at this time whether these ligands and others bind to both forms of tubulin or only to the dimer.

ACKNOWLEDGMENTS

We thank Dr. Lynwood Yarbrough for his comments and suggestions and Dr. Ken Audus for his help in the fluorescence lifetime measurements.

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ADP-ribosylation of Dinitrogenase Reductase in *Rhodobacter capsulatus*†

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Received December 5, 1988; Revised Manuscript Received March 22, 1989

ABSTRACT: In the photosynthetic bacterium *Rhodobacter capsulatus*, nitrogenase is regulated by a reversible covalent modification of Fe protein or dinitrogenase reductase (Rc2). The linkage of the modifying group to inactive Rc2 was found to be sensitive to alkali and to neutral hydroxylamine. Complete release of the modifying group was achieved by incubation of inactive Rc2 in 0.4 or 1 M hydroxylamine. After hydroxylamine treatment of the Rc2 preparation, the modifying group could be isolated and purified by affinity chromatography and ion-exchange HPLC. The modifying group comigrated with ADP-ribose on both ion-exchange HPLC and thin-layer chromatography. Analyses by ^{31}P NMR spectroscopy and mass spectrometry provided further evidence that the modifying group was ADP-ribose. The NMR spectrum of inactive Rc2 exhibited signals characteristic of ADP-ribose; integration of these signals allowed calculation of a molar ratio ADP-ribose/Rc2 of 0.63. A hexapeptide carrying the ADP-ribose moiety was purified from a subtilisin digest of inactive Rc2. The structure of this peptide, determined by amino acid analysis and sequencing, is Gly-Arg(ADP-ribose)-Gly-Val-Ile-Thr. This structure allows identification of the binding site for ADP-ribose as Arg 101 of the polypeptide chain of Rc2. It is concluded that nitrogenase activity in *R. capsulatus* is regulated by reversible ADP-ribosylation of a specific arginyl residue of dinitrogenase reductase.

For a number of phototrophic bacteria, mostly Rhodospirillaceae, rapid inhibition of nitrogenase activity has been reported to occur upon exposure of whole cells to ammonium ions and other sources of combined nitrogen [see Vignais et al. (1985) and Hallenbeck (1987) for reviews]. The mechanism of this regulation has been investigated at the molecular level in *Rhodospirillum rubrum* (Gotto & Yoch, 1982; Preston & Ludden, 1982; Pope et al., 1985a) and *Rhodobacter cap-*

† This work was supported by a research grant from the Centre National de la Recherche Scientifique.
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0006-2960/89/0428-6524$01.50/0

sulatus (formerly *Rhodopseudomonas capsulata*; Imhoff et al., 1984) (Hallenbeck et al., 1982; Michalski et al., 1983; Jouanneau et al., 1983). In both *Rs. rubrum* and *R. capsulatus*, it was demonstrated that inhibition of nitrogenase activity resulted from the inactivation, by covalent modification, of the Fe protein component of nitrogenase, also called dinitrogenase reductase. In the inactive form, the dinitrogenase reductase carries a modifying group covalently bound to one of its subunits; the modified subunit shows a slower migration upon SDS–polyacrylamide gel electrophoresis, which allows molecular distinction between the active and the inactive Fe protein (Preston & Ludden, 1982; Gotto & Yoch, 1982; Jouanneau et al., 1983). The modifying group was shown to consist of adenine, phosphate, and pentose (Ludden & Burris, 1978; Hallenbeck et al., 1982; Nordlund & Ludden, 1983). In *Rs. rubrum*, the modifying group was identified as an ADP-ribose[1] molecule covalently linked to a specific arginyl residue of the dinitrogenase reductase (Pope et al., 1985a). Furthermore, an enzyme activity catalyzing the ADP-ribosylation of dinitrogenase reductase has recently been purified from *Rs. rubrum* (Lowery et al., 1988). The reverse reaction (activation) is catalyzed by an activating enzyme initially isolated as a membrane-bound factor (Ludden & Burris, 1976; Nordlund et al., 1977); this factor was later purified and characterized as a specific arginine-(ADP-ribose) *N*-glycohydrolase (Saari et al., 1984; Pope et al., 1986).

In the past few years, NH_4^+-dependent regulation of nitrogenase activity has been observed in a number of nonphototrophic nitrogen fixing bacteria, including *Azotobacter* (Laane et al., 1980; Cejudo et al., 1984) and *Rhizobium* species (Kush et al., 1985) as well as in a methanotroph (Yoch et al., 1988) and in a cyanobacterium (Reich et al., 1986). However, no evidence for ADP-ribosylation of the dinitrogenase reductase as observed in *Rs. rubrum* has been reported so far. Mechanisms of nitrogenase inhibition other than covalent modification have also been proposed (Laane et al., 1980). In *Rhodobacter sphaeroides*, a close relative of *R. capsulatus*, the apparent absence of modification of the dinitrogenase reductase upon NH_4^+-induced inhibition of nitrogenase suggested the existence of a regulatory mechanism different from dinitrogenase reductase ADP-ribosylation (Yoch et al., 1988).

