Incorporation of adenine into the modifying group of inactive iron protein of nitrogenase from *Rhodospirillum rubrum*

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Adenine was fed to cells of *Rhodospirillum rubrum* grown on glutamate. The adenine was found to be incorporated into the modifying group of the inactive form of iron protein. Adenine labelled in the 8-position ([8-³H]adenine) and the 2-position ([2-³H]adenine) was specifically incorporated into the electrophoretic 'upper-band' subunit of iron protein. Incorporation of label from the 2-position into many proteins was observed if histidine was not present in the medium. Label was removed by the activating enzyme for iron protein.

Whole-cell nitrogenase activity of the non-sulphur photosynthetic bacterium *Rhodospirillum rubrum* is switched off when NH₄⁺, the product of nitrogenase, is added to cells that are actively fixing nitrogen (Gest *et al.*, 1950; Neilson & Nordlund, 1975; Carithers *et al.*, 1979; Sweet & Burris, 1981). Glutamine and asparagine also cause switch-off of nitrogenase activity in whole cells (Neilson & Nordlund, 1975). The biochemical basis for this switch-off is the reversible inactivation of the iron protein of nitrogenase from *Rsp. rubrum* by the attachment of a covalently bound modifying group (Ludden & Burris, 1978, 1979). An activating enzyme for the iron protein has been isolated from the chromatophore fraction of *Rsp. rubrum* extracts (Ludden & Burris, 1976; Nordlund *et al.*, 1977). The known components of the modifying group are phosphate, pentose and an adenine-like compound (Ludden & Burris, 1978). Phosphate on the inactive protein has been measured by colorimetric assays and by incorporation of ³²P into the protein (Ludden & Burris, 1978; Ludden *et al.*, 1982; Gotto & Yoch, 1982). Pentose has been measured by colorimetric assay only (Ludden & Burris, 1978). The evidence for an adenine-like molecule on the iron protein is: (1) the difference spectrum of the protein and (2) a positive test for adenine in Yuki's glyoxyl hydrate assay for adenine (Yuki *et al.*, 1972). Because the λ_{max} of the difference spectrum of inactive minus active iron protein is 268 nm, rather than 260 nm as would be expected for adenine itself or AMP, we

have referred to the compound as adenine-like. In the present paper we show that ³H-labelled adenine is incorporated into the iron protein, that label is incorporated into the electrophoretic 'upper-band' subunit of iron protein only and that the adenine is lost during activation of the iron protein by the activating enzyme *in vitro*. Label from both [8-³H]adenine and [2-³H]adenine is incorporated into the protein.

Experimental

Iron protein labelled with [8-³H]adenine was prepared from cells grown in 500 ml batches on Ormerod's glutamate-containing medium (Ormerod *et al.*, 1961) as described previously for the preparation of ³²P-labelled iron protein (Ludden *et al.*, 1982). The cells were provided with 1 mCi of [8-³H]adenine (25 Ci/mmol)/500 ml of cells 12 h before the cells were harvested. Cells were harvested when whole-cell nitrogenase activity was 1500 nmol of acetylene reduced/h per ml of cells. To ensure that the iron protein obtained would be in the inactive, modified, form, NH₄⁺ and 2-oxoglutarate (final concn. 1 mM) were added to the cells just before harvest. Iron protein was then purified by anaerobic ion-exchange chromatography on DEAE-cellulose and gel electrophoresis as previously described (Ludden *et al.*, 1982).

Analysis of iron protein from small portions of cells was also done. A 20 ml portion of cells was incubated for 12 h in Ormerod's medium (Ormerod *et al.*, 1961) plus 50 μ Ci of [2-³H]- or [8-³H]-adenine under helium at 30°C in stoppered serum vials and shaken in a Gilson illuminated water bath. The

