Purification and Properties of Dinitrogenase Reductase ADP-Ribosyltransferase from the Photosynthetic Bacterium *Rhodospirillum rubrum*

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The enzyme that catalyzes the ADP-ribosylation and concomitant inactivation of dinitrogenase reductase in *Rhodospirillum rubrum* has been purified greater than 19,000-fold to near homogeneity. We propose dinitrogenase reductase ADP-ribosyltransferase (DRAT) as the working name for the enzyme. DRAT activity is stabilized by NaCl and ADP. The enzyme is a monomer with a molecular mass of 30 kDa and is a different polypeptide than dinitrogenase reductase activating glycohydrolase. NAD (K_m = 2 mM), etheno-NAD, nicotinamide hypoxanthine dinucleotide, and nicotinamide guanine dinucleotide will serve as donor molecules in DRAT-catalyzed ADP-ribosylation reaction, and dinitrogenase reductases from *R. rubrum*, *Azotobacter vinelandii*, *Klebsiella pneumoniae*, and *Clostridium pasteurianum* will serve as acceptors. No other proteins or small molecules, including water, have been found to be effective as acceptors. Nicotinamide is released stoichiometrically with formation of the ADP-ribosylated product. DRAT is inhibited by NaCl and has maximal activity at a pH of 7.0.

Biological nitrogen fixation is catalyzed by the two oxygen-labile proteins dinitrogenase and dinitrogenase reductase, which comprise the nitrogenase enzyme complex (1). Electron transfer from dinitrogen reductase to dinitrogenase is coupled to ATP hydrolysis; a theoretical minimum of 16 ATP molecules is consumed per N_2 reduced. In the photosynthetic bacterium *Rhodospirillum rubrum*, nitrogenase activity is regulated in response to various external agents by reversible ADP-ribosylation of dinitrogenase reductase (2-4). Inactivation has been correlated with ADP-ribosylation of arginine 101 on a single subunit of the dinitrogenase reductase homodimer (4). Activation via glycohydrolysis of the ADP-ribosyl-protein linkage is catalyzed by dinitrogenase reductase-activating glycohydrolase (DRAG)² (5, 6). DRAG has been purified greater than R. rubrum. The Escherichia coli phage T4 encodes two transferases, the mod and alt gene products, which both catalyze the ADP-ribosylation of DNA polymerase β-subunit and other cellular proteins (18, 19). The *E. coli* phage N4 carries an enzymatic activity which transfers ADP-ribose from NAD to several host proteins (20). A host-encoded ADP-ribosyltransferase with a number of unidentified cellular targets has also been demonstrated in *E. coli* (21). In all of these cases, the physiological role for the ADP-ribosylation reaction remains the subject of speculation. Thus far, the nitrogenase regulatory system in *R. rubrum* provides the only well-defined example of endogenous enzyme regulation by reversible ADP-ribosylation.

One of our goals is to reconstitute the complete nitrogenase regulatory system in vitro, with purified components, to study how the steady-state level of ADP-ribosylated dinitrogenase reductase is controlled. Toward this end, we have purified DRAT to near homogeneity, examined its substrate specificities for donor and acceptor molecules, and determined some physical properties and kinetic parameters for the enzyme.

EXPERIMENTAL PROCEDURES

*Growth of Bacteria—R. rubrum ATCC 11170 was grown phototrophically at 30 °C, in 100-liter batches. Growth medium was modified Ormerod's medium (22) in which L-glutamate was replaced with 2 g of ammonium chloride per liter of culture. The cells were harvested at an A_600 between 1.5 and 2.0 by tangential flow filtration as previously described (23) and stored at -80 °C.*

*Preparation of Nitrogenase Components—Growth of bacteria and purification of *R. rubrum* active (unmodified) dinitrogenase reductase and dinitrogenase were as previously described (2, 9). Dinitrogenase reductases from *Azotobacter vinelandii* and *Klebsiella pneumoniae*...*
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were also purified using modifications of previously published procedures (9, 24).

Purification of DRAT—The purification was performed at 5 °C except for the final step (Mono Q), which was done at room temperature. All procedures were carried out in the presence of 1 mM dithiothreitol and 1 mM ADP, unless otherwise indicated. One kg of R. rubrum cell paste was thawed in 1 liter of 100 mM MOPS, pH 8.0, containing dithiothreitol and ADP as described above, and 50 μM EDTA. The resuspended cells were incubated with 300 mg of lysozyme and 60 mg each of DNase and RNase for 1 h prior to disruption with a bead-beater (Biospec Products, Bartlesville, OK). The broken cell preparation was centrifuged at 4,400 × g for 5 min to remove polyhydroxybutyrate granules, and then the supernatant was centrifuged at 50,000 × g for 7 h to remove chromatophores.

