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Changes in Amino Acid and Nucleotide Pools of *Rhodospirillum rubrum* during Switch-Off of Nitrogenase Activity Initiated by NH_4^+ or Darkness

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Amino acid and nucleotide pools were measured in nitrogenase-containing *Rhodospirillum rubrum* cultures during NH_4^+- or dark-induced inactivation (switch-off) of the Fe protein. A big increase in the glutamine pool size preceded NH_4^+ switch-off of nitrogenase activity, but the glutamine pool remained unchanged during dark switch-off. Furthermore, methionine sulfoximine had no effect on the rate of dark switch-off, suggesting that glutamine plays no role in this process. In the absence of NH_4^+ azaserine, an inhibitor of glutamate synthate, raised glutamine pool levels sufficiently to initiate switch-off in vivo. While added NH_4^+ substantially increased the size of the nucleotide pools in N-limited cells, the kinetics of nucleotide synthesis were all similar and followed (rather than preceded) Fe protein inactivation. Darkness had little effect on nucleotide pool sizes. Glutamate pool sizes were also found to be important in NH_4^+ switch-off because of the role of this molecule as a glutamine precursor. Much of the diversity reported in the observations on NH_4^+ switch-off appears to be due to variations in glutamate pool sizes prior to the NH_4^+ shock. The nitrogen nutritional background is an important factor in determining whether darkness initiates nitrogenase switch-off; however, no link has yet been established between this and NH_4^+ (glutamine) switch-off.

The nonsulfur photosynthetic bacteria are among a group of N_2-fixing bacteria with nitrogenase activity that is subject to regulation by NH_4^+. Carithers et al. (4) and Nordlund and Eriksson (17) showed that the inhibitory effect of NH_4^+ on nitrogenase activity (switch-off) is related to the covalent modification and concurrent inactivation of the nitrogenase Fe protein extracted from glutamate- and N_2-grown cells (13, 14, 18). Similar to the inactive Fe protein isolated from glutamate-grown cells, Fe protein extracted from NH_4^+-inhibited cells (4, 17) was inactive and could be reactivated by a membrane-bound activating enzyme (13, 18). Switch-off of nitrogenase activity by NH_4^+ catalyzes the covalent modification of one of the two Fe protein subunits (6, 23) with one molecule of ADP-ribose (22).

The role of NH_4^+ as an in vivo signal to activate an Fe protein-inactivating enzyme has been the subject of numerous investigations. Glutamine mimics NH_4^+ (16) and methionine sulfoximine (MSX), an inhibitor of glutamine synthetase (GS), prevents NH_4^+ switch-off in *Rhodospirillum rubrum* (5, 25, 30) and all other N_2 fixers that are capable of switch-off regulation (3, 9). 6-Diazo-5-oxo-L-norleucine (30) and azaserine (7) are inhibitors of glutamate synthase (GOGAT), and both initiate switch-off in the absence of NH_4^+, presumably by blocking the normal flux of nitrogen through the GS-GOGAT pathway. This results in a glutamine buildup sufficient to initiate switch-off.

The first indication that nitrogenase switch-off could be initiated by metabolites other than NH_4^+ or glutamine was obtained by exposure of *R. rubrum* to darkness or to darkness plus an uncoupler. These treatments caused the partial inactivation of nitrogenase (up to 65%) because of covalent modification of the Fe protein (29). Furthermore, NH_4^+ switch-off was faster in cultures exposed to low light than those exposed to high light (30). Dark switch-off of nitrogenase activity caused by enzyme modification was confirmed and extended to show that *R. rubrum* could rapidly reverse this process in the light (10).

It is not readily apparent what glutamine and darkness have in common that leads to the activation of the Fe protein ADP ribosylation (switch-off) system. To determine the factors essential for switch-off, changes in the intracellular pools of both the amino acids and nucleotides were examined during nitrogenase switch-off by NH_4^+ and darkness in cultures with different ages and nutritional backgrounds.

MATERIALS AND METHODS

Strains and media. *R. rubrum* S1-G was grown photoheterotrophically in 1-liter bottles on malate minimal media (20). The nitrogen source was N_2, 2.5 mM NH_4^+ (NH_4-limited cultures), or 5 mM glutamate (25 mM glutamate was used in one experiment for reasons that will be explained below). Cultures (with a 10% inoculum) were used 2 to 3 days after inoculation, unless indicated otherwise. Maximum cellular absorbance (A_{600} of 1.2 to 1.4) was reached between 2 and 3 days of growth on 5 mM glutamate.

Kinetics of nitrogenase switch-off in vivo. *R. rubrum* cultures for NH_4^+ and dark switch-off experiments were forced under argon pressure from the culture bottle into an argon-flushed (300 ml) glass "lollipop" chamber (2 cm in thickness, 15 cm in diameter) into which a stirring bar had been sealed. The cultures were illuminated in this device with four 20-W fluorescent bulbs (two on each side) that were placed 7 cm from the surface for 1 h before the start of all experiments. The total incident light intensity on the chamber was 120 microeinsteins m^{-2} s^{-1}; heating was not a problem. Anaerobic solutions of NH_4^+, glutamate, or inhibitors were added as required. For dark switch-off the chamber was covered with aluminum foil. Cell samples were removed for analysis by forcing liquid up a 20-cm, 18-gauge hypodermic needle into a glass syringe by increasing the argon head pressure in the chamber slightly.

