Comparison of active and inactive forms of iron protein from *Rhodospirillum rubrum*

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The Fe protein of nitrogenase from *Rhodospirillum rubrum* was purified in its active and inactive forms. It is shown that the inactive form exists as a two-subunit modified form of the enzyme as previously reported [Ludden & Burris (1978) *Biochem. J.* 175, 251–259]. In contrast, the active form exists as a single-subunit unmodified form of the enzyme. The upper subunit (on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis) of the inactive form was shown to contain at least the phosphate group of the covalently bound modifying group. The active and inactive forms of the enzyme were shown to be identical proteins on the basis of amino-acid composition, tryptic-digest pattern and immunological cross-reactivity.

Rhodospirillum rubrum exhibits the ability to reduce N_2 to ammonia. As in other organisms with this ability, the reaction is performed by a nitrogenase enzyme complex consisting of a molybdenum- and iron-containing protein and a smaller, iron-containing, protein. The composition of the proteins and their requirements for enzyme activity are similar to those for other nitrogenases, such as those from *Azotobacter vinelandii* and *Klebsiella pneumoniae* (Mortenson & Thorneley, 1979). The regulation of enzyme activity at the genetic level also resembles regulation exhibited by other organisms; both fixed nitrogen and oxygen (P. W. Ludden, unpublished work) repress the synthesis of the enzyme. In addition, Ruvkun & Ausubel (1980) have shown that a high degree of homology exists between the DNA coding for nitrogenase proteins of *Rhsp. rubrum* and *K. pneumoniae*. At the non-genetic level, however, *Rhsp. rubrum* exhibits an unusual type of regulation of nitrogenase activity by covalent modification of the Fe protein (Ludden & Burris, 1979). The addition of NH_4^+ to cells that are actively fixing N_2 results in a loss of enzyme activity in whole cells. Inactivity of the purified enzyme is correlated with covalent modification of the Fe

protein of *Rhsp. rubrum* (Ludden & Burris, 1979). An activating enzyme has been detected for Rr_2 , (Ludden & Burris, 1976; Nordlund *et al.*, 1977; Zumft & Nordlund, 1981). This form of enzyme regulation has been demonstrated in *Rhsp. rubrum* and *Azospirillum lipoferum*, and indirect evidence exists for this type of regulation in *Rhodopseudomonas capsulata*, *Rhps. spheroides* and *Rhps. palustris* (Yoch, 1980; Zumft & Castillo, 1978; Jones & Monty, 1979).

When *Rhsp. rubrum* is grown with glutamate as the nitrogen source, nitrogenase is synthesized and is active *in vivo*. When the Fe protein from glutamate-grown *Rhsp. rubrum* is purified, it is found to be in the inactive form. Carithers *et al.* (1979) have found that when *Rhsp. rubrum* is grown with limiting NH_4^+ as the nitrogen source, crude extracts exhibit nitrogenase activity in the absence of activating conditions for the Fe protein. In the present paper we demonstrate that Fe protein from NH_4^+ -limited cells is active when purified and establish that the enzyme from cells grown under both conditions are the same protein by a number of criteria.

Materials and Methods

Purification of proteins

Dinitrogenase proteins were purified as described by Ludden & Burris (1978), except that cells were lysed (osmotic shock) into buffer containing 100 mM-Mops, pH 7.5, 1 mM-sodium dithionite and 1 mM-dithioerythritol. Previous extractions were performed with buffer containing 100 mM-Tris/HCl, pH 8.3. RNAase is not very active at this pH, and

Abbreviations used: The iron protein of nitrogenase is referred to as the Fe protein and abbreviated Rr_2 ; active Rr_2 obtained from NH_4^+ -limited cells is abbreviated Rr_2^+ ; inactive Rr_2 obtained from glutamate-grown cells is referred to as Rr_2^- ; the iron protein from *Azotobacter vinelandii* is referred to as Av_2 ; the molybdenum/iron protein is abbreviated Rr_1 ; SDS, sodium dodecyl sulphate; Mops, 4-morpholinepropanesulphonic acid; RNAase, ribonuclease; DNAase, deoxyribonuclease.

assays for nucleotides yielded values that were too high.

