ATP-dependent and NAD-dependent Modification of Glutamine Synthetase from *Rhodospirillum rubrum* in Vitro*

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Glutamine synthetase from the photosynthetic bacterium *Rhodospirillum rubrum* is the target of both ATP- and NAD-dependent modification. Incubation of *R. rubrum* cell supernatant with [α-32P]NAD results in the labeling of glutamine synthetase and two other unidentified proteins. Dinitrogenase reductase ADP-riboisyltransferase does not appear to be responsible for the modification of glutamine synthetase or the unidentified proteins. The [α-32P]ATP- and [α-32P]NAD-dependent modifications of *R. rubrum* glutamine synthetase appear to be exclusive and the two forms of modified glutamine synthetase are separable on twodimensional gels. Loss of enzymatic activity by glutamine synthetase did not correlate with [α-32P]NAD labeling. This is in contrast to inactivation by nonphysiological ADP-ribosylation of other glutamine synthetases by an NAD:arginine ADP-ribosyltransferase from turkey erythrocytes (Moss, J., Watkins, P. A., Stanley, S. J., Purnell, M. R., and Kidwell, W. R. (1984) *J. Biol. Chem.* 259, 5100–5104). A 32P-labeled protein spot comigrates with the NAD-treated glutamine synthetase spot when glutamine synthetase purified from *H.332PO4*+-grown cells is analyzed on twodimensional gels. The adenylylation site of *R. rubrum* glutamine synthetase has been determined to be Leu(Asp)-Tyr-Leu-Pro-Pro-Glu-Glu-Leu-Met; the tyrosine residue is the site of modification.

Under nitrogen-fixing conditions in photosynthetic bacteria, the glutamine synthetase (Equation 1)-glutamate synthase (Equation 2) pathway carries out the central reactions of ammonia assimilation:

\[
\text{ATP} + \text{NH}_4^+ + \text{glutamate} \rightarrow \text{glutamine} + \text{ADP} + P_\text{i} + \text{Mg}^{2+} \tag{1}
\]

\[
\text{NADPH} + \text{glutamine} + \alpha - \text{ketoglutarate} \rightarrow 2 \text{glutamate} + \text{NADP}^+ \tag{2}
\]

The regulation of glutamine synthetase by gene expression, feedback inhibition, and covalent modification has been studied extensively in enteric bacteria (1–3). In *Escherichia coli*, glutamine synthetase exists as a dodecamer of 12 identical subunits, each of which can be regulated independently by the reversible adenylylation of a specific tyrosine. Both the adenylylation (glutamine synthetase-inactivating) and deadenylylation (glutamine synthetase-activating) reactions are carried out by one enzyme, adenylyltransferase. Adenylyltransferase in its turn is regulated by the PII protein, which itself is regulated through the reversible uridylylation of a specific tyrosine, carried out by uridylyltransferase. Uridyltransferase responds to the ratio of glutamine to α-ketoglutarate, shifting glutamine synthetase to the more adenylylated state as the ratio of glutamine to α-ketoglutarate increases, and vice versa. This bicyclic cascade provides both signal amplification and the ability to fine-tune the activity of glutamine synthetase in response to the organism’s changing nitrogen needs.

Regulation of glutamine synthetase activity in phototrophic bacteria is similar to that in the enteric bacteria. There is evidence of adenylylation of glutamine synthetase in *Rhodopseudomonas palustris* (4), *Rhodobacter sphaeroides* (5), and *Rhodobacter capsulatus* (6, 7). In the case of *Rhodospirillum rubrum*, adenylylation of glutamine synthetase has also been shown, with some differences from the *E. coli* enzyme. Adenylylated glutamine synthetase from *R. rubrum* shows loss of γ-glutamyltransferase activity in both the presence and absence of 60 mM Mg2+ (8) (contrary to the case in *E. coli*, in which activity is lost only in the presence of 60 mM Mg2+). Treatment of *R. rubrum* glutamine synthetase with snake venom phosphodiesterase fails to remove AMP (even from a glutamine synthetase peptide containing bound nucleotide) or reactivate glutamine synthetase (8) (although Yoch et al. (10) did report a loss of the Mg2+ inhibition after treatment).

In *R. rubrum* growing under N-limiting conditions, the nitrogenase complex reduces dinitrogen to ammonia which is then assimilated by the glutamine synthetase-glutamate synthase pathway. Nitrogenase is also regulated by covalent modification in *R. rubrum* (11). Under conditions of switch-off (12), dinitrogenase reductase is inactivated by ADP-ribosylation of arginine 101 (13). This ADP-ribosylation is carried out by dinitrogenase reductase ADP-ribosyltransferase (DRAT) (14). Dinitrogenase reductase is reactivated by removal of the adenosine diphosphoribose group, a reaction carried out by dinitrogenase reductase activating glycohydrodase (15). The regulation of nitrogenase and glutamine synthetase appears to be coordinated at some point, but how is not clear. Addition of ammonium to cultures grown on glutamate as the N source results in modification of both nitrogenase and glutamine synthetase activities, but under N-limiting conditions, only glutamine synthetase becomes modified (16, 17). Darkness also leads to modification of both enzymes in glutamate-grown cultures and has no effect on...
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either in \textit{N}-limited cultures (16). Methionine sulfoximine, a glutamine synthetase inhibitor, prevents ammonium switch-off of nitrogenase and slows dark switch-off. However, there is no consistent correlation of intracellular glutamine concentration with nitrogenase switch-off (16, 18).

