E. & Nyman, C. J., eds.), pp. 208–233, Washington State University Press, Pullman
- Chen, P. S., Toribara, T. Y. & Warner, H. (1956) *Anal. Chem.* **28**, 1756–1758
- Clark, L. J. & Axley, J. H. (1955) *Anal. Chem.* **27**, 2000–2003
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
- Dische, Z. (1962) in *Methods in Carbohydrate Chemistry* (Whistler, R. L. & Wolfrom, M. L., eds.), vol. 1, p. 484, 505, Academic Press, New York
- Emerich, D. W. (1977) Ph.D. Thesis, University of Wisconsin-Madison
- Goa, J. (1953) *Scand. J. Clin. Lab. Invest.* **5**, 218–222
- Kennedy, C., Eady, R. R., Kondorosoi, E. & Rekosh, D. K. (1976) *Biochem. J.* **155**, 383–389
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Ludden, P. W. & Burris, R. H. (1976) *Science* **194**, 424–426
- Munson, T. O. & Burris, R. H. (1969) *J. Bacteriol.* **97**, 1093–1098
- O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
- Ormerod, J. G., Ormerod, K. J. & Gest, H. (1961) *Arch. Biochem. Biophys.* **94**, 449–463
- Ornstein, L. (1964) *Ann. N.Y. Acad. Sci.* **121**, 321–349
- Reisner, A. H., Nemes, P. & Bucholtz, C. (1975) *Anal. Biochem.* **64**, 509–516
- Shah, V. K. & Brill, W. J. (1973) *Biochim. Biophys. Acta* **305**, 445–454
- Udenfriend, S. (1964) *Fluorescence Assay in Biology and Medicine*, Academic Press, New York
- Van De Bogart, M. & Beinert, H. (1967) *Anal. Biochem.* **20**, 325–334
- Yates, M. G. & Planque, K. (1975) *Eur. J. Biochem.* **60**, 467–476
- Yuki, H., Sempuku, C., Park, M. & Takiura, K. (1972) *Anal. Biochem.* **46**, 123–128