A novel covalent modification of nitrogenase in a cyanobacterium

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Abstract In extracts of the unicellular cyanobacterium *Gloeothece*, the Fe-protein of nitrogenase can be separated by SDS–PAGE into two antigenically identifiable components. Unlike the situation in photosynthetic bacteria such as *Rhodospirillum rubrum*, these two forms do not arise from covalent modification of the protein by ADP-ribosylation. Rather, the Fe-protein of *Gloeothece* nitrogenase is subjected to modification by palmitoylation.

Key words: Covalent modification; Cyanobacterium; Fe-protein; Nitrogenase; Palmitoylation; *Gloeothece*

1. Introduction

Nitrogenase (EC 1.18.6.1) catalyses the reduction of N_2 to ammonium (N_2 fixation) and is widely distributed among bacteria [1]. It consists of two proteins: a MoFe-protein (dinitrogenase) that catalyses N_2 reduction and an Fe-protein (dinitrogenase reductase) that supplies low potential electrons to the MoFe-protein. In several diazotrophs [2,3], the Fe-protein of nitrogenase exists in two forms that can be distinguished by SDS–PAGE and, in photosynthetic bacteria such as *Rhodospirillum rubrum*, it has been demonstrated that the Fe-protein is covalently modified by ADP-ribosylation of one of its two identical subunits [4,5]. In extracts of diazotrophic cyanobacteria, a group of organisms that make a major contribution to global N_2 fixation [6], the Fe-protein of nitrogenase can also be resolved into two components during SDS–PAGE. However, despite considerable research effort, the molecular nature of the two forms of the Fe-protein in cyanobacteria remains to be elucidated. In this paper, we present evidence for a novel palmitoylation of the Fe-protein of nitrogenase in the unicellular cyanobacterium *Gloeothece*.

2. Materials and methods

2.1. Specialised chemicals

All radiolabelled compounds were purchased from Amersham Pharmacia Biotech, Little Chalfont, Bucks., UK. Antiserum to the Fe-protein of nitrogenase from *R. rubrum* was a generous gift from Dr Paul Ludden, Department of Biochemistry, University of Wisconsin (Madison, WI, USA).

2.2. Radiolabelling of cultures

Cultures of *Gloeothece* ATCC 27152 (American Type Culture Collection, Rockville, MD, USA) were grown under alternating 12 h light (30 μmol m^{-2} s^{-1}) and 12 h darkness as described previously [7]. Under these conditions, maximum synthesis of the Fe-protein of nitrogenase occurs between 10 h into the light phase (L10) and 4 h after the onset of darkness (D4) [8]. Radiolabelled compound (60 μCi [^{32}P]orthophosphate, 200 mCi $mmol^{-1}$; 20 μCi [8-^{14}C]adenine, 55 mCi $mmol^{-1}$; 100 μCi [2-^{14}C]acetate, 57 mCi mmol; 20 μCi *RS*-[2-^{14}C]mevalonic acid lactone, 58 mCi $mmol^{-1}$, previously converted to mevalonic acid by addition of 2 mg of $KHCO_3$) was therefore added at L8 to 25 ml samples of culture (concentrated 40-fold) suspended in growth medium [7] containing 5 mM HEPES–NaOH buffer, pH 7.5. At D4, NH_4Cl was added to 2 mM in order to stimulate conversion of Fe-protein to its larger, presumed modified, form [9]. At D6, cells were collected by centrifugation at 10 000×*g*, resuspended in 6 ml of growth medium separated into six equal aliquots, recentrifuged and frozen. In the case of palmitic acid, the procedure was modified as follows. At L8, 25 ml of 40-fold concentrated cells was centrifuged at 700×*g* for 10 min and resuspended in 2 ml of medium. This sample was then mixed with 100 μCi of [9,10(*n*)-^{3}H]palmitic acid (56 mCi $mmol^{-1}$), transferred in 400 μl aliquots to cuvettes with a 2 mm gap and subjected to electroporation at 10 kV cm^{-1} with a time constant of 15 ms. Electroporated cells were then resuspended in 25 ml of buffered growth medium. This treatment had previously been shown to inhibit nitrogenase activity in N_2-fixing cells by no more than 80%, with activity recovering completely during the following 4 h.