Studies of site-specific ADP-ribosylation of eukaryotic proteins by bacterial toxins, for example, glutamine nucleotide-binding proteins by cholera toxin (19) and pertussis toxin (20) and of elongation factor 2 by diphtheria toxin (21) and \textit{Pseudomonas} extoxin A (22), gave rise to the hypothesis that these toxins are interfering in an endogenous regulation. Eukaryotic \textit{nuo}-ADP-ribosyltransferases have since been purified by different groups and their in \textit{vivo} roles are being studied (23, 24). ADP-ribosylation in prokaryotes as diverse as \textit{R. capsulatus} (25) and \textit{Pseudomonas} maltophilia (26) have also been reported recently. ADP-ribosylation may turn out to be as widespread and important a mode of regulation as phosphorylation.

In this paper, further evidence for the adenylation of \textit{R. rubrum} glutamine synthetase is given, and the discovery of an ADP-ribosyltransferase activity capable of modifying glutamine synthetase is reported. \textit{In vitro} ADP-ribosylation of glutamine synthetase and two other proteins, none of which appear to be substrate for DRAT, is also shown.

\textbf{MATERIALS AND METHODS}

\textbf{Preparation of [\textit{a-32P}]NAD—[\textit{o-32P}]NAD} was prepared from [\textit{a-32P}]ATP and nicotinamide mononucleotide, using NAD-pyrophosphorylase (19). [\textit{o-32P}]NAD was purified from the reaction mixture by chromatography on a dihydroxyboronate Bio-Rex 70 column (27). The reaction mixture was diluted with 0.25 mM ammonium acetate buffer, pH 8.6, and loaded onto 1 ml of DHB-Bio-Rex 70. The column was washed with 20 ml of 0.25 M ammonium acetate buffer, pH 8.6, until used. Cell paste (469 g) was thawed in 500 ml of 100 mM imidazole buffer, pH 7.5, and incubated for 1 h with 150 mg of lysozyme, 30 mg of DNase, and 30 mg of RNase. The cells were then broken either by osmotic shock or by sonication under nitrogen using a Heat Systems-Ultrasonics, Inc. model 350 Sonicator and microprobe. A portion of broken cells was frozen as pellets in liquid nitrogen and stored at -80 °C. Portions were thawed either in N-limited cultures (16). Methionine sulfoximine, a glutamine synthetase inhibitor, prevents ammonium switch-off of nitrogenase and slows dark switch-off. However, there is no consistent correlation of intracellular glutamine concentration with nitrogenase switch-off (16, 18).

In some experiments, purified glutamine synthetase was concentrated to 5–8 mg/ml in an Amicon stirred cell ultrafiltration unit, using a 100 K membrane and 100 mM imidazole acetate buffer.

\textbf{Preparation of Crude Extracts for Assay of ADP-Ribosyltransferase Activity}—All steps were carried out anaerobically at 4–8 °C unless stated otherwise. One to five grams of frozen cells grown either in limited ammonium (2 mM), high ammonium (20 mM), or glutamate/malate medium were thawed in equal volumes of 0.1 M dithionite, 1 mM dithioreitol, 1 mM ATP, 50 mM MOPS buffer, pH 7.0 (cell-breaking buffer), together with 0.1 mM of DNPse, 0.1 mg of RNase, and 0.5 mg of lysozyme. The cells were then broken either by osmotic shock or by sonication under nitrogen using a Heat Systems-Ultrasonics, Inc. model 350 Sonicator and microprobe. A portion of broken cells was frozen as pellets in liquid nitrogen and stored at -80 °C. Portions were thawed either in N-limited cultures (16). Methionine sulfoximine, a glutamine synthetase inhibitor, prevents ammonium switch-off of nitrogenase and slows dark switch-off. However, there is no consistent correlation of intracellular glutamine concentration with nitrogenase switch-off (16, 18).

\textit{Incubation of Crab Extracts with [\textit{a-32P}]NAD or [\textit{o-32P}]ATP—} To label proteins with [\textit{a-32P}]NAD, crude extract (broken cells, supernatant solution, or resuspended pellet) containing 100 μg of protein was incubated with [\textit{a-32P}]NAD (5 X 10^6 cpm), 0.2 mM NAD, 4 mM MnCl$_2$, 50 mM imidazole buffer, pH 7.5, and 1 μM of 5'-ADP in a total volume of 200 μl. The reaction mixture was incubated at 30 °C for 60 min, the reaction was stopped, and the incubation was centrifuged at 15,000 × g for 30 min. The supernatant was frozen and stored at -80 °C. Portions were dissolved in 10 μl of two dimensional gel sample buffer.

In some experiments, as noted, the [\textit{a-32P}]NAD incubation was stopped by the addition of 0.5 μl of a stop mixture containing 30 μl of anti-GS antiserum, 50 μl of 10% (v/v) insoluble protein A (Sigma), and 200 μl of 20 mM imidazole buffer.
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and 420 μl of 100 mM MOPS, pH 7.5. After 60 min on ice, the samples were centrifuged for 30 s, and the supernatants were removed. The pellets were resuspended and centrifuged three times in 1 ml of 2 mM NAD, 0.5% (v/v) Nonidet P-40, 100 mM NaN, 25 mM KH₂PO₄, pH 7.0, and 0.2% (v/v) 2-mercaptoethanol, 1% (w/v) 2-mercaptoethanol, for analysis by SDS-PAGE.