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nitrogenase activity was switched off by the addition of NH_4^+ and 2-oxoglutarate to a concentration of 1 mM each before the cells were harvested. When whole-cell nitrogenase activity was greater than 1000 nmol of acetylene reduced/h per ml of cells, NH_4^+ and 2-oxoglutarate were added, and the cells were collected on a glass-fibre filter and quickly frozen in liquid N_2 . The cells were extracted by grinding the glass-fibre filter in a Kontes ground-glass conical tissue grinder. The grinding medium contained 100 mM-Mops (4-morpholinepropanesulphonic acid)/NaOH buffer, pH 7.5, 5 mM- $\text{Na}_2\text{S}_2\text{O}_4$, 1 mM-Methyl Viologen, 1 mM-dithioerythritol, 1 mM- MgCl_2 and 200 mg of 150-mesh carborundum. The grinding tube was flushed with N_2 during the extraction. The extract was treated with 1 μg each of deoxyribonuclease and ribonuclease, and was then centrifuged at 10000g for 1 min in a Beckman Microfuge. A 0.5 ml sample of the supernatant (total volume 1.0 ml) was transferred to a second Microfuge tube containing rabbit anti-(iron protein) antibody. The antibody was incubated with the extract for 3 min and then centrifuged at 10000g for 1 min. The supernatant was discarded, and the iron-protein-containing pellet was resuspended in 100 μl of diluted gel 'cocktail' (Laemmli, 1970). The iron protein was then analysed by electrophoresis on polyacrylamide gels containing sodium dodecyl sulphate (Laemmli, 1970).

Protein bands in gels were stained with Coomassie Brilliant Blue. ^3H -labelled bands were detected by fluorography (Laskey & Mills, 1975). Nitrogenase

activity in whole cells was determined by assay of acetylene reduction (Koch & Evans, 1966). $[8\text{-}^3\text{H}]\text{Adenine}$ and $[2\text{-}^3\text{H}]\text{adenine}$ were obtained from Amersham International.

Activation of $[8\text{-}^3\text{H}]\text{adenine}$ -labelled iron protein was performed in a reaction mixture containing 100 μl of labelled iron protein, 100 μl of activating enzyme, 25 mM- MgCl_2 , 0.5 mM- MnCl_2 and 5.0 mM- $\text{Na}_2\text{S}_2\text{O}_4$ in a total volume of 355 μl . The buffer was 50 mM-Tris/HCl, pH 7.7. Samples (50 μl) of the incubation mixture were taken at various times and quenched in 20 μl of gel 'cocktail' containing sodium dodecyl sulphate. The time samples were analysed by electrophoresis on polyacrylamide gels containing 10% acrylamide and 0.1% sodium dodecyl sulphate. Gels were stained with Coomassie Brilliant Blue. Radioactivity in iron-protein bands was determined by fluorography or by scintillation counting of the radioactivity of the protein after cutting the stained protein bands out of the gel and digestion of the band in aq. $\text{H}_2\text{O}_2/\text{NH}_3$ at 70°C for 3 h.

Activating enzyme was prepared as described by Zumft & Nordlund (1981).

Results and discussion

Fig. 1(a) shows the electrophoretic 'upper-band' and 'lower-band' subunits of the nitrogenase iron protein purified from cells grown in the presence of $[8\text{-}^3\text{H}]\text{adenine}$. Fig. 1(b) shows the fluorogram of the gel and demonstrates that only the 'upper-band' subunit is labelled. We have previously demonstrated that ^{32}P label is incorporated only into the 'upper-band' subunit of the iron protein (Ludden & Preston, 1980; Ludden *et al.*, 1982). During activation of iron protein by the activating enzyme, the 'upper-band' subunit is converted into the 'lower-band' subunit (Preston & Ludden, 1982). Therefore we wanted to know whether the label would be lost during activation of adenine-labelled protein. Fig. 2 shows that during activation of iron protein by the activating enzyme both subunit conversion and loss of label occurs. A duplicate gel to the one shown in Fig. 2 was run and stained with Coomassie Brilliant Blue, and then 'upper-band' and 'lower-band' subunit protein bands for each time point were cut out of the gel. The gel pieces were digested and their radioactivities counted by scintillation counting. Table 1 shows that label lost from the 'upper-band' subunit did not appear in the 'lower-band' subunit. The 'upper-band' and 'lower-band' subunits of the iron protein are thought to be identical peptides (Ludden *et al.*, 1982; S. Nordlund & P. W. Ludden, unpublished work), differing only in the presence of a modifying group on the 'upper-band' subunit. Because label from adenine is incorporated selectively into the 'upper-band' subunit, it can be argued

Fig. 1. Electrophoretogram showing incorporation of $[8\text{-}^3\text{H}]\text{adenine}$ into the 'upper-band' subunit of nitrogenase iron protein

For full experimental details see the text. (a) Protein stain; (b) radioautogram. The arrow indicates the position of the 'upper-band' subunit.