In this study, we present consistent sets of chromatographic and spectroscopic data demonstrating that the dinitrogenase reductase from *R. capsulatus* (Rc2) undergoes ADP-ribosylation. The binding site for ADP-ribose was identified as an arginyl residue located in a highly conserved region of the dinitrogenase reductase.

Experimental Procedures

Chemicals and Biochemicals. Adenosine, 5′-AMP, ADP, ADP-ribose, D-ribose-5-phosphate (barium salt), and snake venom phosphodiesterase (from *Crotalus durissus*) were purchased from Sigma. ATP, DTNB, and subtilisin were from Boehringer. TPCK-treated trypsin (trypsin treated with *N*-tosyl-L-phenylalanine chloromethyl ketone) was from Worthington. DEAE-cellulose (DE52) was from Whatman. Sephadex G-25 and Sephadex G-10 were obtained from Pharmacia. Affigel 601 (phenyl boronate resin) was from Bio-Rad. TFA and acetonitrile, both of HPLC grade, were obtained from Fluka and BDH, respectively. Deuterium oxide 99.8% was from the Commissariat â l'Energie Atomique (CEN Saclay, France). Carrier free [^{32}P]orthophosphoric acid, 10 mCi/mL, was purchased from New England Nuclear. [^{14}C]ADP-ribose was prepared from [*adenosine*-^{14}C(U)]-nicotinamide adenine dinucleotide ([^{14}C]NAD), 600 mCi/mmol, from New England Nuclear, by alkaline treatment and subsequent purification by phenyl boronate affinity chromatography (Payne et al., 1985).

Preparation and Purification of Inactive Rc2. Inactive dinitrogenase reductase Rc2 was prepared essentially as described previously (Hallenbeck et al., 1982; Jouanneau et al., 1983). *R. capsulatus*, strain B10, was grown in 10-L batches with 3 mM glutamate as limiting substrate. Cells were supplemented with 15 mM NH_4Cl 30 min before harvest to inactivate nitrogenase. Rc2 was purified from 300-g batches of packed cells as previously described (Jouanneau et al., 1983) except that a continuous gradient of 0.12–0.4 M NaCl on DEAE-cellulose replaced preparative electrophoresis on polyacrylamide gel. At this stage, the Rc2 preparation contained less than 1% contaminating polypeptides as judged from SDS–PAGE. The preparation was further purified by selectively precipitating Rc2 at pH 4.5 in 0.125 M ammonium acetate. ^{32}P-Labeled Rc2 was prepared and purified according to published procedures (Jouanneau et al., 1983) with the modifications mentioned above.

Purification of the Covalently Modified Peptide from Inactive Rc2. Purified Rc2 was filtered aerobically through a 2.5 × 17 cm column of Sephadex G-25 equilibrated in 0.2 M ammonium acetate, pH 8.2. DTNB was added at a concentration 10 times as high as that of Rc2. The protein solution was supplemented with subtilisin (3% by weight) and stirred at 32 °C for 5 h. After 1 h of digestion, the subtilisin concentration was brought up to 6%. The protein digest was centrifuged, yielding a dark pellet and a clear supernatant that was directly applied to a 2-mL phenyl boronate affinity column (Affigel 601). The column was washed with about 8 bed volumes of buffer, pH 8.2, followed by an equal volume of 0.2% trifluoroacetic acid. The acidic fractions showing adsorption at 260 nm were pooled, concentrated by lyophilization, and chromatographed on a 3.9 mm × 15 cm reverse-phase HPLC column (Nova-Pak C_{18}, Waters), as described in the text.

The ^{32}P radioactive material not retained on the boronate affinity column was concentrated to 5.5 mL by lyophilization and desalted on a Sephadex G-25 column (5 × 24 cm) equilibrated in 50 mM NH_4HCO_3. No radioactivity was detected in salt fractions. The pH was adjusted to 8.5, and further digestion was performed with pronase at 34 °C for 5 h. The digest was concentrated to 3.5 mL by lyophilization and applied to a Sephadex G-25 column (2.5 × 17 cm) coupled in line with a Sephadex G-10 column (2.6 × 26 cm). Both columns were equilibrated in 0.2% TFA. Radioactive fractions were pooled and concentrated to 25–35-μL aliquots that were electrophoresed for 3 h at 1500 V on Whatman 3MM paper in 7.5% acetic acid/2.5% formic acid, pH 1.9. One major radioactive spot (about 80% of total radioactivity) migrating 4–5 cm toward the cathode was eluted in 0.2% TFA. This radioactive eluate was subsequently resolved by reverse-phase HPLC on a 8 mm × 10 cm Nova-Pak C_{18} cartridge (Waters) using a 10-min linear gradient of 0–4% acetonitrile in 0.1% TFA. A major radioactive peptide, eluting at 6 min, was purified and analyzed as described in the text.