Effect of Crude Extract on Glutamine Synthetase Activity and Modification by [α-32P]ATP—Reaction mixtures containing 50 μl of diluted glutamine synthetase (0.5 μg, 0.14 unit of γ-glutamyltransferase activity) in 100 mM imidazole buffer, pH 7.5, and 200 μg of glutamate/malate-grown R. rubrum cell supernatant, [α-32P]NAD (5 × 10⁶ cpm), 0.2 mM NAD, and other additions as noted in 100 mM MOPS buffer, pH 7.0, in 100 μl total volume, were incubated anaerobically at 30 °C. Parallel incubations of glutamate/malate cell supernatant, glutamine synthetase, and glutamine synthetase with 0.015 unit purified DRAT, and glutamine synthetase alone were also carried out. After 20 min, 40 μl was assayed for γ-glutamyltransferase activity, 40 μl for bioisotopic activity, and the remaining 20 μl was frozen in liquid nitrogen and stored at −80 °C, to be precipitated with trichloroacetic acid later for two-dimensional gel electrophoresis and autoradiography.

Adenylylation of Glutamine Synthetase Activity in Crude Extracts—The experimental conditions are described in the legend for Fig. 1. SDS-PAGE, Two-dimensional Gels, Immunoblots, Autoradiograms—SDS-PAGE was carried out according to Laemmli (84) using a 4.5% stacking gel and a 10% resolving gel. Two-dimensional gel electrophoresis was carried out as described (35, 36). Each gel had a second dimension. Gels were fixed in formaldehyde-stained (37) washed with distilled, deionized H₂O, then stained with Coomassie blue and dried under vacuum onto filter paper. Silver-staining was carried out as described by Morrissey (38). Immunoblots were carried out essentially as described by Towbin et al. (39), with 0.1% Tween 20 added to the wash buffers. Cross-reacting material was visualized using 4-chloro-1-napthol and horseradish peroxidase conjugated to goat anti-rabbit IgG. Autoradiograms of gels and immunoblots used preflashed Kodak X-OMAT AR film exposed at −80 °C with an intensifying screen.

A Zeinco soft laser scanning densitometer was used to estimate the relative degree of radio-labeling of proteins in the two-dimensional gels. Each spot was scanned in two directions and the absorption values averaged in terms of arbitrary units. The units derived in this manner should be used only to compare the relative degree of radio-labeling between proteins within the same experiment, and not to compare separate experiments.

Preparation of Anti-glutamine Synthetase Antiserum—A male Hazleton rabbit was injected subcutaneously along the back with 0.7 mg of purified R. rubrum glutamine synthetase in complete Freund’s adjuvant and boosted 3 weeks later with 0.7 mg of glutamine synthetase in incomplete Freund’s adjuvant. Bleeding was started 14 days after the boost.

Preparation of Anti-DRAT Antiserum—Fractions of partially purified DRAT (14) were pooled and electrophoresed on an 11% (total), 5% (cross-linker) SDS-polyacrylamide gel. Proteins were visualized by soaking the gel in ice-cold 0.25 M KCl, 1 mM diithiothreitol (40), and the DRAT band was cut out. The location of DRAT in the gel was confirmed by Coomassie staining one end of the gel containing a small portion of the pooled DRAT sample and a lane with molecular weight standards. DRAT was eluted from the gel slices into 50 mM NH₄HCO₃, 0.1% SDS buffer using a model 422 Electro-Eluter from Bio-Rad. Fifty-three micrograms of protein were recovered and found to be pure by SDS-PAGE with silver-staining (38).

Twenty micrograms of DRAT in complete Freund’s adjuvant was injected into a male Hazleton rabbit by multiple intradermal injections (41). The rabbit was boosted 1 month later with 1.1 μg of DRAT in incomplete Freund’s adjuvant, and bleeding started 10 days after the boost.

DRAT Inhibition by DRAT Antiserum—DRAT antiserum and normal rabbit serum were incubated at 56 °C for 20 min and centrifuged. DRAT was incubated in a 1:10 dilution of either heat-treated and DRAT-serum-normal rabbit serum in 100 mM MOPS buffer, pH 7.0, and in 100 mM MOPS buffer, pH 7.0, alone for either 4 h on ice or 15 min at 30 °C. The treated DRAT was then made anaerobic and tested for activity in a radioactive filter assay (14), using Klebsiella pneumoniae dinitrogen reductase as the substrate.

To test the inhibition of DRAT activity by antibodies in crude extracts, extracts of limited ammonium- and high ammonium-grown cells were incubated anaerobically with 1:10 dilutions of heat-treated anti-DRAT serum (control incubations used normal rabbit serum in 100 mM MOPS buffer, pH 7.0, and 100 mM MOPS buffer, pH 7.0, alone in place of anti-DRAT serum) for 4 h on ice. The treated extracts were then incubated for 20 min at 30 °C with [α-32P]NAD (5 × 10⁶ cpm), 0.2 mM NAD, and 100 μg of glutamate/malate-grown R. rubrum cell supernatant, without adding 1 mM ADP + 5 mM MgCl₂. They were then precipitated with trichloroacetic acid and subjected to two-dimensional gel electrophoresis, and the gels were stained with Coomassie Blue, dried, and autoradiographed.