Azotobacter vinelandii Fe protein was purified as described by Hageman (1979).

Cells to be used to prepare inactive Rr2 were grown with glutamate as the nitrogen source as described by Ludden & Burris (1978). Cells to be used to prepare active Rr2 were grown on the NH_4^+ -limited medium described by Carithers *et al.* (1979).

Amino-acid analyses

Amino-acid compositions of Rr2_i and Rr2_a were determined on a Beckman amino-acid analyser after hydrolysis of the proteins in 6M-HCl at 100°C under a vacuum for 24 h. Cysteine was determined as cysteic acid after performic acid oxidation.

Subunit compositions

Subunit compositions of Rr2_i and Rr2_a were determined by electrophoresis on SDS/10% (w/v)-polyacrylamide gels using Laemmli's (1970) method.

Proteinase digests

Partial proteinase digests were prepared by treating 750 µg of either Rr2_a or Rr2_i with 37 µg of trypsin (Sigma, type VII) for 10 min in a total volume of 250 µl. The incubation mixture was buffered with 100 mM-Tris/HCl, pH 8.0, and the reaction was quenched after 10 min by the addition of 50 µl of the concentrated SDS cocktail solution described by Laemmli (1970) to adjust the SDS concentration to 1% (w/v). Peptides were analysed by SDS/12% (w/v)-polyacrylamide gels.

U.v.-visible spectra

Rr2_i and Rr2_a to be used for u.v.-visible spectra were treated for 10 min with 10 µg each of DNAase and RNAase in 1.0 ml of 100 mM-Mops buffer, pH 7.5, at room temperature; 0.5 mg of each protein was used. The proteins were then precipitated with 1% HClO_4 and resuspended in 200 µl of 25 mM- NH_3 . The pH was adjusted to 8.0 by the addition of 1.0 ml of 100 mM-Tris/HCl, pH 8.0. The spectra of the redissolved proteins and the difference spectra of Rr2_i and Rr2_a were determined on a Cary 118 spectrophotometer.

Modifying-group assays

The presence of pentose, phosphate and the adenine-like compound on the proteins was determined as described by Ludden & Burris (1978). Autoradiography of ^{32}P -labelled proteins was performed with DuPont Cronex or Kodak XAR-5 film. Kodak developer and fixer were used. The time of film exposure to gel depended on the extent of labelling and varied from 10 h to 2 days.

Preparation of ^{32}P -labelled Rr2 was accomplished by growing cells in 500 ml batches in a specially designed 500-ml fermenter. Washed stock culture cells were added to the medium so that the initial A_{600} of the cell culture was 0.2. The medium was the same as that for large cultures of glutamate-grown cells, except that the phosphate concentration was lowered to 0.05 g/litre and 1 mCi of carrier-free $^{32}\text{PO}_4$ was added. Mops buffer (10 g/litre) was added to restore buffering capacity to the medium. The cells were grown under a helium atmosphere. When the whole cell nitrogenase activity (acetylene reduction) was high (more than 1000 nmol of acetylene reduced/h per ml of cells), cells were collected anaerobically and lysed by osmotic shock. The extract was spun for 60 min at 50 000 g and the supernatant was placed on a small (0.5 cm × 4 cm) anaerobic DEAE-cellulose column. The column was washed with buffer containing 50 mM-Tris/HCl, pH 7.7, 1 mM- $\text{Na}_2\text{S}_2\text{O}_4$ and 150 mM-NaCl. Rr1 and Rr2 were then eluted from the column by increasing the buffer NaCl concentration to 400 mM. The brown nitrogenase-containing fraction was desalted on a Sephadex G-25 column and then applied to a 3.0 mm-thick polyacrylamide slab gel; the gel contained no protein denaturing agents. The slab gel had been pre-run in the presence of 1 mM- $\text{Na}_2\text{S}_2\text{O}_4$ to make it anaerobic. The gel was electrophoresed at 150 V for 2 h. The brown Rr2 band was sliced out of the gel with a razor blade and the gel slice was macerated so that the protein could be eluted easily from the gel with water. The eluted protein solution was separated from fine pieces of polyacrylamide by passing it through a Gelman disposable filter (0.2 µm pore size) and concentrated by freeze-drying. The protein was resuspended in the SDS cocktail for analysis on SDS-containing gels.

