JOURNAL OF BACTERIOLOGY, Dec. 1997, p. 7872–7874 Vol. 179, No. 24
0021-9193/97/$04.00+0

Dinitrogenase Reductase-Activating Glycohydrolase Can Be Released from Chromatophores of *Rhodospirillum rubrum* by Treatment with MgGDP

AGNETA NORÉN AND STEFAN NORDLUND*

Department of Biochemistry, Arrhenius Laboratories for Natural Sciences, Stockholm University, S-106 91 Stockholm, Sweden

Received 16 July 1997/Accepted 13 October 1997

Dinitrogenase reductase-activating glycohydrolase (DRAG), involved in the regulation of nitrogenase activity in *Rhodospirillum rubrum*, is associated with chromatophore membranes in cell extracts. We show that DRAG can be specifically released by treatment with MgGDP; other nucleotides studied had no effect. The DRAG activity released corresponds to the release of DRAG protein.

In the nitrogen-fixing phototroph *Rhodospirillum rubrum*, some other phototrophs, and some *Azospirillum* species, nitrogenase, the enzyme catalyzing the reduction of dinitrogen to ammonia (2), is regulated on both the genetic and the metabolic levels. In phototrophs this metabolic control is manifested as a reversible decrease in nitrogenase activity, the "switch-off" effect (14), when effectors such as ammonium, asparagine, or glutamine are added or when cells are transferred to the dark (5). At the molecular level it has been established that the switch-off effect is due to mono-ADP-ribosylation of dinitrogenase reductase (11), one of the two proteins comprising the nitrogenase complex. This reaction is catalyzed by dinitrogenase reductase ADP-ribosyltransferase (DRAT), whereas the activation, i.e., the removal of the ADP-ribose moiety, is catalyzed by dinitrogenase reductase-activating glycohydrolase (DRAG). Upon addition of switch-off effectors, DRAT is activated and DRAG is inactivated (5). We have previously suggested that an increase in NAD^+ concentration could be the internal signal for DRAT activation, leading to modification (inactivation) of dinitrogenase reductase (8, 9, 12). The identity of the internal signal regulating DRAG activity is, however, still an unresolved question. When cells are disrupted, the DRAG protein is found to be associated with the chromatophore membrane fraction, but it can be released by the addition of 0.5 M NaCl (4, 7). To investigate if this association is of physiological significance, we have studied the effect of nucleotides on the association of DRAG with the membranes.

R. rubrum cells were grown under nitrogen-fixing conditions with N_2 as the nitrogen source (10) and subjected to switch-off by the addition of 2 mM ammonium chloride 30 min prior to harvest. After being harvested, the cells were resuspended in 50 mM Tris-HCl, pH 7.8, that had been evacuated and flushed with N_2. A crude cell extract was prepared by disintegration of the cells in a Ribi press and removal of cell debris by centrifugation. No reducing agents, such as dithionite or dithiothreitol, were added, in order to maintain redox conditions as close to the in vivo situation as possible. DRAG has been considered to be oxygen labile, but Nielsen and coworkers (6) have shown this inactivation to be due to radical formation when manganese is exposed to dithionite in air. Manganese was earlier shown to stabilize DRAG and therefore was used during the purification of DRAG (15).

Chromatophores were obtained by centrifugation of the crude extract at 100,000 × *g* for 90 min, after which they were pelleted and kept in liquid nitrogen until being used. The activity of DRAG was measured indirectly by the acetylene reduction assay as the activation of inactive dinitrogenase reductase. Two different preparations of nitrogenase were used: preparation I, the supernatant from the 100,000 × *g* centrifugation (extract), and preparation II, a concentrated fraction (DEAE pool) containing a crude mixture of the two nitrogenase proteins, obtained by loading the supernatant on a DEAE-Sepharose ion exchange column (equilibrated with 0.1 M NaCl in 50 mM Tris-HCl [pH 7.8] containing 1 mM dithionite and 2 mM dithiothreitol), washing with 0.15 M NaCl, and eluting nitrogenase with 0.5 M NaCl (in the same buffer). Antibodies (dilution, 1:500) raised against DRAG were used to identify the DRAG protein after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (3) and Western blotting (13). Enhanced chemiluminescence was used for detection (second antibody dilution, 1:5,000). Chromatophore suspensions, used to study conditions for dissociation of DRAG from the membranes, were obtained by suspending 5 g (wet weight) of chromatophores in 10 ml of anaerobic 50 mM Tris-Cl (pH 7.6). One-milliliter aliquots of this chromatophore suspension were transferred to anaerobic vials and incubated for 15 min at room temperature with the additions specified for each experiment, the incubation mixtures were centrifuged (143,000 × *g*, 60 min), and the supernatants obtained were examined for DRAG activity.