3P Labeling of Cells—Cells were grown in glutamate/malate medium in 500-m1 cultures as described previously (8). One mg of carrier-free H₃P0₄, was added 8–12 h before the cells were harvested.

Digestion of Glutamine Synthetase and Purification of Peptides—In vivo 3P-labeled glutamine synthetase (2.9 mg) was trichloroacetic acid-precipitated together with 1.1 mg of glutamine synthetase purified from glutamate/malate-grown cells. The protein pellet was suspended in 1 ml of 100 mM ammonium acetate buffer, pH 8.5, and the pH adjusted to 8.0. Subtilisin digestion (1% w/v) was performed overnight at room temperature. The digest was diluted 10-fold with 0.25 M ammonium acetate buffer, pH 8.6, and loaded onto a 1-ml Bio-Rex 70 column at room temperature, equilibrated with the same buffer. The column was washed with equilibrium buffer and then with distilled, deionized H₂O. All of the peptides eluted through and wash. The flow-through and wash were pooled, then concentrated and washed with distilled, deionized H₂O in a Savant Speed Vac. The sample was then loaded onto a 4.1 × 100-mm Synchropak RPP C18 column on a Waters HPLC system. The column was washed with 0.2% formic acid for 6 min at a flow rate of 1 ml/min, followed by an elution linear gradient of 0–60% (v/v) 2- propanol in 0.1% formic acid. The column was monitored for absorbance at 254 nm, and a peak containing 97% of the eluted 3P appeared at the end of the gradient. Pooled fractions were concentrated and washed with distilled, deionized H₂O in a Savant Speed Vac. This sample was dissolved in 20 mM ammonium acetate buffer, pH 6.0, and applied to a 4.1 × 100-mm Synchropak AX-300 ion-exchange column. The column was washed with 20 mM ammonium acetate buffer, pH 6.0, for 5 min at 2 ml/min, followed by a 20-min linear gradient of 20–500 mM ammonium acetate buffer, pH 6.0. A peak that contained 91% of the eluted 3P began to appear 14.0 min into the gradient (at about 370 mM ammonium acetate). Five min after the end of the gradient the column was washed with 1 M ammonium acetate buffer, pH 6.0, for 15 min. No further UV-absorbing peaks were eluted (as determined by monitoring at 254 nm.) The peak fractions were pooled, concentrated, and washed with distilled, deionized H₂O in a Savant Speed Vac. Amino acid analysis and sequencing of the purified nucleotide peptide were carried out by Gary Hathaway of the Biotechnology Instrumentation Facility, University of California-Riverside.

RESULTS

Adenylyltransferase activity in crude supernatants of high ammonium-grown R. rubrum cells was demonstrated by incubation with purified glutamine synthetase and [α-32P]ATP under conditions described in the legend for Fig. 1. Glutamine synthetase was strongly and specifically labeled (Fig. 1). No adenylyltransferase activity was found in the cell pellet.

The adenylylation site of R. rubrum glutamine synthetase was determined by analysis of a proteolytic digest of the in vivo-labeled protein. Four mg of glutamine synthetase (2.9 mg of 3P-labeled in vivo + 1.1 mg of unlabeled) purified from cells grown in glutamate/malate medium was precipitated
with trichloroacetic acid and digested with subtilisin. The digest was chromatographed on a DHB-Bio-Rex 70 column to remove any ADP-ribosylated peptides, and further purified by reverse-phase and ion-exchange HPLC as described under "Methods and Materials." The amino acid sequence of the 32P-labeled peptide is Leu-Asp-Tyr-Leu-Pro-Pro-Glu-Glu-Leu-Met, although the identification of the aspartate is uncertain due to interference by ammonium in the sample buffer. That the tyrosine residue is the site of modification was deduced from the observation that position 3 of the sequence was blank and the sequence lacked a tyrosine residue even though one tyrosine was detected unambiguously in the amino acid analysis. The sequence of the adenylylation site of E. coli glutamine synthetase is Asn-Leu-Tyr-Asp-Leu-Pro-Pro-Glu-Ala-Lys (residues 395-405); the tyrosine is adenylylated (45). An absorption spectrum of the purified peptide from R. rubrum glutamine synthetase shows an absorption maximum at 260 nm (Fig. 2), as seen previously in a partially purified glutamate/malate-grown cells were incubated with [α-32P]NAD, [α-32P]NAD (5 X 10^6 cpm) in 100 mM MOPS buffer, pH 7.0, as described under "Materials and Methods." The spectrum was obtained using a Shimadzu UV-160 spectrophotometer.

Incubation of crude extracts of R. rubrum (broken cells, supernatant, and pellet) with [α-32P]NAD resulted in labeling of three proteins besides dinitrogenase reductase, as observed by autoradiography of two-dimensional gels (Fig. 3). These proteins were labeled most strongly in the supernatant fraction, so all further experiments were carried out using the supernatant fraction. No labeling unique to the membrane fraction (pellet) was observed under the conditions tested.