2.3. SDS–PAGE/Western blotting

Frozen samples were resuspended in 0.5 ml of extraction buffer and subjected to sonication [10]. For immunoprecipitation, frozen samples were resuspended in 0.5 ml of phosphate-buffered saline, sonicated [10] and centrifuged at 3000×*g* for 3 min. The supernatant was then incubated with gentle mixing for 60 min at 25°C with 60 μl of antiserum to the Fe-protein of nitrogenase from *R. rubrum*. To this mixture was added 10 mg of protein A Sepharose CL43, suspended in 0.1 ml of phosphate-buffered saline and, after incubation for a further 15 min, the sample was centrifuged at 10 000×*g* for 2 min. The supernatant was carefully removed with a syringe and the precipitate washed twice with 1 ml of phosphate-buffered saline containing 2% (w/v) bovine serum albumin and 1% (v/v) Triton X-100 and four times with 1 ml of phosphate-buffered saline. The precipitate was then suspended in 100 μl of extraction buffer and prepared for SDS–PAGE/Western blotting [8].

2.4. Detection of radiolabelled proteins

Radiolabelled proteins were visualised in blots by autoradiography [10]. In the case of samples prepared from cultures incubated with [9,10(*n*)-^{3}H]palmitic acid, the nitrocellulose filter was first presoaked in 20% (w/v) 2,5-diphenyloxazole (PPO) in toluene and then air-dried [11]. After autoradiography, PPO was removed by washing the filter three times in toluene and the Fe-protein of nitrogenase located immunologically [8]. For quantification of radioactivity on blots, strips corresponding to particular extracts were carefully cut into 1 mm segments and subjected to scintillation counting.

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Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; HPTLC, high performance thin layer chromatography; MALDI, matrix-assisted laser desorption; PAGE, polyacrylamide gel electrophoresis; PPO, 2,5-diphenyloxazole; TOF, time of flight

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2.5. Transesterification and analysis of fatty acid methyl esters

After immunoprecipitation and SDS–PAGE of some extracts of cells that had been radiolabelled with [2-^{14}C]acetate, the bands corresponding to the Fe-protein of nitrogenase were excised and incubated with 0.1 M KOH in methanol for 4 h at 25°C after which the fatty acid methyl esters were extracted three times with 1 ml of hexane [12]. The hexane extracts were then subjected to reverse phase HPTLC [13] on C^{18}-silica, using chloroform/methanol/water (15:45:3, v/v) as solvent. Methyl esters of laurate (12:0), myristate (14:0), palmitate (16:0) and stearate (18:0) served as standards. Radiolabelled material was located by scintillation counting after scraping off 0.5 mm segments from strips containing the chromatographed hexane extract.

3. Results and discussion

Gloeothece ATCC 27152 is a unicellular cyanobacterium that is capable of aerobic N_2 fixation [14]. Nitrogenase in *Gloeothece* consists of an MoFe-protein and an Fe-protein and, as in several other cyanobacteria [15–22], the Fe-protein of *Gloeothece* nitrogenase can be resolved into two different, though antigenically similar, forms by SDS–PAGE. In *Gloeothece*, these two forms have apparent M_r values of 38 500 and 40 000 respectively [8] and their relative concentrations vary according to prevailing environmental conditions. For example, formation of the larger form of the protein is favoured by exposure of cultures to elevated concentrations of O_2 [8], to elevated temperature [23], to illumination after a 12 h period of darkness [24] or to a source of combined nitrogen [9]. Since an increase in the larger form of the Fe-protein occurs in the presence of chloramphenicol, an inhibitor of de novo protein synthesis in *Gloeothece* [10], it is likely that this protein arises by modification of the smaller form of the Fe-protein. It is also likely that the larger form of the Fe-protein is catalytically inactive, at least under aerobic conditions of growth [8], so interconversions between the two forms of this protein may regulate nitrogenase activity in *Gloeothece*.