The high-speed supernatant (850 ml) was applied to a 5.0 × 20-cm DE52-celulose column which was equilibrated with 100 mM MOPS, pH 7.0, containing 50 μM EDTA and 50 mM NaCl. The column was washed with 700 ml of the equilibration buffer and 250 ml of the same buffer with the NaCl increased to 100 mM. The DRAT activity was then eluted with a linear gradient of NaCl (100–300 mM, 2 liters total) in 100 mM MOPS, pH 7.0, containing 50 μM EDTA. Fractions containing DRAT activity (near the middle of the gradient) were pooled and applied to a 1.5 × 50-cm column of Cibacron Blue F3GA-cross-linked agarose which was equilibrated with 250 mM NaCl in 100 mM MOPS, pH 7.0. The column was washed with 150 ml of 500 mM NaCl in 100 mM MOPS, pH 7.0, and then DRAT activity was eluted with 50 ml of 10 mM ADP and 2.5 mM NaCl in 100 mM MOPS, pH 7.0. The Cibacron Blue column eluate was concentrated to 10-fold using an immersible CX-30 ultrafiltration unit (Millipore, Bedford, MA). The concentrated enzyme was then exchanged onto a 2.5 × 82-cm Sephacryl S-200 HR column which was equilibrated with 250 mM NaCl in 100 mM MOPS, pH 7.0. Fractions of 5.0 ml were collected and assayed for DRAT activity. The S-200 fraction with the highest activity was loaded onto a 2.5 × 82-cm Sephacryl S-200 HR column which was equilibrated with 100 mM NaCl in 100 mM MOPS, pH 7.0. Fractions of 5.0 ml were collected and assayed for DRAT activity. The S-200 fraction with the highest activity was then diluted 2-fold with 100 mM MOPS, pH 7.0, and chromatographed on a 0.5 × 8.0-cm Mono Q column (Pharmacia LKB Biotechnology Inc.) which was equilibrated with 100 mM MOPS, pH 7.0. After loading, the column was washed with 2 ml of 30 mM sodium phosphate in 100 mM MOPS, pH 7.0. A linear sodium phosphate gradient (20 ml, 30-300 mM) was then run at 1 ml min" for 7 h to remove chromatophores.'

The results of a typical purification of DRAT, starting from 1 kg of cell paste, are shown in Table I. Assuming that DRAT represents approximately 40% of the protein in Mono Q fractions (see Fig. 1A), the yield from 22 g of soluble protein was 4 μg of DRAT. Thus DRAT constitutes approximately 0.002% of soluble cell protein. If additional S-200 fractions of lower specific activity are also chromatographed on the Mono Q column, the total activity yield is increased about 5-fold. The yield from the Mono Q step is somewhat unpredictable; it ranges from 15 to 50% recovery of the DRAT activity in S-200 fractions. However, this step is necessary because it removes several contaminants (see Fig. 1A, lanes 1 and 2) and results in a 3–5-fold increase in DRAT specific activity.

Stability—One of the major problems encountered during the purification of DRAT was its extremely low stability in both crude extracts and after partial purification. The initial specific activities in extracts of cells grown on ammonia ranged from 0.003 to 0.007 nmol min" mg" and from 0.007 to 0.009 nmol min" mg" in extracts from glutamate-grown cells. However, loss of DRAT activity in extracts from glutamate-grown cells. However, loss of DRAT activity in extracts from glutamate-grown cells.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Conc.</th>
<th>Protein</th>
<th>Total Activity</th>
<th>Specific Activity</th>
<th>Yield</th>
<th>Purification</th>
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<td>mg/ml</td>
<td>mg</td>
<td>%</td>
<td>units/mg</td>
<td>Total units</td>
<td></td>
<td>-fold</td>
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<td>22,440</td>
<td>0.004</td>
<td>90</td>
<td>100</td>
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<td>840</td>
<td>0.096</td>
<td>81</td>
<td>90</td>
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<td>5.6</td>
<td>36</td>
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<td>45</td>
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<td>0.010</td>
<td>77.2</td>
<td>0.77</td>
<td>0.9</td>
<td>19,300</td>
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The [α-32P]NAD was prepared enzymatically from [α-32P]ATP and nicotinamide mononucleotide and purified using a combination of reverse-phase HPLC and phenyl boronate affinity chromatography. Dinitrogenase reductase from A. vinelandii was used as substrate in DRAT assays during the course of the purification. For other experiments the source of dinitrogenase reductase is indicated in the figure legends. One unit of DRAT activity is defined as 1 nmol of ADP-ribosyltransferase to dinitrogenase reductase min" mg".
mate-grown cells was twice as fast as the rate of decay in extracts from cells grown on ammonia (t1/2 = 10 and 18 h, respectively). We found that, even after elution from the DE52 column, DRAT activity was significantly more stable if ammonia-grown cells were used as a source of the enzyme rather than glutamate-grown cells. The rate of activity loss was unaffected by protease inhibitors. Purification of DRAT from glutamate-grown cells using the protocol described here results in a final yield of less than 0.2%. Thus ammonia-grown cells were used as a source of DRAT despite the lower initial specific activity.

