ADP-ribosylation of dinitrogenase reductase from *Clostridium pasteurianum* prevents its inhibition of nitrogenase from *Azotobacter vinelandii*

Scott A. MURRELL, Robert G. LOWERY and Paul W. LUDDEN*

Department of Biochemistry and the Center for the Study of Nitrogen Fixation, College of Agriculture and Life Sciences, University of Wisconsin-Madison, 420 Henry Mall, Madison, WI 53706-1569, U.S.A.

The effect of ADP-ribosylation of dinitrogenase reductase on its binding to dinitrogenase was investigated. Dinitrogenase reductase from *Clostridium pasteurianum* (Cp2) was a substrate for the ADP-ribosyltransferase and the dinitrogenase-reductase-activating glycohydrolase from *Rhodospirillum rubrum*. ADP-ribosylation inactivated Cp2 and prevented its formation of a tight complex with dinitrogenase from *Azotobacter vinelandii* (Av1). The complex between Cp2 and Av1 could not be ADP-ribosylated once it formed.

INTRODUCTION

Dinitrogenase reductase from the purple photosynthetic bacterium *Rhodospirillum rubrum* is regulated by the reversible ADP-ribosylation of a specific arginine residue (Pope *et al.*, 1984; Lowery *et al.*, 1986). An ATP- and M^{2+} (bivalent cation)-dependent glycohydrolase that activates dinitrogenase reductase by removing ADP-ribose has been identified and purified (Ludden & Burris, 1976; Nordlund *et al.*, 1977; Saari *et al.*, 1984) and is referred to as 'dinitrogenase-reductase-activating glycohydrolase' (DRAG; formerly 'AE'). An NAD^+ -dependent, ADP-ribosyltransferase has been identified and is referred to as 'inactivating enzyme' or 'dinitrogenase reductase ADP-ribosyltransferase' (DRAT) (Lowery *et al.*, 1986). The ADP-ribosylated form of dinitrogenase reductase from *Rhodospirillum rubrum* will bind MgATP, can be oxidized and reduced, and exhibits a normal e.p.r. spectrum; however, it is incompetent with respect to electron transfer to dinitrogenase, and the ADP-ribosylated form does not inhibit the unmodified form of dinitrogenase reductase (Ludden *et al.*, 1982). A reasonable hypothesis has been that the ADP-ribosylated form of the enzyme is unable to bind to dinitrogenase.

Although most heterologous crosses of nitrogenase proteins from different organisms result in effective nitrogenases, the dinitrogenase reductase of *Clostridium pasteurianum* (Cp2) and the dinitrogenase of *Azotobacter vinelandii* (Av1) form a tight, catalytically inactive, complex (Emerich & Burris, 1976; Emerich *et al.*, 1978). Cp2 is known to have an arginine residue at the position analogous to the site of modification of the *R. rubrum* enzyme (Tanaka *et al.*, 1977). The effect of ADP-ribosylation of Cp2 on its ability to inhibit *Azotobacter* nitrogenase was examined in order to test the hypothesis that ADP-ribosylation prevents binding between dinitrogenase and dinitrogenase reductase.

MATERIALS AND METHODS

Purification of proteins

Av1 and Av2 were purified as described by Shah & Brill (1973). Cp1 and Cp2 were purified by modifications of the method of Tso *et al.* (1972). N_2 -grown cells (100 g fresh wt.) were disrupted with a bead beater after lysozyme treatment in buffer containing 100 mM-Mops, pH 7.5; operations were carried out in an anaerobic glove box (Vacuum Atmospheres, Hawthorne, MA, U.S.A.) with an N_2 atmosphere containing less than 1 p.p.m. of O_2 . Preparation of enzymes in the glove box permitted chromatography in the presence of low levels of dithionite; the presence of a very active hydrogenase in crude extracts of *C. pasteurianum* makes column chromatography difficult in the presence of dithionite (Erbes & Burris, 1978). The broken cells were centrifuged at 50 000 *g* for 3 h, and the supernatant was applied to a column (1.5 cm $\times$ 10 cm) of DE-52 DEAE-cellulose. The column was washed with 150 mM-NaCl in 100 mM-Mops buffer (this removes hydrogenase), and nitrogenase proteins were then eluted with 450 mM-NaCl in buffer. The nitrogenase-containing fraction was diluted 2-fold with buffer containing 1 mM-dithionite applied to an anaerobic DE-52 column (1.5 cm $\times$ 14 cm) that had been pre-equilibrated with buffer containing 90 mM-NaCl and 1 mM-dithionite. Proteins were eluted with a salt gradient from 100 to 450 mM-NaCl in buffer containing 1 mM-dithionite. Fractions containing Cp2 were concentrated on a third anaerobic DEAE-cellulose column after dilution. The Cp1 fraction was used without dilution or further purification.