[1] Abbreviations: ADP-ribose, adenosine 5′-diphosphoribose; DEAE-cellulose, (diethylaminoethyl)cellulose; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; Rc2, *R. capsulatus* dinitrogenase reductase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TLC, thin-layer chromatography.

Thin-Layer Chromatographic and Electrophoretic Analyses. Adenine nucleotides, ribose 5-phosphate, and phosphate were separated by chromatography on 20 × 20 cm thin-layer silica sheets precoated with fluorescent indicator (Merck 5554) under conditions already described (Bronnikov & Zahkarov, 1983). Nucleotides were visualized under UV light. Phosphate and phosphate-containing compounds derived from ^{32}P-labeled Rc2 or peptides were detected by autoradiography. Ribose 5-phosphate was visualized after it was sprayed with a solution of glacial acetic acid/aniline/water (1:1:1 by volume).

Adenine nucleotides, adenine, adenosine, and ribose 5-phosphate were separated by high-voltage electrophoresis on thin-layer cellulose sheets (Merck 5577) in 7.5% acetic acid/2.5% formic acid, pH 1.9. Compounds were detected as described above.

^{31}P NMR Analysis. Phosphorus 31 NMR spectra were obtained in the pulse Fourier transform mode with a Bruker AM400 spectrometer operating at 162 MHz. Samples (2.3 mL) were in 10-mm (o.d.) analytical tubes maintained at 283 K during analysis. D_2O (13%, v/v) provided the signal used to lock and shim the magnetic field. The free induction decays were acquired on 8K with a spectral width of 5200 Hz in the broad-band decoupling mode. They were Fourier transformed on 16K after exponential multiplication. The longitudinal relaxation time of free ADP-ribose in solution, measured with the inversion recovery method, has been taken as an estimation of the actual relaxation time of the ADP-ribose group bound to Rc2 or to the hexapeptide. Repetition times (RT) used in the experiments were chosen in accordance with this assumption. When inorganic phosphate (P_i) was present, RT were increased to allow full recovery of the P_i magnetization. The chemical shifts are referred to the position of 85% phosphoric acid resonance taken as an external standard.

SDS–PAGE. Analytical electrophoresis was performed in 12% polyacrylamide slab gels in the presence of 0.1% (w/v) SDS, as previously described (Laemmli & Favre, 1973).

Amino Acid Analysis and Sequencing. Samples were analyzed on an HPLC gradient system (Waters) according to the manufacturer's specifications. Briefly, the method involved hydrolysis of vacuum-dried samples in 6 N HCl vapor, derivatization of amino acids with phenyl isothiocyanate, and reverse-phase chromatographic separation of derivatized amino acids. For determination of protein concentration of purified Rc2, an internal standard of norleucine was used to take into account losses during preparation of amino acid derivatives. Amino acid sequencing was performed on a Model 470 gas-phase sequencer (Applied Biosystems).

Results

Identification of ADP-ribose Covalently Bound to Inactive Rc2. Previous studies indicated that inactive Rc2 carried a phosphorylated molecule covalently bound to one of its subunits (Hallenbeck et al., 1982; Jouanneau et al., 1983). ^{32}P-Labeled Rc2 was prepared by in vivo labeling with [^{32}P]orthophosphate; it was confirmed that radioactivity was incorporated into the slower moving subunit of Rc2 on SDS–polyacrylamide gels (Jouanneau et al., 1983). The purification procedure included a gradient chromatography on DEAE-cellulose instead of the electrophoresis step on native polyacrylamide gels described earlier (Jouanneau et al., 1983) which we found more convenient to purify large amounts of protein (100–200 mg). The resulting preparation contained minor amounts of contaminating polypeptides (less than 1% on SDS gel) that were easily removed by selective precipitation of Rc2 under mild acidic conditions (see Experimental Procedures). No loss of radioactivity was detectable at this stage, confirming that the phosphate label was entirely linked to inactive Rc2. Although irreversibly inactivated by this acidic treatment, the Rc2 preparation appeared homogeneous on SDS gels and was appropriate for the purposes of this study.

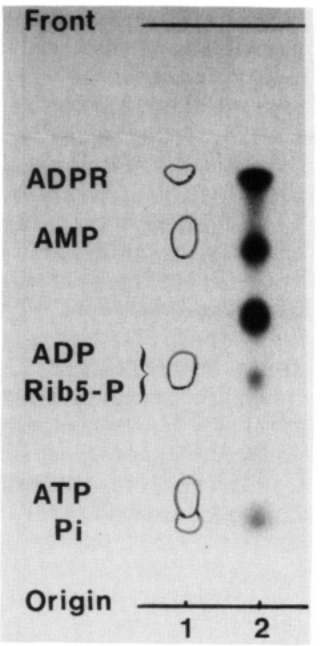

FIGURE 1: Thin-layer chromatographic analysis of the labeled compounds released upon alkaline treatment of inactive ^{32}P-labeled Rc2. Inactive ^{32}P-labeled Rc2 (1.6×10^5 cpm) was precipated in 25% trichloroacetic acid, washed in ethanol, and then incubated in 0.1 M KOH at 37 °C for 3 h. Protein was precipitated with a slight excess of perchloric acid, and the supernatant fluid, containing 35% of the initial radioactivity, was analyzed by TLC and autoradiographed. (Lane 1) 10 nmol each of AMP, ADP, ATP, ADP-ribose (ADPR), and (ribose 5-P) was applied. Pi refers to [^{32}P]orthophosphate run on a separate lane. (Lane 2) 3 μL of supernatant fluid from KOH-treated ^{32}P-labeled Rc2 was applied (2500 cpm).