DRAT activity in desalted DE52 column fractions from ammonia-grown cells decreased by 90% after 18 h of storage on ice (data not shown). Addition of NaCl (>200 mM) resulted in a 2-4-fold increase in stability and addition of 1 mM ADP plus NaCl almost completely prevented loss of activity (data not shown). Protease inhibitors had no effect on DRAT stability. After the Cibacron Blue step, DRAT stability in the absence of ADP was greatly increased. However, even after the final purification step, DRAT stability to freeze-thaw treatment is affected by ADP. One freeze-thaw cycle results in approximately 50% loss of activity in the absence of ADP, but only a 15% loss when 1 mM ADP is present. ADP also affects DRAT elution from the Mono Q column, increasing its separation from the low molecular weight impurity (see Fig. 1). We are currently exploring the possibility that ADP binds to DRAT. The effect of NaCl on the stability of the purified enzyme cannot be accurately assessed because all desalting procedures tried with Mono Q fractions have caused almost complete, irreversible loss of activity.

Molecular Properties—Lane 2 of Fig. 1A shows the SDS-polyacrylamide gel profile of the DRAT activity peak after elution from the Mono Q column. The 29-kDa band is believed to be DRAT for the following reasons. 1) The 29-kDa polypeptide is the only one which is present in all fractions which contain DRAT activity. 2) DRAT activity elutes from a gel filtration column with a Kav of 0.41, which indicates a relative molecular mass of 30,900 (Fig. 2), very close to the Mr of 29,000 estimated by SDS-polyacrylamide gel electrophoresis (Rf = 0.43, Fig. 2). 3) When equal amounts of protein from DRAT fractions of different specific activities are electrophoresed on an SDS-polyacrylamide gel and then scanned with a laser densitometer, the staining intensity of the 29-kDa band is closely correlated with DRAT specific activity (Fig. 3). The coincidence of the 29-kDa band and DRAT activity can be visually assessed in Fig. 1B. SDS-polyacrylamide gel profiles of successive Mono Q fractions are shown and the amount of DRAT activity eluted in each fraction is given in the figure legend. This column profile was chosen because it clearly shows the lack of coincidence of the major low molecular weight impurity with DRAT activity; the fraction shown in Fig. 1A is more typical of our highly purified DRAT fractions.

In a number of covalent regulatory systems, the modification and demodification reactions are catalyzed by a single, bifunctional enzyme (33-36). We previously presented evidence which suggested that DRAG and DRAT are separate polypeptides (9). In Fig. 1, lanes 2 and 4, it can be seen that...
DRAT and DRAG migrate similarly, but not identically on SDS-polyacrylamide gels and, in lanes 5 and 6, that there is no cross-reactivity of DRAT with polyclonal antiserum raised against purified DRAG. Thus we can state definitively that DRAG and DRAT are distinct polypeptides.