FIG. 1. Autoradiogram of SDS-PAGE of glutamine synthetase 32P-adenylated by R. rubrum high ammonium cell supernatant. For each sample, 50 µg of glutamine synthetase (GS) was incubated with 10 µl of supernatant from high ammonium-grown cells in a reaction mixture containing 1 µCi [α-32P]ATP, 1 mM ATP, 1 mM glutamine, 1 mM MgCl2 in 50 mM imidazole buffer, pH 7.5. Total volume was 100 µl. Lane 1, 0 min; lane 2, 30-min incubation. Reactions were stopped by the addition of 1 ml of 50 mM imidazole buffer, pH 7.5, and precipitated with 0.4 ml of 20% (w/v) trichloroacetic acid. The pellet was washed with 5% (w/v) trichloroacetic acid, then subjected to SDS-PAGE and autoradiography as described under "Materials and Methods." Lanes 3 and 4, same as lanes 1 and 2, with 25 mM ATP in the dilution buffer.

FIG. 2. UV absorption spectrum of purified 32P-labeled peptide from subtilisin digest of R. rubrum glutamine synthetase. The spectrum was obtained using a Shimadzu UV-160 spectrophotometer.

FIG. 3. Two-dimensional gel and autoradiogram of [α-32P]NAD-labeled glutamate/malate-grown R. rubrum cell supernatant. The incubation was carried out anaerobically with 0.2 mM NAD, [α-32P]NAD (5 X 10^6 cpm) in 100 mM MOPS buffer, pH 7.0, as described under "Materials and Methods." Top, two-dimensional gel; bottom, autoradiogram; GS, glutamine synthetase.

Spot A appears on the autoradiogram over a protein that is present in very low quantity, but it is labeled relatively strongly. Spot B is not labeled very strongly. The conditions under which labeling of Spot B increases roughly parallel those for the labeling of glutamine synthetase, although the conditions for Spot A labeling are generally the opposite of those for glutamine synthetase labeling (Tables I and III). In extracts of cells grown on glutamate/malate and high ammonium media, glutamine synthetase was labeled most strongly when ADP and ATP were omitted from the incubation. In extracts of N-starved cells, glutamine synthetase was not labeled strongly, even when ADP and ATP were omitted. Thus, either some factor that is required for [α-32P]NAD labeling of glutamine synthetase is lacking in extracts of N-starved cells, or some inhibitor is present. Dinitrogenase reductase labeling was as expected in these experiments. In limited ammonium cell supernatant, dinitrogenase reductase is labeled strongly when ADP and MgCl2 (required for dinitrogenase reductase modification by DRAT) are added to the incubation. In glutamate/malate cell supernatants, much of the dinitrogenase reductase is already modified, so the dinitrogenase reductase is labeled only faintly. Additional DRAT increases the labeling of dinitrogenase reductase in limited ammonium cell supernatants. Aerobic incubation decreases the amount of labeled dinitrogenase reductase because O2-denatured dinitrogenase reductase is no longer a substrate for DRAT (46). In high ammonium-grown cells, dinitrogenase reductase is not synthesized.

To test for nonenzymatic addition of [α-32P]adenosine di-phosphoribose (ADP-ribose) to proteins, extracts from glutamate/malate-grown cells were incubated with [α-32P]NAD together with unlabeled ADP-ribose (Table II). The labeling of glutamine synthetase was unaffected. The labeling of protein Spot B was decreased, either by nonenzymatic addition of ADP-ribose or possibly by inhibition of a transferase activity. The labeling of protein Spot A was increased by the addition of ADP-ribose, perhaps by the same mechanism by which ADP and ATP enhance the labeling of protein Spot A.

The identification of glutamine synthetase as one of the proteins labeled by [α-32P]NAD was carried out as follows.
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TABLE I

| Crude extract | Incubation conditions | Relative amount of label |
|---------------|-----------------------|--------------------------|
|               |                       | A spot | B spot | Glutamine synthetase | R. rubrum dinitrogenase reductase |
| Glutamate/malate supernatant | Anaerobic, 1 mM ADP, 5 mM MgCl₂ | 63 | 0.5 | 3 | 4 |
|               | Anaerobic, 1 mM ADP, 5 mM MgCl₂, DRAT | 92 | - | 4 | 3 |
|               | Anaerobic | 28 | 11 | 98 | - |
|               | Aerobic, 1 mM ADP, 5 mM MgCl₂ | 140 | - | 33 | - |
| High NH₄Cl supernatant | Anaerobic, 1 mM ADP, 5 mM MgCl₂ | 110 | - | 10 | - |
|               | Anaerobic, 1 mM ADP, 5 mM MgCl₂, DRAT | 98 | - | 6 | - |
|               | Anaerobic | 26 | 14 | 105 | - |
|               | Aerobic, 1 mM ADP, 5 mM MgCl₂ | 169 | - | 27 | - |
| Limited NH₄Cl supernatant | Anaerobic, 1 mM ADP, 5 mM MgCl₂ | 118 | 3 | 18 | 314 |
|               | Anaerobic, 1 mM ADP, 5 mM MgCl₂, DRAT | 158 | - | 3 | >600 |
|               | Anaerobic | 32 | 7 | 11 | 49 |
|               | Aerobic, 1 mM ADP, 5 mM MgCl₂ | 193 | - | 15 | 43 |

a — not detected.