Antibody preparation

Antibodies against Rr2 were raised in rabbits by injecting 1 mg of inactive Rr2 per rabbit in an emulsion containing 50% Freund's complete adjuvant (Colorado Serum Companies Laboratories, Denver, CO, U.S.A.) and double-distilled water in the hind legs in a series of three injections over a period of 3 days. This procedure was repeated about every 6–10 weeks to boost the antibody titre. About 30 ml of whole blood was drawn from each rabbit and allowed to clot at 4°C for 2–5 h. After centrifuging at 8000 g for 20 min, the supernatant was frozen for future use as a crude preparation of antibodies.

Precipitation of Rr2 by antibodies using either purified enzyme or crude extracts was demonstrated by the Ouchterlony double-immunodiffusion method (Munoz, 1971). Slides were incubated at 37°C.

Nitrogenase assays

Nitrogenase activity was determined by acetylene reduction (Ludden & Burris, 1976). High concentrations of Mg^{2+} and Mn^{2+} were used when measuring $Rr2_i$; 10mM- Mg^{2+} but no Mn^{2+} was present in the assay when $Rr2_a$ was measured. Acetylene was separated from ethylene by gas chromatography on a 180cm $\times$ 1.8mm (int.diam.) column of Porapak R. A Varian 940 flame-ionization gas chromatograph was used. Acetylene for the assays was generated from CaC_2 .

Reagents

ATP, creatine phosphate, creatine phosphokinase (EC 2.7.3.2), Tris buffer (Trizma), Mops buffer and dithioerythritol were obtained from Sigma. Dithionite was obtained from J. T. Baker Co., Phillipsburg, NJ, U.S.A. Gases were from Matheson, Joliet, IL, U.S.A., and were further purified by passing them over catalyst R3-11 from Chemical Dynamics Corp., South Plainfield, NJ, U.S.A. Acrylamide and bisacrylamide were obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Whatman DE-52 DEAE-cellulose was used for ion-exchange chromatography of proteins.

Results and discussion

$Rr2$ from NH_4^+ -limited cells can be purified from extracts by using the same purification scheme as that employed for the inactive enzyme from glutamate-grown cells. Furthermore, $Rr2_a$ and $Rr2_i$ co-electrophorese on native (i.e. non-SDS-containing) analytical gels (results not shown). They also show identical polymerization patterns on native gels if exposed to O_2 before running the gel. Fig. 1 shows the subunit pattern seen for $Rr2_a$ and $Rr2_i$ on SDS/polyacrylamide gels. $Rr2_i$ shows two subunit types as previously described (Ludden & Burris,

Fig. 1. Subunit composition of $Rr2$ as determined by SDS/polyacrylamide-gel electrophoresis
A 10 μ g portion of each protein was run on a 10% (w/v) polyacrylamide/SDS gel. Lane 1, Av2; lane 2 $Rr2_i$ from glutamate-grown cells; lane 3, $Rr2_a$ from NH_4^+ -limited cells; lane 4, $Rr2_i$ from NH_4^+ -limited cells treated with 2mM- NH_4Cl before harvesting.

1978); $Rr2_a$, however, shows a single major subunit type equivalent to the lower subunit of $Rr2_i$, and a lesser amount of an upper subunit equivalent to the upper subunit of $Rr2_i$. If NH_4^+ -limited cells are treated with NH_4Cl before harvesting to switch off the whole cell nitrogenase activity, a two-subunit $Rr2$ is obtained. Fe protein from *Azotobacter vinelandii* is included in Fig. 1 for comparison.