Fig. 2. Electrophoretogram showing loss of label from the 'upper-band' subunit of the nitrogenase iron protein during activation by activating enzyme

For full experimental details see the text. (a) Protein stain; (b) radioautogram. Time of activation: track 1, 0 min; track 2, 30 min; track 3, 60 min; track 4, 90 min; track 5, 120 min. The arrow indicates the position of the 'upper-band' subunit on the gel and radioautogram.

Fig. 3. Radioautogram of an electrophoretogram of nitrogenase iron protein purified from cells grown with $[8\text{-}^3\text{H}]\text{adenine}$ and/or $[2\text{-}^3\text{H}]\text{adenine}$

Extracts were prepared from 10 ml of cells. For full experimental details see the text. Track 1, $[8\text{-}^3\text{H}]\text{adenine}$ -labelled iron protein from cells grown in the absence of histidine; track 2, $[8\text{-}^3\text{H}]\text{adenine}$ -labelled iron protein from cells grown in the presence of histidine; track 3, $[2\text{-}^3\text{H}]\text{adenine}$ -labelled iron protein from cells grown in the presence of histidine; track 4, mixture of $[2\text{-}^3\text{H}]\text{adenine}$ - and $[8\text{-}^3\text{H}]\text{adenine}$ -iron protein from cells grown in the presence of histidine. Arrows indicate the positions of the 'upper-band' subunit (u) and the 'lower-band' subunit (l) of iron protein and the dye front (f).

Table 1. Loss of adenine from the 'upper-band' subunit of nitrogenase iron protein during activation of the iron protein by activating enzyme

For full experimental details see the text.

Time of activation (min)	Radioactivity (c.p.m.)	
	'Upper-band' subunit	'Lower-band' subunit
0	1481	114
30	592	101
60	435	85
90	285	100
120	197	85

that the adenine has not been metabolized to amino acids and incorporated into the iron protein. Thus appearance of an adenine-like molecule in the modifying group of *Rsp. rubrum* nitrogenase iron protein is confirmed.

Because the adenine is labelled in a specific position, the 8-position, the proton on the 8-position must be retained in the adenine of the modifying group. Thus no linkages or modifications of the adenine ring structure can be attached to the C-8 position. We sought to repeat these experiments with adenine labelled at the C-2 position. When we did this experiment, we found that both subunits of iron

protein became labelled. Further analysis showed that all proteins in the extract became labelled. In the bacteria that have been studied, adenine is a precursor for histidine; the C-2 proton of adenine, but not the C-8 proton, is conserved in the histidine molecule (Brenner & Ames, 1971). Although no information is available about the biosynthesis of histidine in photosynthetic bacteria (see review by Datta, 1978), this experiment and the following one imply that adenine is a precursor to histidine in *Rsp. rubrum*.

To overcome the problem with histidine, we tried to eliminate the non-specific incorporation of label from $[2\text{-}^3\text{H}]\text{adenine}$ into protein by including 1 mM-histidine in the growth medium. Fig. 3 shows the result of this experiment. Label from $[8\text{-}^3\text{H}]\text{adenine}$ is incorporated into the 'upper-band' subunit of nitrogenase iron protein both in the presence and in the absence of histidine in the growth medium. Label from $[2\text{-}^3\text{H}]\text{adenine}$ is also incorporated specifically into the 'upper-band' subunit of the protein if histidine is present in the medium. A mixture of $[2\text{-}^3\text{H}]\text{-}$ and $[8\text{-}^3\text{H}]\text{-adenine}$ also shows incorporation.

Because the protons on the C-2 and C-8 positions

of the adenine ring are conserved during incorporation into the modifying group, no linkage can be through these positions. The lack of any modification of the purine ring at the C-2 position also rules out guanosine as a constituent of the modifying group. Previously it has been demonstrated that the modified iron protein gives a positive reaction in Yuki's assay for adenine (Ludden & Burris, 1978). Yuki's assay requires that the 6-amino group on adenine is not modified for the reaction to take place (Yuki *et al.*, 1972). Positions 4 and 5 of the purine ring are not available for bonding, and thus it can be concluded that attachment of the adenine-like molecule to the protein or to the rest of the modifying group must be through one of the ring nitrogen atoms.

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