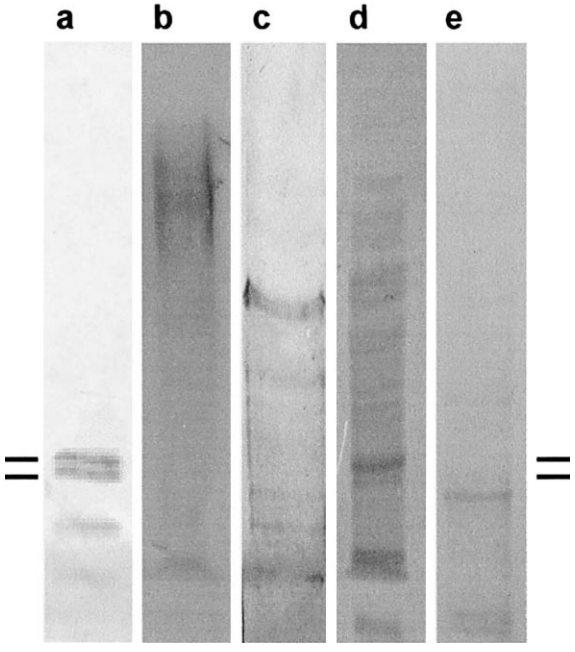

Fig. 1. SDS–PAGE of radiolabelled extracts of *Gloeothece*. a: Western blot showing the two forms of the Fe-protein of *Gloeothece* nitrogenase (marked). The upper band (M_r = 40 000) is the assumed modified form of the protein, the lower band has an apparent M_r of 38 500. b–e: Autoradiograph of extracts from cultures radiolabelled by incubation with (b) [^{32}P]orthophosphate, (c) [8-^{14}C]adenine, (d) [2-^{14}C]acetate and (e) [2-^{14}C]mevalonic acid.

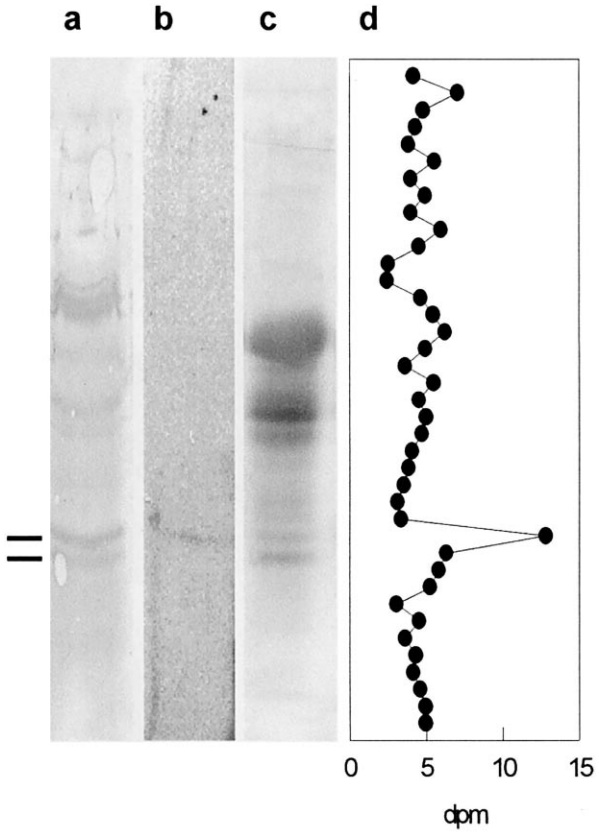

Fig. 2. SDS–PAGE of extracts of *Gloeothece* radiolabelled by incubation with [9,10(*n*)-^{3}H]palmitic acid. a: Western blot showing the upper (modified) and lower (unmodified) forms of the Fe-protein of *Gloeothece* nitrogenase (marked). b: Autoradiograph of extract from culture incubated with [9,10(*n*)-^{3}H]palmitic acid. c: Western blot of *Gloeothece* extract after immunoprecipitation with antisera to the Fe-protein of nitrogenase from *R. rubrum*. The additional bands result from reaction of the staining reagent with protein A and IgG present in the antiserum used for immunoprecipitation. d: Location of radioactivity in immunoprecipitated extract.

The two forms of the Fe-protein that can be detected in extracts of *Gloeothece* behave very similarly during SDS–PAGE to the Fe-protein in extracts of the photosynthetic purple bacterium *R. rubrum*. In *R. rubrum*, the Fe-protein of nitrogenase undergoes reversible covalent modification by ADP-ribosylation [5]. This is not, however, the case in *Gloeothece*. For example, incubation of *Gloeothece* with either [^{32}P]phosphate or [8-^{14}C]adenine failed to generate any radioactive Fe-protein (Fig. 1b,c), though other proteins became radiolabelled. Furthermore, antibodies raised against ADP-ribose [25] failed to cross-react with the Fe-protein from *Gloeothece*, though they did react with Fe-protein from *R. rubrum*. We therefore conclude that the Fe-protein is not subject to phosphorylation, adenylylation (or any other form of nucleotidylation) or to ADP-ribosylation. In addition, after SDS–PAGE the protein did not react with periodic acid-Schiff reagent [26]. It therefore also seems unlikely that the Fe-protein is modified by glycosylation.