Fig. 2 shows the subunit pattern for $Rr2$ obtained from glutamate-grown cells grown in the presence of $^{32}PO_4$. Comparison of the protein stain and the autoradiogram clearly shows that only the upper band is labelled. Note that the faint band seen on the autoradiogram below the upper band of $Rr2_i$ does not correspond to the lower band of $Rr2_i$; the identity of the faint band is not known. No label is seen in $Rr2_a$ if $Rr2_a$ is purified from NH_4^+ -limited cells grown in the presence of $^{32}PO_4$. On the basis of this result it is clear that only the upper subunit carries the modifying group.

Table 1 shows the comparison of the amounts of modifying-group components for $Rr2_i$ and $Rr2_a$. In a previous paper (Ludden & Burris, 1978), it was reported that there were two each of pentose, PO_4

Fig. 2. ^{32}P -labelling of subunits of $Rr2_i$
 $Rr2_i$ was isolated from cells grown in the presence of $^{32}PO_4$. (a) The two subunits of $Rr2_i$ stained for protein; (b) autoradiogram of the gel.

Table 1. Analysis of $Rr2_a$ and $Rr2_i$ for modifying-group component

	Content (nmol/nmol of $Rr2$)		
	Pentose	Phosphate	Adenine-like molecule
$Rr2_i$	1.32	1.26	0.63
$Rr2_a$	0.23	0.4	0.09

and the adenine-like compound on Rr2_i. In the present study we find one of each component of the modifying group per protein dimer. We believe that this results from the improved procedure for RNAase treatment described in the Materials and methods section of the present paper. The value shown for the adenine-like molecule is lower than the value for pentose and phosphate. This is in part due to lower background for the adenine assay. In addition, it is possible that the fluorescence yield for the adenine-like compound differs from the fluorescence yield for the adenine used as a standard. Rr2_a shows low levels of each component. The amounts found on Rr2_a could be interpreted as representing the amount of inactive protein present plus some unavoidable contamination.

The u.v.-visible spectra of Rr2_i and Rr2_a also indicate a difference in modification (Fig. 3). Rr2_a lacks the shoulder at 265–268 nm that is seen with Rr2_i. A difference spectrum of Rr2_i minus Rr2_a shows a peak at 265–268 nm (Fig. 4). This is similar to the difference spectrum for Rr2_i minus Rr2_a that was obtained when Rr2_a was prepared by activation *in vitro* using the activating enzyme (Ludden & Burris, 1979). In the previous study it was shown that the removal of the adenine-like compound from Rr2_i resulted in activation.

If the interconversion of Rr2_i and Rr2_a is to be approached as a reversible covalent-modification

system, it must be demonstrated that Rr2_a and Rr2_i are identical proteins. This has been tested in several ways. Table 2 shows the amino-acid compositions of Rr2_a and Rr2_i and, for comparison, the amino-acid composition for Av2 (Hageman, 1979). Although there is some variation, it is clear that the amino-acid compositions are essentially identical. Similarly,

Fig. 3. U.v.-visible spectra of Rr2_i and Rr2_a

Fig. 4. Difference spectrum of Rr2_i minus Rr2_a

Table 2. Amino-acid composition of Rr2_i, Rr2_a and Av2

	Composition (mol/mol of protein dimer)		
	Rr2 _i	Rr2 _a	Av2
Lys	28	31	32
His	8	8	6
Arg	18	19	28
Asp	61	63	60
Thr	15	15	24
Glu	67	70	70
Ser	29	29	20
Pro	14	13	18
Gly	91	95	56
Ala	75	77	56
Cys	24	26	14
Val	32	32	50
Met	14	15	26
Ile	8	9	40
Leu	33	34	46
Tyr	12	11	18
Phe	8	8	14

analysis of partial tryptic digests on SDS/polyacrylamide gels shows that an identical set of peptides is generated from $Rr2_i$ and $Rr2_a$ (Fig. 5).