Dinitrogenase-reductase-activating glycohydrolase (DRAG) was purified as described by Saari *et al.* (1984). Dinitrogenase reductase ADP-ribosyltransferase (DRAT) was isolated as described by Lowery *et al.* (1986) and purified (Lowery & Ludden, 1988). Synthesis and

Abbreviations used: DRAG, dinitrogenase-reductase-activating glycohydrolase; DRAT, dinitrogenase reductase ADP-ribosyltransferase; PAGE, polyacrylamide-gel electrophoresis; Cp1 and Cp2, Av1 and Av2, dinitrogenases, and dinitrogenase reductases, from *Clostridium pasteurianum* and *Azotobacter vinelandii* respectively.

* To whom correspondence and reprint requests should be sent.

Fig. 1. Time course of Cp2 modification

Cp2 was modified in a reaction mixture that contained 0.2 mM-NAD⁺, 0.3 μ Ci of [³²P]NAD⁺, 5 mM-MgCl₂, 0.4 mM-ADP, 380 μ g of Cp2 and 5 μ l of DRAT (0.031 mg/ml); the total volume was 25 μ l. At various time points, 5 μ l samples were taken and quenched into SDS cocktail buffer. Samples were then analysed on an SDS/polyacrylamide gel. (a) lane 1, *M_r* standards (bovine serum albumin, ovalbumin, carbonic anhydrase and myoglobin); lanes 2–6, time points as indicated on the Fig.; lane 7, a control reaction (C) in which DRAT was omitted. The lower part of the Figure, (b), shows the autoradiogram of the gel shown in the upper part of the Figure, (a).

purification of [³²P]NAD⁺ was performed as described by Lowery *et al.* (1986). SDS/PAGE and autoradiography were performed as described by Lowery *et al.* (1986). Nitrogenase activity was assessed by acetylene reduction (Stewart *et al.*, 1968). Protein concentration was determined by the microbiuret method of Goa (1953), with bovine serum albumin as standard.

ADP-ribosylation reactions

ADP-ribosylations were carried out in microcentrifuge tubes inside stoppered glass serum vials (Lowery *et al.*, 1986). The atmosphere inside the vial was made anaerobic by evacuating and flushing with N₂; a 100 mM-dithionite solution was added to the vial outside the microcentrifuge tubes to trap any traces of oxygen. Dithionite reduces the NAD⁺ and renders it ineffective as a substrate

Table 1. Effect of ADP-ribosylation on Cp2 activity

A 380 μ g portion of Cp2 was incubated in a total volume of 50 μ l containing 2 mM-NAD⁺, 0.8 μ Ci of [³²P]NAD⁺, 5 mM-MgCl₂ and 0.2 mM-ADP, and 0.31 μ g of DRAT was added. After 20 min, 43 μ l of the mixture were transferred to an acetylene-reduction assay mixture (0.5 ml total volume) containing 250 μ g of Cp1, 10 mM-sodium dithionite, an ATP-regenerating system, 25 mM-MgCl₂, 0.5 mM-MnCl₂ and (where added) 5 μ g of DRAG. Acetylene reduction was assayed at 5, 10 and 15 min as indicated in column headings.

Treatment	Time (min)...	Acetylene reduction (nmol/assay)		
		5	10	15
None		62	152	246
DRAT		9.6	30.2	62
DRAT + DRAG		22	101	219

Table 2. Effect of Cp2 and Cp2 ADP-ribose on the activity of *A. vinelandii* nitrogenase

Incubations were carried out as described in the legend to Fig. 1, except that a 100 μ l total volume was used. An 85 μ l portion was transferred to the acetylene-reduction assay mixture (0.5 ml total vol.). Where indicated, 230 μ g of Av1 or 50 μ g of Cp1 was added.

Additions to incubation mixture	Additions to acetylene assay	Acetylene reduced (nmol/5 min assay)
1. None	Av1,Av2	141
2. Cp2	Av1,Av2	26
3. Cp2	DRAG,Av1,Av2	22
4. Cp2,DRAT	Av1,Av2	112
5. Cp2,DRAT	DRAG,Av1,Av2	38
6. DRAT	Av1,Av2	134
7. Cp2	Cp1	237
8. Cp2,DRAT	Cp1	37
9. DRAT	Cp1	0

for DRAT and thus cannot be present at concentrations usually provided for protection of dinitrogenase reductase.

RESULTS AND DISCUSSION

That Cp2 is a substrate for DRAT is shown in Fig. 1. [³²P]NAD⁺-dependent labelling of Cp2 is observed, and the extent of labelling is time-dependent. Modification of Cp2 does not result in differential migration of the subunits on SDS/PAGE in the gel system used here. Cp2 is the smallest of the dinitrogenase reductase proteins described to date and migrates much faster than Rr2 or Av2 on SDS/PAGE. The modification reaction is specific for Cp2, as seen in Fig. 1. Fig. 1(a) shows the protein composition of partially purified Cp2 and Fig. 1(b) the autoradiogram of the gel. Only Cp2 became labelled in the DRAT-dependent reaction shown here. The labelled band was identified as Cp2 on an immunoblot using anti-

Fig. 2. Autoradiogram showing presence or absence of [^{32}P]ADP-ribose on Cp2 used in acetylene assays of Av1/Av2

Protein from samples of incubations 2–5 in Table 2 was precipitated and subjected to SDS/PAGE, and an autoradiogram was prepared from the gel. Lanes 1–4, protein was precipitated immediately after the incubation: lanes 1 and 2 (incubations 2 and 3 respectively), Cp2 incubated in the absence of DRAT; lanes 3 and 4 (incubations 4 and 5 respectively), Cp2 incubated in the presence of DRAT; lanes 5–8, protein was precipitated after Av1/Av2 acetylene assays: lanes 5 and 6 (assays 2 and 3 of Table 2 respectively), Cp2 incubated in absence of DRAT and added to Av1/Av2 assays in the absence (lane 5) and in the presence (lane 6) of DRAG; lanes 7 and 8 (assays 4 and 5 of Table 2 respectively), Cp2 incubated in the presence of DRAT and added to Av1/Av2 assays in the absence (lane 7) and in the presence (lane 8) of DRAG.