We attempted to isolate the phosphorylated group by subjecting a preparation of ^{32}P-labeled inactive Rc2 to various treatments. Heat treatment at 60 °C as described for the *Rs. rubrum* Fe protein (Dowling et al., 1982; Pope et al., 1985b) resulted in the precipitation of Rc2 and little release of radioactive material. When ^{32}P-labeled Rc2 was incubated under alkaline conditions (0.1 M KOH), a proportion varying from 30 to 40% of the radioactivity was released from the protein. Analysis of the released products by thin-layer chromatography revealed three major radioactive spots, two of which comigrated with 5′-AMP and ADP-ribose (Figure 1). When incubated under similar alkaline conditions (0.1 M KOH at 37 °C for 3 h), control ADP-ribose underwent 60–70% hydrolysis as judged from HPLC analysis. AMP was the main byproduct detected. These results suggested that ADP-ribose was released from inactive Rc2 but was partially hydrolyzed by alkali.

Extensive cleavage of the phosphorylated group from inactive Rc2 was achieved upon incubation with hydroxylamine at neutral pH (Figure 2). After about 1 h of initial lag time, release of the labeled modifying group followed first-order kinetics with a half-time of about 3 h. Analysis of protein samples by SDS–PAGE at time intervals during incubation showed that the modified, slower moving subunit of Rc2 disappeared at the same rate as the bound radiolabel (Figure 2). A control sample incubated in the absence of hydroxylamine showed no significant release of radioactivity and no disappearance of the modified subunit of Rc2 (Figure 2, C). Hence, the unmodified form of Rc2 was generated upon hydroxylamine treatment. However, the catalytic activity of Rc2

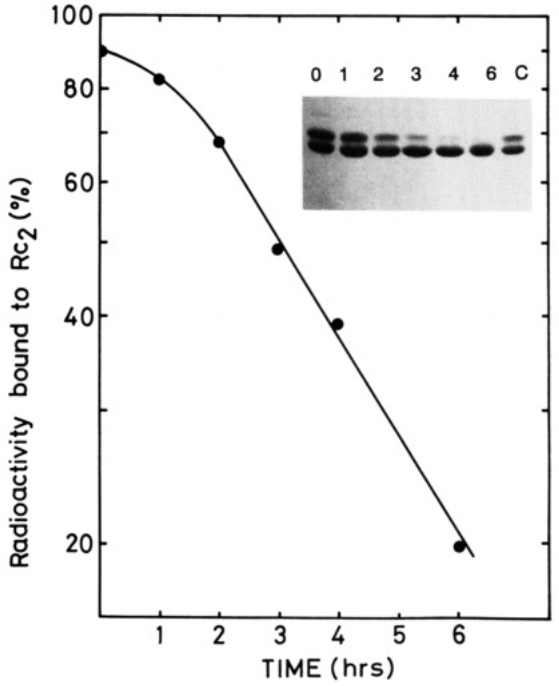

FIGURE 2: Release of the labeled modifying group from inactive ^{32}P-labeled Rc2 upon hydroxylamine treatment. A sample of purified ^{32}P-labeled Rc2 (42 000 cpm) was incubated at 37 °C in 50 mM Tris-HCl, 0.4 M hydroxylamine, and 1 mM dithionite under argon. At time intervals, aliquots were withdrawn and precipitated with 25% trichloroacetic acid. Protein samples were centrifuged, washed in 100% ethanol, and analyzed for bound radioactivity and by SDS–PAGE; 100% radioactivity corresponds to total counts in an unprecipitated aliquot (6500 cpm). Results of SDS–PAGE are shown in the insert; numbers correspond to sampling times (in hours). C refers to a control sample incubated under the same conditions for 6 h but in the absence of hydroxylamine.