Nitrogenase activity was measured in vivo by acetylene

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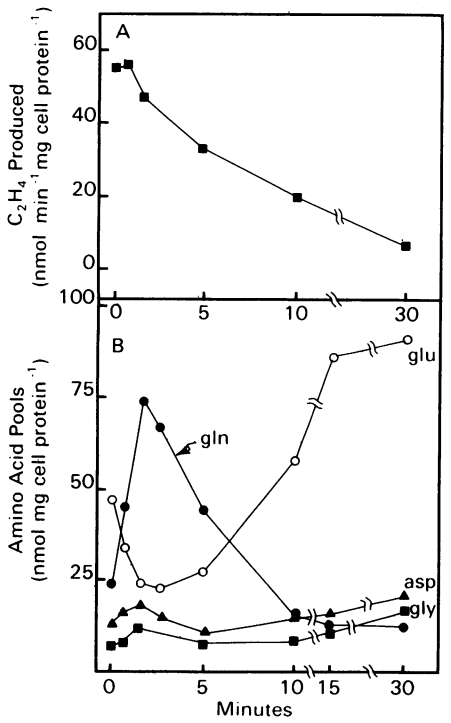

FIG. 1. Effect of NH_4^+ on nitrogenase activity and amino acid pools. NH_4Cl (final concentration, 10 mM) was added anaerobically at time zero to a 3-day-old culture of *R. rubrum* grown on glutamate as a nitrogen source. Nitrogenase activity was measured for 2 min.

reduction of 2-ml fractions in 6.5-ml Fernbach flasks that were degassed and illuminated for 15 min as described previously (28). The time points in the nitrogenase switch-off experiments (see Fig. 2 to 6) represent averages over a 15-min period, which was the time used to establish the rate. When nitrogenase activity was switched off by darkness, 10-ml fractions of the culture were removed in the dark, placed in 25-ml Erlenmeyer flasks, and illuminated for 2 min. Incubation times of longer than 2 min resulted in substantial reactivation of the Fe protein, as noted previously. (10). To determine the kinetics of switch-off by NH_4^+ in relation to the glutamine pool buildup (Fig. 1), 10-ml fractions were also assayed for 2 min.

Extraction of amino acids and nucleotides. At each time point, 10 ml of cells was withdrawn anaerobically (as described above) from the lollipop chamber in a glass syringe (so the cells would remain illuminated) and injected onto a Whatman GF/C glass microfiber filter (diameter, 4.25 cm; Millipore Corp., Bedford, Mass.). The anaerobic environment of the cells on the filter was maintained by previously covering the reservoir above the Millipore filter with Parafilm (American Can Co., Greenwich, Conn.) and flowing high-purity argon through it and the filter. When nitrogenase switch-off was initiated by darkness, the syringe and entire Millipore apparatus was covered with aluminum foil, and the cells were injected through the foil onto the filter. Within 15 s the cells on the filter were washed with 10 ml of degassed phosphate buffer (pH 6.8) and placed in 8 ml of 80% ethanol (80°C). This procedure was used to extract both amino acids and nucleotides.

Analysis of amino acids and nucleotides. The ethanol extracts of *R. rubrum* were dried in a rotary evaporator at 40°C under reduced pressure. For amino acid analysis the residue was suspended in 1.2 ml of a 1:1 mixture of 5% sulfosalicylic acid–sodium citrate amino acid dilution buffer (Beckman Instruments Inc., Palo Alto, Calif.) and allowed to stand overnight at 5°C to ensure solubilization of amino acids and precipitation of any protein solubilized in the ethanol extraction step. The samples were then centrifuged for 5 min (15,600 × *g*) in an Eppendorf microcentrifuge (Brinkmann Instruments Inc., Westbury, N.Y.). Controls showed that glutamine was stable under these conditions. Amino acids were analyzed on an analyzer (model 119C; Beckman Instruments, Inc., Fullerton, Calif.) equipped with an automatic sample injector (1-ml storage coils) and a computing integrator (AA; Beckman). Amino acids were quantitated by comparison of integrated peak areas with those of authentic standards of known concentration. Our column did not separate glutamine and serine, but acidified controls showed serine to be less than 10% of the size of the glutamine pool at any time; the correction was made for each time point.

For nucleotide analysis the dried ethanol extract was solubilized in 1 ml of high-performance liquid chromatographic buffer (HPLC; 10% HPLC grade acetonitrite [J. T. Baker Chemical Co., Phillipsburg, N. J.]–0.03 M KH_2PO_4–10 mM tetrabutylammonium phosphate [Eastman Kodak Co., Rochester, N.Y.]; the pH was adjusted earlier to 2.65 with 1 M phosphoric acid). Nucleotides were analyzed isocratically on a high-performance liquid chromatograph (model 110A; Beckman) equipped with an ion pair column (250 by 4.6 mm; C_{18} Ultrasphere; Altex, Berkeley, Calif.) eluted at 0.5 ml min^{-1} with the buffer described above. Nucleotides were identified spectrophotometrically by comparing retention times and by cochromatography with authentic standards; all nucleotides purchased from Sigma Chemical Co., St. Louis, Mo.