Table 3. Effect of Av1 on ADP-ribosylation of Cp2

The reaction mixture contained 0.2 mM-NAD⁺ (total radioactivity 945795 c.p.m./assay), 0.3 mM-ADP and 5 mM-MgCl₂. Where indicated, 0.2 µg of DRAT, 260 µg of Cp1, 190 µg of Cp2, 110 µg of Av1 or 100 µg of Av2 was added. Reactions were allowed to proceed at 30 °C for 50 min, then quenched with acid and the precipitated protein collected by filtration. The washed filters were then subjected to liquid-scintillation counting. Values given are averages for duplicate samples. Variation was less than 5%.

Additions to assay mixture	Acid-precipitated radioactivity (c.p.m./assay)
1. None	262
2. DRAT, Cp2	18235
3. Cp1, Cp2	250
4. DRAT, Cp1, Cp2	14620
5. Av1, Cp2	215
6. DRAT, Av1, Cp2	260
7. DRAT, Av2	10434
8. DRAT, Av1, Av2	14544

(dinitrogenase reductase) as the specific antibody (results not shown).

That DRAT treatment results in loss of Cp2 activity is shown in Table 1. Samples of Cp2 were treated with DRAT in an assay mix such as that described in the legend to Fig. 1. At 20 min, samples of the inactivation mix were taken for acetylene-reduction assays. In the absence of DRAT, Cp2 showed a nearly linear time course of activity (Table 1, row 1). Pretreatment with DRAT resulted in greatly diminished acetylene-reduction activity (Table 1, row 2). Cp2 that had lost activity by DRAT treatment could be re-activated by treatment with DRAG (Table 1, row 3); thus ADP-ribosylated Cp2 is a substrate for DRAG. The time course for acetylene reduction is non-linear, as expected for the activation of Cp2, and the activity approaches that of the unmodified Cp2. Interestingly, the activity of Cp2 ADP-ribose in the absence of DRAG (Table 1, row 2) also increases non-linearly; this non-linear increase above background

activity might be due to a glycohydrolase activity in *C. pasteurianum* or an inherently unstable glycosidic bond between ADP-ribose and Cp2.

The effect of ADP-ribosylation of Cp2 on its ability to inhibit the activity of the *A. vinelandii* nitrogenase proteins is shown in Table 2. Row 1 of Table 2 shows the activity of Av1 plus Av2 in the absence of any Cp2. Row 2 shows the effect of Cp2 on Av1 plus Av2: an 82% inhibition of acetylene reduction is observed. If a sample of this Cp2 is ADP-ribosylated and the same amount of Cp2 protein added, acetylene reduction of Av1 and Av2 activity recovers to 79% of the control (Table 2, row 4). When DRAG is added to the assay mix, to remove ADP-ribose from Cp2, the inhibitory behaviour of Cp2 is recovered (Table 2, row 5). DRAT alone had no effect on the activity of Av1 + Av2 (Table 2, row 6), and DRAG alone had no effect on the inhibitory activity of Cp2 (Table 2, row 3).

Demonstration that Cp2 became modified during the incubation with DRAT and unmodified upon DRAG treatment is shown in Fig. 2, where samples taken from the assays described in Table 2 were analysed by autoradiography of an SDS/polyacrylamide gel.

The effect of Av1 on the modification of Cp2 was also tested. Table 3 shows that the modification of Cp2 is greatly inhibited in the presence of Av1. By contrast, the modification of Av2 was not inhibited by the presence of Av1; in fact, some stimulation was observed in these experiments. Likewise, modification of Cp2 was not significantly inhibited in the presence of Cp1. The presence of ATP did not affect any of the assays (results not shown). These results suggest that the site of modification on Cp2 is buried within the complex between Av1 and Cp2. This is in contrast with the result obtained for ATP binding (Emerich *et al.*, 1978), where neither the affinity nor the stoichiometry of ATP binding to Cp2 was affected by Av1.

The experiments described here provide evidence in support of the hypothesis that ADP-ribosylation of dinitrogenase reductase prevents binding between dinitrogenase reductase and dinitrogenase. It is also noteworthy that *C. pasteurianum* is not known to exhibit modification of dinitrogenase reductase *in vivo*, yet dinitrogenase reductase serves as a substrate for both DRAT and DRAG. Furthermore, the modification of

this heterologous dinitrogenase reductase by DRAG results in the loss of its activity with its homologous dinitrogenase protein.

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