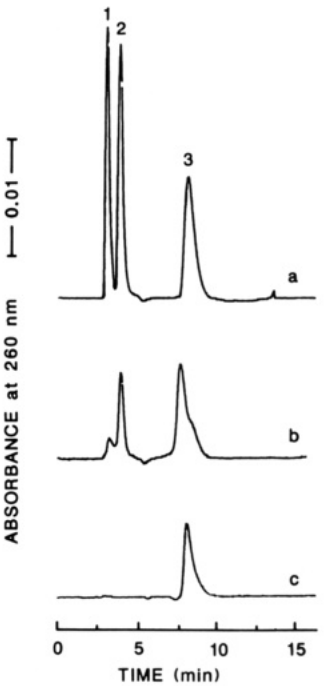

FIGURE 3: Ion-exchange HPLC analysis of the compounds released upon hydroxylamine treatment of inactive Rc2. Analysis was performed on a 8 × 100 mm SAX anion-exchange cartridge (Waters) protected by a 3.2 × 15 mm RP8 guard cartridge (Brownlee). Chromatography was run isocratically at 1 mL/min with 0.1 M KH_2PO_4 adjusted to pH 2.6 with HCl. (a) Separation of reference adenosine (1), AMP (2), and ADP-ribose (3), 1.0 nmol each. (B) Soluble extract from hydroxylamine-treated Rc2. (c) Modifying group released from hydroxylamine treated Rc2 as purified by phenyl boronate chromatography and anion-exchange HPLC; conditions were as outlined above.

was not recovered because of its rapid and irreversible denaturation in 0.4 M hydroxylamine. Denaturation that occurred during the first hour of incubation might actually favor the cleavage of the bound radiolabel since the rate of ^{32}P release increased during this period of time (Figure 2). Accordingly, when Rc2 was denatured by acidic precipitation prior to incubation with hydroxylamine, release of the radiolabel followed first-order kinetics (data not shown). Selective precipitation of Rc2 at pH 4.5 (see Experimental Procedures) also allowed removal of minor contaminants including a nucleotidase. Omitting this precipitation step before hydroxylamine treatment caused the nucleotidase activity to hydrolyze the modifying group after its release from Rc2. Two of the degradation products of the modifying group by the nucleotidase were identified as adenosine and phosphate (not shown).

To isolate the modifying group, the precipitated Rc2 was resuspended in 5% trichloroacetic acid and then dissolved in 0.1 M KOH, and immediately thereafter the solution was adjusted to pH 8.5 by adding neutral hydroxylamine at 1 M final concentration. After incubation for 5 h at 37 °C, 90–95% of the radiolabel was released from ^{32}P-labeled Rc2. Proteins were eliminated by centrifugation, and the soluble fraction was purified on a phenyl boronate affinity column. The radioactive fractions eluted from the column with trifluoroacetic acid also displayed adsorption at 260 nm. The modifying group contained in these fractions was further purified by anion-exchange HPLC using the conditions outlined in the legend to Figure 3. HPLC analysis of the purified product revealed a single peak with a retention time identical with that of authentic ADP-ribose (Figure 3c). Moreover, analysis of the soluble extract obtained immediately after hydroxylamine treatment of Rc2 showed a major peak eluting at 7 min, 40 s and representing approximately 70% of the total absorption at 260 nm (Figure 3b). The retention time of this peak was about 30 s shorter than that of ADP-ribose and was identical with that of the hydroxamate derivative of ADP-ribose, a derivative formed upon incubation of ADP-ribose with hydroxylamine (Moss et al., 1983). After purification by phenyl boronate affinity chromatography and ion-exchange HPLC, the retention time of the modifying group derivative became 30 s longer and eventually matched that of free ADP-ribose (Figure 3c). No appreciable loss of radioactivity was noticed during this conversion; under similar conditions, ADP-ribose hydroxamate was also converted to free ADP-ribose (not shown).

The purified modifying group was analyzed by thin-layer chromatography (Figure 4). A UV-absorbing spot comigrating with ADP-ribose was the major detected compound. A small amount of material corresponding to AMP was also observed that might come from some decomposition of the modifying group during the freeze-drying step that preceded the analysis.

The purified modifying group was also subjected to ^{31}P NMR spectroscopy. The expanded ^{31}P NMR spectrum is given in Figure 5. In spite of appreciable base-line noise, a complex set of signals was obtained, characterized by a main quadruplet with chemical shifts at −11.06, −11.19, −11.38, and −11.50 ppm. This pattern was similar to that obtained for authentic ADP-ribose with respect to the positions of the resonance signals as well as their relative intensities.

The modifying group was finally analyzed by mass spectrometry after Sephadex G-10 filtration and vacuum drying over sodium hydroxide pellets. The negative-ion fast atom

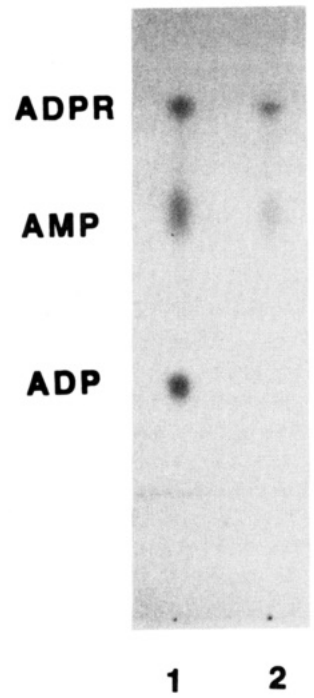

FIGURE 4: Thin-layer chromatographic analysis of purified modifying group. (Lane 1) 5 nmol each of AMP, ADP, and ADP-ribose was applied. (Lane 2) 5 nmol of purified modifying group was applied. Spots were revealed by their absorption under UV light.