As shown in Table 1, incubation with 10 mM MgGDP led to the release of DRAG activity into the supernatant (about 40% of the activity obtained when chromatophores were treated with 0.5 M NaCl). The total recovery of DRAG activity in the 0.5 M NaCl supernatant was about 50% of that in the chromatophore suspension. The activity obtained with the other nucleotides studied, ATP, ADP, and GTP, was no higher than that obtained when only buffer was added. Furthermore, magnesium ions alone had no effect, whereas the addition of only GDP in some experiments led to detectable but much lower release of activity (data not shown), which could be due to the presence of manganese in the buffer or possibly to magnesium ions bound to the chromatophores. If the release of DRAG was due merely to the increase in ionic strength caused by the

* Corresponding author. Mailing address: Department of Biochemistry, Arrhenius Laboratories for Natural Sciences, Stockholm University, S-106 91 Stockholm, Sweden. Phone: 46 8 16 29 32. Fax: 46 8 15 77 94. E-mail: stefan@biokemi.su.se.

TABLE 1. Effect of nucleotides on the release of DRAG from chromatophores[a]

Addition[b]	Nitrogenase activity (nmol of C_2H_4 formed)
50 mM Tris-Cl, 0.5 mM $MnCl_2$	22
0.5 M NaCl (0.5 M)	212
10 mM MgGTP (0.11 M)	20
10 mM MgGDP (0.09 M)	88
10 mM MgATP (0.11 M)	24
10 mM MgADP (0.09 M)	24

[a] The acetylene reduction assay was run for 15 min with 2.3 mg of nitrogenase (extract).

[b] Values in parentheses show the calculated additional ionic strength caused by the additions. The charge used for the calculations was 4− for ATP and GTP and 3− for ADP and GDP.

additions, it would be expected that ADP and GDP would have similar effects and that ATP and GTP would be more efficient, neither of which is the case. There was also no effect by NAD^+ or NADH (data not shown).

Since DRAG activity is measured indirectly as the capability to activate inactive dinitrogenase reductase, the effect of MgGDP on the in vitro assay was investigated. At concentrations higher than 1 mM, which is the highest concentration of GDP that was present during activity measurements due to carryover from the incubation mixture, GDP inhibited the in vitro activity (Table 2). To investigate if the stimulatory effect of GDP was due to interaction with some other protein(s) solubilized from the chromatophores, GDP was added to the supernatant after centrifugation instead of before. An acetylene reduction assay was run for 30 min with 1.2 mg of nitrogenase (DEAE pool), and nitrogenase activity (nanomoles of C_2H_4 formed) was as follows: with no additions, 26 nmol; with 50 mM Tris-Cl (pH 7.8), 43 nmol; with 10 mM MgGDP added to the supernatant after centrifugation, 36 nmol; and with 10 mM MgGDP added before centrifugation, 532 nmol. As can be seen, no DRAG activity was obtained, indicating that the effect of GDP can be attributed to solubilization of DRAG from the chromatophores. Taken together these results suggest that DRAG was released from the membranes upon addition of GDP. To verify that the DRAG protein was indeed released, the supernatants were subjected to SDS-PAGE and DRAG was identified by Western blotting. As shown in Fig. 1, a clear band was detected in the supernatant from GDP-treated chromatophores, although it was not as strong as that in the sample from chromatophores treated with 0.5 M NaCl, the standard procedure for solubilization of DRAG. No DRAG signal was obtained with ATP or ADP. In some experiments a faint band was also detected when GTP was used, which could possibly be due to hydrolysis of GTP or contamination by GDP in the GTP solution. The major slower-migrating band and the other faint

TABLE 2. Effect of MgGDP on nitrogenase in vitro activity assay[a]

GDP in assay (mM)	Nitrogenase activity (nmol of C_2H_4 formed)
0	35
0.1	38
0.5	43
1.0	37.5
2.0	13.9
5.0	2.1

[a] The acetylene reduction assay was run for 15 min with 1.2 mg of nitrogenase (DEAE pool).