Substrate Specificity—DRAT is extremely specific with respect to acceptor substrate. The only acceptor which we have been able to identify is native dinitrogenase reductase. DRAT catalyzes the ADP-ribosylation of dinitrogenase reductases from a number of other species of bacteria besides *R. rubrum* including *A. vinelandii, K. pneumoniae* (Table II), and *Clostridium pasteurianum.* This is not surprising since there is extensive structural and functional homology between the nitrogenase proteins from different species (37, 38). However, using DRAT we could not detect ADP-ribosylation of any other protein commonly used as substrate for other arginine-specific ADP-ribosyltransferases (Table II). Nor could we detect any transfer of ADP-ribose to arginine, arginine derivatives, or water using DRAT (Table II). More surprisingly, the hexapeptide Gly-Arg-Gly-Val-Ile-Thr, which was generated by subtilisin digestion of *R. rubrum* dinitrogenase reductase, and which contains the DRAT target amino acid Arg-101, was not a substrate for DRAT (Table II). The ADP-ribosylated form of this hexapeptide is a good substrate for DRAG (8). In the experiments using arginine and arginine derivatives and in the NAD glycohydrolase assay the sensitivities were such that we would have detected less than 0.3% of the DRAT specificity observed with dinitrogenase reductase as the substrate. Cholera toxin or ADP-ribosyltransferase from turkey erythrocytes was used as a positive control in the experiments with alternative acceptors.

DRAT will use several pyridine nucleotides besides NAD as donor molecules (Table III). Suitability as a donor was tested by preincubation of the NAD analogs with DRAT and dinitrogenase reductase and subsequent assay of dinitrogenase reductase activity. The enzyme exhibits higher specificity for the nicotinamide portion of the donor molecule than for the purine portion. Of the analogs tested, those in which the adenine portion of NAD is replaced with guanine, hypoxanthine, or 1-N6-ethenoadenine are effective donors, but those in which the nicotinamide portion is altered such as the 3-aminopyridine, acetoxypridine, or nicotinic acid derivatives will not donate ADP-ribose in the DRAT reaction. Both the reduced and the phosphorylated forms of NAD are inhibitors of DRAT (data not shown), but are not effective donors (Table III). DRAT is also inhibited by nicotinamide and 3-aminobenzamide (data now shown). The *K*ₘ for NAD, which is believed to be the physiological donor, is 2 mM (Fig. 4).

![Diagram](image-url)
by analysis of similar incubation mixtures (with carbonyl-\(^{13}C\)) with 1 ml of cold 20 mM potassium phosphate, pH 5.6, with 1 mM NAD and "[^{14}C]nicotinamide present. Identification of the product as nicotinamide was corroborated in separate experiments with the other half was diluted into dinitrogenase reductase and release of 

Nicotinamide is released colinearly with transfer of ADP-ribose to dinitrogenase reductase by DRAT, and the ratio of products is very close to 1 (Fig. 5). Thus there is no detectable NAD glycohydrolase activity even in the presence of the protein acceptor.

DRAT activity is reversibly inhibited by salts; the \( I_{50} \) for NaCl is 175 mM (Fig. 6). Less purified DRAT fractions (DE52) are similarly inhibited by NaCl. The inhibitory effect of KCl is similar to that of NaCl; NaBr inhibits much more strongly (data not shown). Thus the salt inhibition is due to specific ion effects rather than total ionic strength. The pH optimum for the DRAT reaction is approximately 7.0 (data not shown).

**DISCUSSION**

We have presented a procedure which reproducibly yields highly purified DRAT. The purification protocol is relatively simple, involving only four steps of column chromatography. The high level of acceptor specificity exhibited by DRAT is unusual for an arginine-specific ADP-ribosyltransferase. To the best of our knowledge, all ADP-ribosyltransferases which have been tested exhibit some level of NAD glycohydrolase activity and most will use some proteins and/or small molecules other than their physiological targets as acceptors (19-21, 26). We have not excluded the possibility that DRAT may have an extremely low level of NAD glycohydrolase activity which is below the limits of detection of our assays. However, other ADP-ribosyltransferases have glycohydrolase activities ranging from 5 to 20% of their activity in the presence of guanidino acceptors (26); we would have detected NAD glycohydrolase activity by DRAT at levels less than 0.3% of the activity seen with dinitrogenase reductase as substrate. The high acceptor specificity of DRAT also contrasts with the substrate specificity of DRAG, which will use several ADP-ribosylated arginine analogs (8) and oxygen-denatured ADP-ribosylated dinitrogenase reductase as substrates. An interesting possibility which would explain our inability to detect any ADP-ribose transfer with DRAT in the absence of the physiological acceptor protein is that the enzyme is activated by binding to dinitrogenase reductase; this would effectively prevent ADP-ribosylation of proteins other than dinitrogenase reductase in the cell, thus obviating the need for DRAG to have a strict substrate specificity.