TABLE II

Effect of adenosine diphosphoribose (ADP-ribose) on [α-32P]NAD labeling of proteins in glutamate/malate cell supernatant

Duplicate samples of glutamate/malate cell supernatant were incubated anaerobically with [α-32P]NAD (5 x 10⁶ cpm) in 0.2 mM NAD, with [ADP-ribose] as indicated below and described under "Materials and Methods." Two-dimensional gels, autoradiograms, and densitometry were carried out as described under "Materials and Methods."

| [ADP-ribose] | Glutamine synthetase A spot | B spot |
|--------------|----------------------------|-------|
| 0 mM         | 160 ± 57                   | 18 ± 10 | 24 ± 5 |
| 0.02 mM      | 160 ± 27                   | 14 ± 1.5 | 11 ± 2 |
| 0.2 mM       | 203 ± 34                   | 32 ± 3  | 3.7 ± 0.1 |
| 2.0 mM       | 143 ± 15                   | 100 ± 17 | - |

a — not detected.

Extracts of glutamate/malate-grown cells were incubated with [α-32P]NAD and electrophoresed on two-dimensional gels as described under "Materials and Methods." The proteins were transferred to nitrocellulose and antigens detected by the appropriate antibodies. The developed blots were then autoradiographed. Comparison of the proteins recognized by the various antisera with the location of the [α-32P]NAD-labeled proteins gave negative results for anti-R. rubrum dinitrogenase and anti-R. rubrum ribulose bisphosphate carboxylase oxygenase and positive results for anti-R. rubrum glutamine synthetase and anti-R. rubrum dinitrogenase reductase (Fig. 4). In the case of glutamine synthetase, the radiolabeled spot corresponded to the most acidic portion of the spot recognized by the anti-glutamine synthetase serum.

When an extract from glutamate/malate-grown cells is incubated with 0.2 mM NAD and [α-32P]ATP (5 x 10⁶ cpm) instead of [α-32P]NAD, neither the A protein spot nor glutamine synthetase are labeled (Fig. 5). The B protein spot does appear to be labeled, however. Incubation with 1 mM ADP, 5 mM MgCl₂, 0.2 mM NAD, and [α-32P]ATP (5 x 10⁶ cpm) causes the B protein spot and glutamine synthetase to be labeled strongly (presumably the addition of MgCl₂ to the reaction is required for glutamine synthetase to be labeled by [α-32P]ATP). However the [α-32P]ATP-labeled glutamine synthetase does not coelectrophorese with the [α-32P]NAD-labeled glutamine synthetase.
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Fig. 5. Autoradiograms of two-dimensional gels of [α-32P]ATP-labeled extracts of glutamate/malate-grown R. rubrum cells. Top, incubation of 100 μg of supernatant with 0.2 mM NAD, [α-32P]ATP (5 x 10^6 cpm), in 100 mM MOPS buffer, pH 7.0. Bottom, incubation as in the top, with the addition of 1 mM ADP and 5 mM MgCl₂. GS, glutamine synthetase.

Fig. 6. In vivo 32P-labeled glutamine synthetase. A, two-dimensional gel of in vivo 32P-labeled glutamine synthetase purified from R. rubrum grown in glutamate/malate medium. Arrows point to the 32P-labeled proteins. B, autoradiogram.

labeled glutamine synthetase. The [α-32P]ATP-labeled glutamine synthetase (presumably adenylylated glutamine synthetase) is the major protein spot between unmodified glutamine synthetase and the [α-32P]NAD-labeled glutamine synthetase. The hazy streak in the autoradiogram in Fig. 5 is also seen in some autoradiograms of [α-32P]NAD reactions with cell supernatant, and it can be reduced or eliminated by the addition of RNase and DNase either before or after the reaction.

32P-Labeled glutamine synthetase purified from cells grown in glutamate/malate medium appears as three spots in a two-dimensional gel (Fig. 6). The center and rightmost spots are radiolabeled. In a two-dimensional gel, in vivo 32P-labeled glutamine synthetase comigrates with the proteins identified as [α-32P]ATP- and [α-32P]NAD-labeled glutamine synthetase in glutamate/malate-grown cell supernatant (data not shown).

Glutamine synthetase purified from glutamate/malate-grown cells migrates as two bands on SDS-PAGE, while glutamine synthetase purified from limited ammonium-grown cells migrates as one band, as was previously shown by Soliman and Nordlund (47) (Fig. 7). In a two-dimensional gel, the glutamine synthetase purified from limited ammonium grown cells migrates as two spots (Fig. 7). It is possible, but not yet shown, that these two spots correspond to unmodified and NAD-modified glutamine synthetase.

To investigate whether glutamine synthetase, protein Spot A, or protein Spot B were substrates for DRAT, inhibition of modification by antibody against DRAT was tested. Treatment of DRAT with a 1:10 dilution of anti-DRAT antiserum for 15 min at 30 °C (or 4 h on ice) reduced the ADP-ribosylation of dinitrogenase reductase by 89–95%, as compared to control DRAT incubated in buffer alone. DRAT incubated with a 1:10 dilution of normal rabbit serum showed activities of 121–143% of the control DRAT. This increase in activity may be due to stabilization of the DRAT activity by serum proteins, because anti-DRAT and normal rabbit serum incubated with dinitrogenase reductase without DRAT had background activities of only 1–2% of the control DRAT activity.