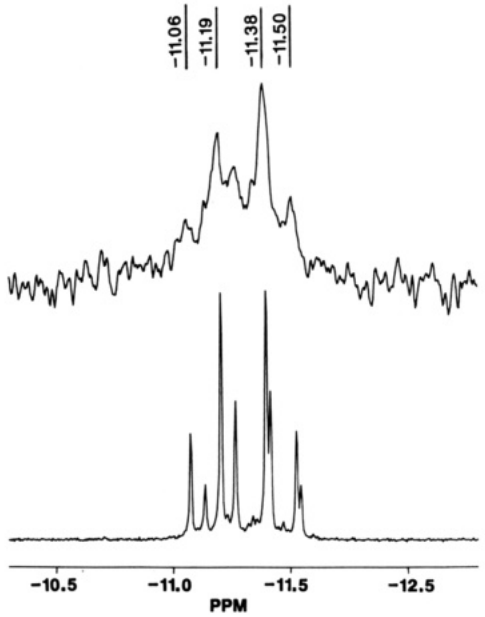

FIGURE 5: ^{31}P NMR of the modifying group purified from inactive Rc2. (Upper trace) The spectrum of the purified modifying group (50 μM) is the result of 22 300 scans acquired with a pulse angle of 60° and a repetition time of 4 s; an exponential multiplication of 2 Hz was used. (Lower trace) The spectrum of reference ADP-ribose (10 mM) was obtained by direct Fourier transformation of 256 scans.

bombardment spectrum exhibited a characteristic signal at m/z 558 corresponding to (ADP-ribose − H)$^-$. Peaks at m/z 309, 346, and 426 corresponding to the main fragmentation products of ADP-ribose, namely (ribose-PP_i − H)$^-$, (AMP − H)$^-$, and (ADP − H)$^-$, respectively, were also observed (data not shown).

The presence of the modifying group bound to native Rc2 could be detected by direct ^{31}P NMR analysis of the whole purified dinitrogenase reductase. The spectrum of purified Rc2 recorded at 162 MHz is presented in Figure 6. Two sets of signals were observed with chemical shifts around +4 and

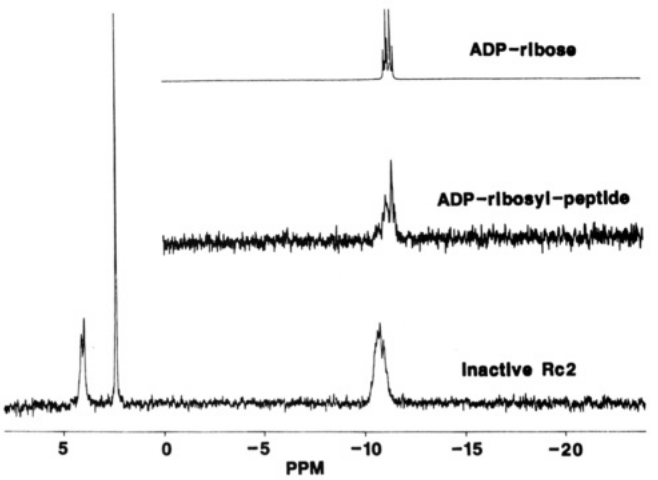

FIGURE 6: ^{31}P NMR spectrum of inactive Rc2, the ADP-ribosylated hexapeptide, and ADP-ribose. The sample of inactive Rc2 contained 40.8 mg/mL protein (0.63 mM) on the basis of amino acid analysis. Orthophosphate (0.5 mM) was added as an internal standard (signal at 2.35 ppm) for signal intensity measurement. The sample was made 5 mM with EDTA and was maintained under anaerobic conditions (argon atmosphere; 2 mM dithionite) during measurement. The spectrum was obtained from 3600 scans acquired with a pulse angle of 90° and a repetition time of 10 s; an exponential multiplication of 1 Hz was used. The ADP-ribosylated peptide was 50 μM on the basis of the absorption of adenine measured at 260 nm. The spectrum is the result of 36 800 scans acquired with a pulse angle of 60° and a repetition time of 4 s; an exponential multiplication of 2 Hz was used. The reference spectrum of ADP-ribose (10 mM) was obtained as in Figure 5.

−10.8 ppm, indicative of the presence of phosphomonoester and phosphodiester linkages, respectively. Orthophosphate was added to the preparation as an internal standard (signal at 2.35 ppm) to estimate the concentration of phosphorus corresponding to each set of signals. Values of 0.89 and 1.26 mol of phosphorus/mol of Rc2 (dimer) were calculated for the +4 and −10.8 ppm signals, respectively. The broad signal at −10.8 ppm appeared similar to that of ADP-ribose, although slightly shifted downfield by about +0.4 ppm compared to the reference. The spectrum presented in Figure 6 was reproducible with respect to the chemical shifts and the ratio of the signals at 4 and −10.8 ppm for another preparation of inactive Rc2 (data not shown).