The only major contaminant visible in silver-stained gels is a low molecular weight polypeptide which we do not believe plays a role in the dinitrogenase reductase regulatory system. The low molecular weight impurity is a very abundant cellular protein, present in at least 100-fold excess over DRAT as judged by relative staining intensity in SDS gel profiles of fractions from early steps in the purification; the amount of the impurity remaining in our highly purified DRAT fractions is only a small fraction of the amount originally present. Further manipulation of Mono Q fractions in attempts to completely remove the low molecular weight impurity results in unacceptable losses in activity; even rapid desalting of Mono Q fractions results in less than 20% recovery of DRAT activity. The data in Figs. 1B and 3 clearly demonstrate the coincidence of the amount of the 29-kDa polypeptide with DRAT activity and the lack of such a correlation for the low molecular weight impurity.

**Fig. 5. Time course of formation of products in the DRAT reaction.** Incorporation of [\(^{32}P\)]ADP-ribose (C) from [\(~^{32}P\)]NAD into dinitrogenase reductase and release of [\(^{14}C\)]nicotinamide (B) from (carbonyl-[\(^{14}C\)]NAD was monitored simultaneously. A *vinelandii* dinitrogenase reductase (250 \(\mu\)g) was incubated as described under "Experimental Procedures" with 0.22 \(\mu\)Ci each of the \(^{32}P\) and the \(^{14}C\) radionuclides. At the indicated times, half of each incubation mixture was treated as described for determination of [\(^{32}P\)]ADP-ribose incorporation into dinitrogenase reductase. The other half was diluted with 1 ml of cold 20 mM potassium phosphate, pH 5.6, with 1 mM NAD and 5 mM nicotinamide added. Diluted samples were filtered to remove protein and nicotinamide was purified by reverse-phase HPLC (20 mM potassium phosphate, pH 5.6, 3% methanol, isocratic, on a 0.46 \(\times\) 25 cm C-18 column). Fractions eluting in or near the nicotinamide peak were counted in a liquid scintillation counter to determine the amount of [\(^{14}C\)]nicotinamide present. Identification of the product as nicotinamide was corroborated in separate experiments by analysis of similar incubation mixtures (with [carbonyl-[\(^{14}C\)]NAD as the only radionuclide) by two different thin layer chromatography systems (45).

**Fig. 6. Effect of varying the NaCl concentration on DRAT activity with *R. rubrum* dinitrogenase reductase as acceptor.**
catalysis to occur. More specifically, the replacement of the amide nitrogen of the nicotinamide moiety with either carbon or oxygen yields an ineffective donor molecule. Thus, this NH₂ group may be involved in catalysis or in positioning the donor substrate in the active site of DRAT. A series of elegant studies from John Collier's laboratory have shown that the γ-methylene carbon of an essential glutamate residue (Glu-148) of diphtheria toxin becomes covalently bound to C-6 of the nicotinamide moiety of NAD upon UV irradiation of the toxin-NAD complex (39-41). These data place the carboxylate side chain of glutamate 148 in close proximity to the N-glycosidic bond that is broken during catalysis, but there was no evidence for the involvement of the amide nitrogen of nicotinamide in catalysis, as suggested by our data with DRAT.

The Kₐ of 2 mM for NAD in the DRAT reaction is at least an order of magnitude higher than the values reported for any of the other arginine-specific mono-ADP-ribosyltransferases, except for cholera toxin, which has a Kₐ of 3.6 mM for NAD (21, 26). Given the high Kₛ and the fact that NADH will not serve as a donor molecule in the DRAT reaction, it is reasonable to predict that DRAT activity may be sensitive to changes in the ratio of oxidized to reduced pyridine nucleotides in vivo. However, no obvious correlations have been found between cellular pyridine nucleotide levels and the inactivation or activation of ADP-ribosylated dinitrogenase reductase (42).

The dinitrogenase reductase ADP-ribosylation cycle in _R. rubrum_ provides an excellent system for elucidating the role of mono-ADP-ribosylation in the regulation of cellular processes. Currently, we are using DRAT, DRAG, and several dinitrogenase reductases to examine the mechanism of inhibition of dinitrogenase reductase by ADP-ribosylation. Also the effects of adenine nucleotides on the DRAT reaction are being investigated. ATP stimulates and ADP inhibits the activation of ADP-ribosylated dinitrogenase reductase by DRAG (6). The same nucleotides have the opposite effects on the intact nitrogenase regulatory system in _vitro_ (42). Most importantly, with DRAT now purified, we can begin a rigorous analysis of the intact nitrogenase regulatory system in _vitro_.

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REFERENCES