Anaerobic treatment of crude supernatants from limited ammonium- and high ammonium-grown cells with anti-DRAT before incubation with [α-32P]NAD strongly inhibited modification of dinitrogenase reductase in the limited ammonium supernatant, as expected. Labeling of the A and B proteins and of glutamine synthetase, however, was not affected in either supernatant (data not shown).

DRAT did not ADP-ribosylate glutamine synthetase under conditions in which glutamate/malate cell supernatant modifies glutamine synthetase in vitro (Table III). Labeling of glutamine synthetase with [α-32P]NAD did not correlate with a loss of enzyme activity under standard assay conditions. Inactivation of glutamine synthetase by the addition of ADP/
Glutamine Synthetase from R. rubrum

Effect of incubation conditions on \([\alpha-^{32}P]NAD\) labeling and enzymatic activity of glutamine synthetase

Incubations and assays were carried out as described under “Materials and Methods.” In samples marked no additions, no \([\alpha-^{32}P]NAD\) was added. Numbers in parentheses are percentage of control glutamine synthetase activity. [NAD] = 0.2 mM; [ADP], [ATP], [glutamine], \([NH_4Cl]\) = 1 mM; [MgCl₂] = 5 mM.

| Sample | Incubation conditions | Relative amount of label | GS activity |
|--------|-----------------------|--------------------------|-------------|
|        |                       | A spot | B spot | \(\gamma\)-Glutamyl-transferase | Biosynthetic |
| Glutamate/malate supernatant | No additions | --- | --- | --- | --- |
|        | NAD, ADP, MgCl₂ | 57 | 41 | 0.104 (185) | 0.0089 (216) |
|        | NAD, ADP | 62 | 47 | 0.073 (39) | 0.0090 (33) |
|        | NAD, ATP, MgCl₂ | 45 | 19 | 0.108 (130) | 0.012 (185) |
|        | NAD, ATP | 48 | 11 | 0.059 (0) | 0.0089 (0) |
|        | NAD, MgCl₂ | 132 | 9 | 0.106 (86) | 0.012 (135) |
|        | NAD, glutamine | 216 | 1 | 0.116 (150) | 0.011 (181) |
|        | NAD, NH₄Cl | 188 | 6 | 0.087 (167) | 0.0097 (155) |
|        | NAD | 190 | 5 | 0.073 (57) | 0.0094 (170) |
| GS + glutamate/malate supernatant | No additions | --- | --- | --- | --- |
|        | NAD, ADP, MgCl₂ | 14 | 13 | 0.084 | 0.0073 |
|        | NAD, ADP | 11 | 11 | 0.078 | 0.0096 |
|        | NAD, ATP, MgCl₂ | 21 | 9 | 0.059 | 0.0086 |
|        | NAD, ATP | 15 | 26 | 0.081 | 0.0089 |
|        | NAD, MgCl₂ | 70 | 3 | 0.08 | 0.0081 |
|        | NAD, glutamine | 111 | 5 | 0.064 | 0.0079 |
|        | NAD, NH₄Cl | 111 | 2 | 0.062 | 0.008 |
|        | NAD | 129 | 5 | 0.065 | 0.0077 |
| GS + DRAT | No additions | --- | --- | --- | --- |
|        | NAD, ADP, MgCl₂ | 14 | 13 | 0.064 | 0.0085 |
|        | NAD, ADP | 11 | 11 | 0.078 | 0.0096 |
|        | NAD, ATP, MgCl₂ | 21 | 9 | 0.059 | 0.0086 |
|        | NAD, ATP | 15 | 26 | 0.081 | 0.0089 |
|        | NAD, MgCl₂ | 70 | 3 | 0.08 | 0.0081 |
|        | NAD, glutamine | 111 | 5 | 0.064 | 0.0079 |
|        | NAD, NH₄Cl | 111 | 2 | 0.062 | 0.008 |
|        | NAD | 129 | 5 | 0.065 | 0.0077 |

*GS, glutamine synthetase.

**Not detected; ND, not determined.

MgCl₂ or ATP/MgCl₂ to the incubation is most likely due to adenyllylation.

In attempts to scale up the in vitro labeling of glutamine synthetase with \([\alpha-^{32}P]NAD\), it was found that when 100 μg of desalted glutamine synthetase was added to the normal 50 μl (total volume) incubation mixture, the amount of glutamine synthetase labeled was much less than when 10 μg of this glutamine synthetase was added to the mixture. This inhibition was removed if the glutamine synthetase was dialyzed against 100 mM MOPS, pH 7.0 buffer. We suspected that ADP or MnCl₂ from the purification procedure which was not removed by the desalting column might be responsible for the inhibition. To test this hypothesis, reactions were performed in the presence of 1 mM ADP, MnCl₂, MgCl₂, and EDTA (Fig. 8). One mM ADP, MnCl₂, and EDTA all inhibited the labeling of glutamine synthetase by extracts from glutamate/malate-grown cells, while 1 mM MgCl₂ had no effect or enhanced the labeling slightly. One mM ADP together with 1 mM MgCl₂ was also inhibitory.