On the other hand, radioactivity from [2-^{14}C]acetate was efficiently incorporated into the Fe-protein of *Gloeothece* nitrogenase (Fig. 1d). Although this may simply reflect the con-

version of acetate to metabolically related amino acids, the fact that more radioactivity appears in the larger form of the Fe-protein than in the smaller form implies that acetate might be rather more specifically involved in the modification that results in the apparent increase in M_r. This could be acetylation or acylation of the Fe-protein. In contrast, [2-^{14}C]mevalonic acid was not incorporated into the Fe-protein, though it was incorporated into a protein with a similar M_r (Fig. 1e). This observation rules out the possibility that the Fe-protein is modified by prenylation.

Fe-protein that had been radiolabelled with [2-^{14}C]acetate was isolated by immunoprecipitation, purified using SDS–PAGE and then subjected to alkaline transesterification in order to detach ester-linked fatty acids from the Fe-protein. About 0.014% of the radioactivity added to cultures could be recovered in purified Fe-protein and, after transesterification, about 27% of this could then be extracted into hexane. Reverse phase HPTLC of the hexane extract gave a single radioactive spot of $R_F = 0.45$, corresponding to the methyl ester of palmitic acid (C16:0). This could, however, account for no more than 38% of the total radioactivity extracted into hexane, so it is possible that other acetate-based modifying groups were also present on the Fe-protein.

Incubation of cultures of *Gloeothece* with [9,10(*n*)-^{3}H]palmitic acid resulted in an accumulation of radioactivity in the larger form of the Fe-protein of nitrogenase (Fig. 2b,d). This protein therefore appears to undergo palmitoylation. The lower efficiency of incorporation of radioactivity from [9,10(*n*)-^{3}H]palmitic acid into the Fe-protein (0.004%) relative to that from [2-^{14}C]acetate can be explained by poor uptake of palmitic acid, even using electroporation.

Based on their behaviour during SDS–PAGE, the two forms of the Fe-protein of *Gloeothece* nitrogenase have apparent M_r values of 38 500 and 40 000, a difference in M_r of 1500 [8]. However, MALDI-TOF mass spectrometry of immunoprecipitated Fe-protein [27] shows *m*/*z* peaks at 36 098 and 38 459, which may correspond to the two forms of this protein. This would give a difference in M_r of 2361. Since the M_r for palmitoyl is 239, 7–10 modifying groups per molecule of Fe-protein would be needed to account for the difference in M_r between modified and unmodified Fe-protein. Such a degree of palmitoylation seems unlikely, so it is possible that palmitoylation is not the sole form of modification of the Fe-protein of nitrogenase that occurs in *Gloeothece*.

Palmitoylation of proteins involved with signal transduction is well documented in eukaryote cells [28] and, more recently, evidence has emerged that certain prokaryote proteins can be palmitoylated [29]. Whether palmitoylation of *Gloeothece* nitrogenase has any role in signal transduction remains to be demonstrated. However, palmitoylation of the Fe-protein of nitrogenase may well promote interaction with membranes in *Gloeothece*. Such interactions may facilitate a link between N_2 fixation and membrane-located phenomena such as respiration and photosynthesis which could then supply ATP and/or reductant directly to nitrogenase, thereby stimulating activity. Association with membranes could also limit the damaging effect of O_2 on nitrogenase, as has been proposed for the modified form of Fe-protein in *Anabaena* CA [15]. However, there is no evidence that the higher molecular weight form of the Fe-protein shows either increased catalytic activity or stability to O_2 [8]. To the contrary, the modified form of the Fe-protein in *Synechococcus* sp. strain RF-1 has been proposed to be preferentially targeted for proteolytic degradation [19]. However, in extracts of *Gloeothece*, both forms of the Fe-protein appeared to be equally sensitive to degradation, both in vivo [8,9] and in vitro [30]. The functional significance of the observed palmitoylation of the Fe-protein of *Gloeothece* nitrogenase therefore remains unclear.

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