Ouchterlony double immunodiffusion of $Rr2_a$ and $Rr2_i$ against rabbit antiserum raised against $Rr2_i$ was also used to test the similarity of the proteins. A single band is seen for $Rr2_i$ and $Rr2_a$ both against crude extracts and for the purified forms of the enzyme (Fig. 6). From this we conclude that a single protein molecule accounts for all Fe protein and that $Rr2_i$ and $Rr2_a$ are the same protein, differing only by the covalent modification of $Rr2_i$.

$Rr2_a$ isolated from NH_4^+ -limited cells is highly

Fig. 5. Peptides of $Rr2_a$ and $Rr2_i$ generated by partial digestion with trypsin

Lane 1, untreated $Rr2_i$; lane 2, trypsin only; lane 3, 30 μ g of trypsin-treated $Rr2_i$; lane 4, 150 μ g of trypsin-treated $Rr2_i$; lane 5, 30 μ g of trypsin-treated $Rr2_a$; lane 6, 150 μ g of trypsin-treated $Rr2_a$.

Fig. 6. Ouchterlony immunodiffusion assay of $Rr2_i$ and $Rr2_a$

Antibody was raised against purified $Rr2_i$. A, anti- $Rr2_i$; B, normal rabbit serum; 1 and 5, crude extract from glutamate-grown cells; 2, purified $Rr2_i$; 3 and 6, purified $Rr2_a$; 4 and 7, crude extract from NH_4^+ -limited cells.

active when $Rr1$, MgATP and reductant are added. Specific activities as high as 1600 nmol of acetylene reduced/min per mg of protein have been achieved. Unlike $Rr2$ from glutamate-grown cells, this activity is not dependent on activation by activating enzyme or on the presence of 0.5 mM- Mn^{2+} , which is the preferred metal cofactor for activating enzyme (Ludden & Burris, 1976). Although nitrogenase activity is expressed in glutamate-grown cells, $Rr2$ is switched off at some point during cell collection or lysis; apparently the switch-off does not occur with NH_4^+ -limited cells.

Yoch & Cantu (1980) have proposed that there are three forms of nitrogenase in *Rhsp. rubrum*: an active form that cannot be switched off, which they refer to as 'nitrogenase A' and a form that can be switched on and off, which they refer to as 'nitrogenase R'. We have prepared nitrogenase from NH_4^+ -limited cells, which should yield nitrogenase A, and from glutamate-grown cells, which should yield nitrogenase R. We have demonstrated that the $Rr2$ protein is identical except for the covalent modification of the inactive form. We propose that there are two forms of the enzyme *in vivo*: an active, unmodified form and an inactive, modified form.

Several groups have observed that NH_4^+ -limited cells switch off very slowly, or not at all, when NH_4^+ is added to this medium (Ormerod *et al.*, 1961; Carithers *et al.*, 1979; Sweet & Burris, 1981). It seems likely that this results from a depleted intracellular pool of the modifying-group donor; it is not due to an alternative protein for $Rr2$. From the present study we have learned that the subunit composition of $Rr2$ reflects its activity, with the presence of the upper band indicating that the enzyme is in the inactive form. Because the upper band is also labelled with phosphate, it also appears that only the upper band carries the covalent modification. Because the amino-acid composition, tryptic map and antigenic specificity of the two forms of the protein are identical, we conclude that Fe protein from *Rhsp. rubrum* has a single type of subunit, and that the difference between the two subunit types of $Rr2_i$ seen on SDS/polyacrylamide gels results from the covalent modification of one subunit but not the other. It is not known if conditions exist under which both subunits are modified. Although the small molecular-weight difference resulting from the addition of the modifying group would not be expected to result in a change in mobility on SDS/polyacrylamide gels, it should be noted that the mobility of proteins on SDS/polyacrylamide gels is sometimes affected by properties other than molecular weight. For example, Noel *et al.* (1979) have found that a single amino-acid change in an *Escherichia coli* membrane protein results in altered mobility of the protein on SDS/polyacrylamide gels.

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