Localization of the Binding Site for ADP-ribose on Inactive Rc2. Identification of the target amino acid involved in Rc2 ADP-ribosylation required the isolation of an ADP-ribosylated peptide. For this purpose, we subjected a preparation of ^{32}P-labeled Rc2 to proteolytic digestion and chromatographed the peptide mixture on a phenyl boronate affinity column. Best results, in terms of recovered radioactivity, were obtained by using duration of proteolysis of 5 h with 6% subtilisin (by weight) as described under Experimental Procedures. Under these conditions, 2 μmol of a mixture of cold Rc2 and ^{32}P-labeled Rc2 was subjected to subtilisin digestion, followed by purification of the digest on a phenyl boronate column. Fractions retained on the column contained approximatively 25% of the radioactivity and 0.45 μmol of adenine-containing compounds on the basis of absorption at 260 nm. These fractions were pooled and analyzed by reverse-phase HPLC on a 4.6 × 150 mm Nova-Pak C_{18} column (Waters) developed with a 15-min linear gradient of 0–12% acetonitrile in 0.1% TFA. UV absorption recording at 260 nm revealed a major nonradioactive peak eluting at 10 min and a minor peak associated with radioactivity eluting at 15 min. The major peak was identified as adenosine by coelution with an authentic standard under the same HPLC conditions as well as co-

electrophoresis on thin-layer cellulose at pH 1.9. The second peak was purified on a shallower gradient of acetonitrile on the same HPLC column and analyzed for its amino acid composition. The purified product was identified as a hexapeptide showing the following amino acid composition: Gly_2, Arg, Thr, Val, Ile. The sequence of this peptide was determined by Edman degradation and is NH_2-Gly-X-Gly-Val-Ile-Thr-COOH. From the amino acid composition given above, it can be deduced that X represents a modified arginyl residue.

The ^{31}P NMR spectrum of the modified hexapeptide is presented in Figure 6. A complex set of signals with two major peaks at −11.1 and −11.4 ppm was obtained that closely matched the spectrum characteristic of authentic ADP-ribose. Chemical treatment of the modified peptide with hydroxylamine as described in the preceding section, followed by thin-layer chromatography and autoradiography, also confirmed that ADP-ribose was covalently bound to the hexapeptide (data not shown).

As mentioned above, some of the radioactivity was not retained on the phenyl boronate affinity column; presumably, the unbound radioactive material corresponded to peptides carrying products of degradation of the ADP-ribosyl residue. The possibility was also considered that radioactivity might be associated with a molecule bound at another site on the Rc2 polypeptide. The ^{32}P-labeled peptides not retained on phenyl boronate were purified by a combination of gel filtration on Sephadex G-25 and G-10 columns, paper electrophoresis, and reverse-phase HPLC, as detailed under Experimental Procedures. Most of the radioactivity was associated with a single peptide, the amino acid composition of which was identical with that of the ADP-ribosylated hexapeptide. The peptide displayed no absorption at 260 nm and released its radioactive label upon hydroxylamine treatment. The released phosphorylated molecule was analyzed by thin-layer chromatography and autoradiography as in Figure 1. A single spot was detected at an R_f intermediate between those of AMP and ADP. The data indicate that this peptide carried a breakdown product of ADP-ribose.

Discussion

Previous studies had established that *R. capsulatus* is endowed with a regulatory system that can reversibly inactivate nitrogenase by covalent modification of component 2 of the enzyme (Rc2) (Hallenbeck et al., 1982; Jouanneau et al., 1983). We have now provided evidence that Rc2 is covalently modified by ADP-ribosylation. This conclusion is based on the identification of ADP-ribose, as released from inactive Rc2 upon hydroxylamine treatment, by ^{31}P NMR spectroscopy and mass spectrometry as well as chromatographic techniques. During incubation with hydroxylamine, a clear correlation was observed between the loss of ^{32}P label from Rc2, conversion of Rc2 to the unmodified form [single subunit on SDS–PAGE (Figure 2)], and accumulation of a radioactive UV-absorbing compound (Figure 3). That compound, identified as ADP-ribose hydroxamate, represented 70% of the released products (on the basis of UV absorption and HPLC analysis). The release of ADP-ribose from Rc2 by hydroxylamine followed first-order kinetics with a half-time of about 3 h (Figure 2); this is comparable to the breakdown kinetics of the ADP-ribose conjugates of histones and agmatine in neutral hydroxylamine (Moss et al., 1983). The two later conjugates are known to contain N-glycosydic linkages between ADP-ribose and the guanidino group of arginine or agmatine. The relative instability of this linkage in hydroxylamine was used as a criterion to distinguish it from the hydroxylamine stable linkages, ADP-ribose–diphthamide and ADP-ribose–cysteine (Payne et al., 1985). Hence, the ADP-ribose–Rc2 linkage exhibited stability characteristics of an ADP-ribose–arginine conjugate, which is in accordance with the identification of an arginyl residue as the binding site for ADP-ribose on Rc2. In the course of this study, a 5′-nucleotidase was discovered as a contaminant in the Rc2 preparation, whose activity hindered detection of intact ADP-ribose in early experiments. The nucleotidase hydrolyzed free-ADP-ribose to adenosine, phosphate, and ribose 5-phosphate; on the other hand, it had no apparent effect on ADP-ribosylated Rc2. This rules out any functional participation of that enzyme in the regulation of nitrogenase activity.