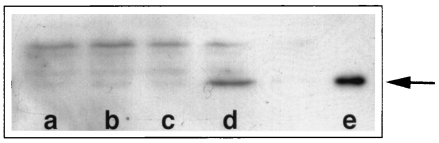

FIG. 1. Identification of DRAG by Western blotting of supernatants from chromatophores incubated with nucleotides. Ten microliters of supernatant was loaded per lane. The arrow indicates the migration of DRAG. Lane a, 10 mM MgATP; lane b, 10 mM MgADP; lane c, 10 mM MgGTP; lane d, 10 mM MgGDP; lane e, 0.5 M NaCl.

bands (Fig. 1, lanes a to d) have not been identified and were not present to the same degree in all experiments. Most likely they are due to nonspecific reactions with the antibodies, which were raised against a synthesized N-terminal peptide of DRAG and not the purified protein. The results shown in Fig. 1 are in good agreement with the activities shown in Table 1.

Our results show for the first time that DRAG can be released by treatment with a low-molecular-weight compound of physiological significance. Furthermore, the fact that neither GTP, ADP, nor ATP has the same effect shows that the release of DRAG is not due to a nonspecific effect, e.g., an increase in ionic strength. It is thus possible that the association of DRAG with the chromatophores is a physiological phenomenon and not an artifact resulting from breaking the cells. It could be hypothesized that the association of DRAG with the membranes is in fact the mode by which DRAG is rendered inactive during switch-off conditions. In this context it is interesting that the specific effect of GDP was not as clear when chromatophores produced in the presence of reducing agents, i.e., dithionite and dithiothreitol, were used or when the solubilization was done under aerobic conditions. This could indicate that the redox status of the membranes is part of the mechanism regulating the association of DRAG. The role of GTP- and GDP-binding proteins in the regulation of metabolic processes in eukaryotic cells is well documented (1); however, a similar possible role for GDP in the switch-off regulation of nitrogenase in phototrophic bacteria remains to be demonstrated.

This work was supported by grants to S.N. from the Swedish Natural Science Research Council.

REFERENCES

1. **Bourne, H. R., D. A. Saunders, and F. McCormick.** 1990. The GTPase superfamily, a conserved switch for diverse cell functions. Nature **348:**125–132.
2. **Burris, R. H.** 1991. Nitrogenases. J. Biol. Chem. **266:**9339–9342.
3. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
4. **Ludden, P. W., and R. H. Burris.** 1976. Activating factor for the iron protein of nitrogenase from *Rhodospirillum rubrum*. Science **194:**424–426.
5. **Ludden, P. W., and G. P. Roberts.** 1989. Regulation of nitrogenase activity by reversible ADP-ribosylation. Curr. Top. Cell. Regul. **30:**23–55.
6. **Nielsen, G., Y. Bao, G. P. Roberts, and P. W. Ludden.** 1994. Purification and characterization of an oxygen-stable form of dinitrogenase reductase activating glycohydrolase from *Rhodospirillum rubrum*. Biochem. J. **302:**801–806.
7. **Nordlund, S., U. Eriksson, and H. Baltscheffsky.** 1977. Necessity of a membrane component for nitrogenase activity in *Rhodospirillum rubrum*. Biochem. Biophys. Acta **462:**187–195.
8. **Norén, A., and S. Nordlund.** 1994. Changes in the NAD(P)H concentration caused by addition of nitrogenase "switch-off" effectors in *Rhodospirillum rubrum* G-9, as measured by fluorescence. FEBS Lett. **356:**43–45.
9. **Norén, A., A. Soliman, and S. Nordlund.** 1997. The role of NAD^+ as a signal during nitrogenase switch-off in *Rhodospirillum rubrum*. Biochem. J. **322:** 829–832.
10. **Ormerod, J. G., K. S. Ormerod, and H. Gest.** 1961. Light-dependent utilization of organic compounds and photoproduction of molecular hydrogen by photosynthetic bacteria; relationships with nitrogen metabolism. Arch. Biochem. Biophys. **94:**449–463.
11. **Pope, M. R., S. A. Murrell, and P. W. Ludden.** 1985. Covalent modification

of the iron protein of nitrogenase from *Rhodospirillum rubrum* by adenosine diphosphoribosylation of a specific arginyl residue. Proc. Natl. Acad. Sci. USA **82:**3173–3177.
12. **Soliman, A., and S. Nordlund.** 1992. Studies on the effect of NAD(H) on nitrogenase activity in *Rhodospirillum rubrum*. Arch. Microbiol. **157:**431–435.
13. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
14. **Zumft, W. G., and F. Castillo.** 1978. Regulatory properties of the nitrogenase from *Rhodopseudomonas palustris*. Arch. Microbiol. **117:**53–60.
15. **Zumft, W. G., and S. Nordlund.** 1981. Stabilization and partial characterization of the activating enzyme for dinitrogenase reductase (Fe-protein) from *Rhodospirillum rubrum*. FEBS Lett. **127:**79–82.