The time course of \([\alpha-^{32}P]NAD\) labeling of glutamine synthetase in extract from glutamate/malate-grown cells was followed as shown in Fig. 9. Labeling was complete by 60 min as determined by densitometry of the autoradiogram.

DISCUSSION

The amino acid sequence of the adenyllylation site of R. rubrum glutamine synthetase is similar to that of E. coli
glutamine synthetase, with tyrosine as the modified residue in both cases.

The \([\alpha-\text{\textsuperscript{32}}P]\)NAD labeling studies were undertaken originally to discover if there were any other protein substrates for DRAT besides dinitrogenase reductase in \(R.\ rubrum\). Unlike cholera toxin and turkey erythrocyte ADP-ribosyltransferase, which are also NAD:arginine transferases, DRAT is highly specific in the substrates it will modify. Thus far only native dinitrogenase reductase from \(R.\ rubrum, K.\ pneu-
moniae, Azotobacter vinelandii,\) and Clostridium pasteurianum have been shown to be modified by DRAT (14). Three other proteins besides dinitrogenase reductase were labeled in crude extracts of \(R.\ rubrum\) incubated with \([\alpha-\text{\textsuperscript{32}}P]\)NAD. Although it has not been demonstrated chemically, our working hypothesis is that these proteins are ADP-ribosylated.

Preincubation of the crude extract with antibody against DRAT greatly reduced labeling of dinitrogenase reductase, but did not affect labeling of the other three proteins. From this result it appears that these other three proteins are not substrates for DRAT and that there must be at least one more ADP-ribosyltransferase in \(R.\ rubrum\).

Finding that \(R.\ rubrum\) glutamine synthetase could be ADP-ribosylated as well as adenylylated \emph{in vitro} was surprising but not unprecedented. Moss et al. (48) have shown that ovine brain glutamine synthetase and chicken heart glutamine synthetase are inactivated by ADP-ribosylation carried out by a NAD:arginine ADP-ribosyltransferase from turkey erythrocytes. More recently, \(E.\ coli\) glutamine synthetase was also found to be a substrate for this NAD:arginine ADP-ribosyltransferase (49). ADP-ribosylation of a specific arginine in \(E.\ coli\) glutamine synthetase resulted in the loss of both biosynthetic and \(\gamma\)-glutamyltransferase activities. In view of these results, it is interesting that we did not see a loss of \(R.\ rubrum\) glutamine synthetase activity together with NAD-dependent modification. There are two possibilities. One is that the effect of ADP-ribosylation on \(R.\ rubrum\) glutamine synthetase activity is more subtle, a small shift in pH optimum or affinity for substrates for example, than we could detect under our assay conditions. Also, the amino acid ADP-ribosylated in \(R.\ rubrum\) glutamine synthetase is not necessarily the same as that ADP-ribosylated in the other glutamine synthetases by the erythrocyte transferase. The other possibility is that ADP-ribosylation of \(R.\ rubrum\) glu-
tamine synthetase does not affect the activity directly, but may be involved in coordination of nitrogenase and glutamine synthetase regulation. In limited ammonium cell supernatant, glutamine synthetase was not labeled by \([\alpha-\text{\textsuperscript{32}}P]\)NAD as strongly as in high ammonium and glutamate/malate cell supernatants.

The adenylylation and ADP-ribosylation of \(R.\ rubrum\) glutamine synthetase appear to be exclusive reactions. In the two-dimensional gel autoradiograms, there was no glutamine synthetase spot that could be labeled by both \([\alpha-\text{\textsuperscript{32}}P]\)ATP and \([\alpha-\text{\textsuperscript{32}}P]\)NAD. Also, adenylylation of glutamine synthetase required added MgCl\(_2\) in the incubation, while ADP-ribosylation did not. The modification sites may be near enough to each other to prevent double modification by steric hindrance or a change in protein conformation after one site is modified may conceal the second site.

Work is underway to determine the site of NAD-dependent modification of \(R.\ rubrum\) glutamine synthetase and confirm the identity of the modifying group. The putative glutamine synthetase ADP-ribosyltransferase will also be purified and characterized.

Acknowledgment—We would like to thank Robert Lowery for helpful advice and discussions, and for providing some of the DRAT used in these experiments.

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FIG. 9. Time course of \([\alpha-\text{\textsuperscript{32}}P]\)NAD labeling of glutamine synthetase in supernatant from glutamate/malate-grown \(R.\ rubrum\) cells. One hundred \(\mu l\) of supernatant from glutamate/malate-grown \(R.\ rubrum\) cells was incubated in 0.2 \(\text{nM}\) NAD, \([\alpha-\text{\textsuperscript{32}}P]\)NAD (50 \(\times\) 10\(^{5}\) cpm), 100 \(\text{mM}\) MOPS buffer, pH 7.0, 250 \(\mu l\) total volume. Twenty-five-\(\mu l\) aliquots were removed at times shown (minutes), and glutamine synthetase was precipitated with anti-glutamine synthetase serum as described under "Materials and Methods." Pellets were washed and subjected to SDS-PAGE and autoradiography. The autoradiogram is shown.
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