An ADP-ribosylated hexapeptide has been isolated from inactive Rc2 and its structure determined by amino acid analysis and sequencing. Its sequence, Gly-(ADP-ribose-Arg)-Gly-Val-Ile-Thr, corresponds exactly to the sequence of amino acid residues 100–105 of Rc2, the primary structure of which has been deduced from the nucleotide sequence (Schumann et al., 1986). An identical peptide has been isolated from the inactive dinitrogenase reductase of *Rs. rubrum* and identified as the ADP-ribose binding region (Pope et al., 1985a). This region is highly conserved among Fe proteins of other nitrogen-fixing bacteria (Eady, 1986). The arginyl residue identified as the ADP-ribose binding site is separated by only two amino acids from a cysteine considered as one probable ligand of the iron–sulfur cluster (Hausinger & Howard, 1983). A further indication of the proximity of the FeS center is given by the ^{31}P NMR analysis of inactive Rc2; the observed slight shift by 0.4 ppm downfield of the ^{31}P NMR resonance of ADP-ribose linked to Rc2 compared to that of free ADP-ribose (Figure 6) can be viewed as an interaction between the phosphorus nucleus and the paramagnetic iron–sulfur cluster. Accordingly, no resonance shift was noticeable in the ^{31}P NMR spectrum of the ADP-ribosylated hexapeptide.

The intensity of the ^{31}P resonance signals in the NMR spectrum of inactive Rc2 can be used to estimate the ratio of bound P-containing molecule per Rc2. From the data presented in Figure 6, a ratio of 0.63 mol of ADP-ribose/mol of Rc2 can be calculated. Besides, the split signal at approximately 4 ppm indicates that at least one phosphomonoester was also present in the Rc2 preparation. A likely explanation for the presence of phosphomonoesters bound to Rc2 is that they originate from the breakdown of the ADP-ribose moiety. As judged from the complete release of radioactivity from hydroxylamine-treated ^{32}P-labeled Rc2, the putative phosphomonoester–Rc2 conjugate appears equally as sensitive to hydroxylamine as the ADP-ribose conjugate. Furthermore, the kinetics of ^{32}P release shows a single slope, indicating that the phosphomonoester(s) and ADP-ribose were probably bound to Rc2 through the same kind of linkage. On the other hand, a labeled peptide with no absorption at 260 nm was also isolated from ^{32}P-labeled Rc2. This peptide was identical with the ADP-ribosylated hexapeptide with respect to the amino acid content and carried a labeled compound bound through a hydroxylamine-sensitive linkage. These observations indicate that the labeled peptide carried a degradation product of ADP-ribose and suggest that Rc2 was initially ADP-ribosylated at a single site. Attempts to identify the radioactive compound bound on this peptide or the phosphomonester linked to Rc2 and detected by ^{31}P NMR were precluded by the instability of these products. Assuming that the phosphomonoester detected on Rc2 by ^{31}P NMR was a breakdown product of ADP-ribose, the question arises as to whether the partial degradation of the bound ADP-ribosyl group occurred

during the purification of Rc2 or in intact cells. In the latter case, ADP-ribose degradation would be catalyzed by an endogenous enzyme. The only known enzyme capable of attacking the ADP-ribose–dinitrogenase reductase conjugate is the activating enzyme isolated from *Rs. rubrum*, which is a specific *N*-glycohydrolase that removes the entire ADP-ribosyl group (Saari et al., 1984; Pope et al., 1986). However, removal of only part of the ADP-ribosyl group (at least the adenine ring) was observed when *Rs. rubrum* dinitrogenase reductase was incubated with a crude preparation of activating enzyme (Ludden & Burris, 1979). Hence, in addition to the *N*-glycohydrolase, there may exist, in *Rs. rubrum* and *R. capsulatus*, endogenous enzymes that remove ADP-ribose by sequential degradation as observed in other regulatory systems (Smith et al., 1985).

ACKNOWLEDGMENTS

We thank Dr. M. H. Bruschi (Laboratoire de Chimie Bactérienne, CNRS, Marseille) for determining the sequence of the ADP-ribosylated peptide, Dr. J. Ulrich (Service d'Etudes Analytiques, CEN Grenoble) for mass spectrometric analyses, Dr. J. C. Willison for critical reading of the manuscript, and J. Boyer for secretarial assistance.

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