Immunoblotting. To visualize the modification of the Fe protein in cell cultures treated with azaserine or placed in the dark, cell protein was extracted, electrophoresed, Western blotted, and immunostained. Specifically, 2 ml of cells was taken anaerobically from the lollipop vessel and injected onto a glass fiber filter as described above. Liquid nitrogen was immediately poured on the filter, which was then removed, broken into pieces, and put into 0.6 ml of sodium dodecyl sulfate (SDS) sample preparation buffer at 100°C (11). After microcentrifugation for 2 min (15,600 × *g*), samples were electrophoresed on SDS-polyacrylamide gels (11). Protein bands were electroblotted onto nitrocellulose in a cold room at 30 V (70 mA) overnight by the method of Towbin et al. (26). The immunobinding of Fe protein with anti-Fe protein immunoglobulin G (IgG) was followed by the addition of goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad Laboratories, Richmond, Calif.), as described by Hawkes et al. (8).

RESULTS

Amino acid and nucleotide pool changes during NH_4^+ switch-off: glutamate-grown cells. Nitrogenase switch-off by NH_4^+ occurs most reliably in glutamate- (and N_2-) grown *R. rubrum* cultures. The rate of switch-off and the changes in the amino acid pools were compared after the addition of NH_4^+ (Fig. 1). Nitrogenase inactivation was preceded by a rapid increase in the pool size of glutamine, which peaked at 1.5 min. The kinetics of the change in pool size of glutamine and glutamate (Fig. 1) are consistent with suggestions by Nagatani et al. (15) and Weare and Shanmugam (27) that *R. rubrum* utilizes the GS-GOGAT pathway of NH_4^+ assimilation. All the other amino acids, of which aspartate and

glycine are indicative, had lower concentrations that began to increase slowly after 5 min.

Because there have been indications that the degree of NH_4^+ switch-off varies with culture age, we determined if there was any correlation between the rate of switch-off and the amino acid pool sizes as the culture aged. The rate of switch-off in glutamate-grown cultures was most rapid at 2 days (84% inhibition within 7 min after the addition of NH_4^+) and decreased with culture age (Fig. 2). The glutamine pool size decreased in parallel with the rate of switch-off. Both the rate of switch-off and the size of the glutamine pool, however, appeared to be controlled by the intracellular concentration of glutamate (the precursor of glutamine). Because the concentration of glutamate decreased with culture age, it appears from the data shown in Fig. 2 that less glutamine can be synthesized after the NH_4^+ shock, resulting in a slower rate of nitrogenase switch-off.

In Fig. 3 is shown the relationship between nitrogenase activity during two cycles of switch-off and switch-on and the intracellular pool sizes of various nucleotides. The glutamate-grown cultures were treated with 150 μM NH_4^+ which was sufficient to switch off nitrogenase activity, but only for about 30 min, at which time switch-on occurred; this cycle was repeated a second time (Fig. 3A). About half the nucleotides (or nucleotide precursors) separated on our HPLC system increased dramatically in concentration (>100%) following the addition of NH_4^+ (Fig. 3B), while others changed little and some changed not at all (data not shown). It is assumed that if a nucleotide served as a switch-off signal (either as a derivative or along with glutamine), its rise in concentration would precede the inactivation of nitrogenase. In these studies we found no

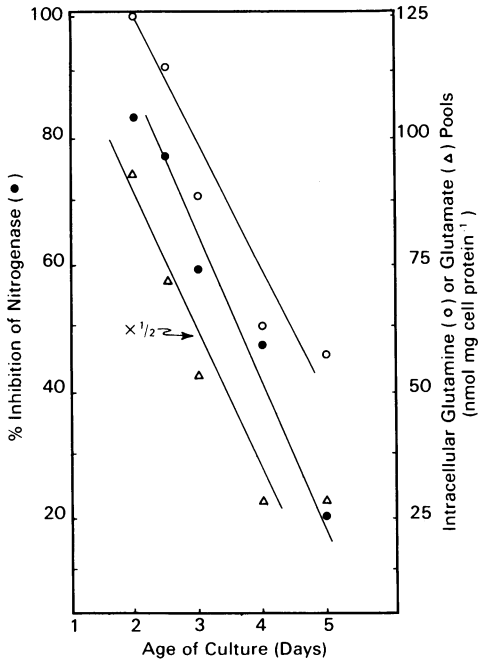

FIG. 2. Relationship between rate of nitrogenase switch-off and intracellular pool sizes of glutamate and glutamine. These parameters were measured as a function of culture age (in days) in glutamate-grown cells (A_{600} = 1.51). The percent inhibition of nitrogenase activity was measured 7 min after 10 mM NH_4Cl was added to the culture. Intracellular glutamate pools were measured at time zero (before the NH_4^+ was added), and glutamine pools were measured 2 min after the addition of NH_4^+.

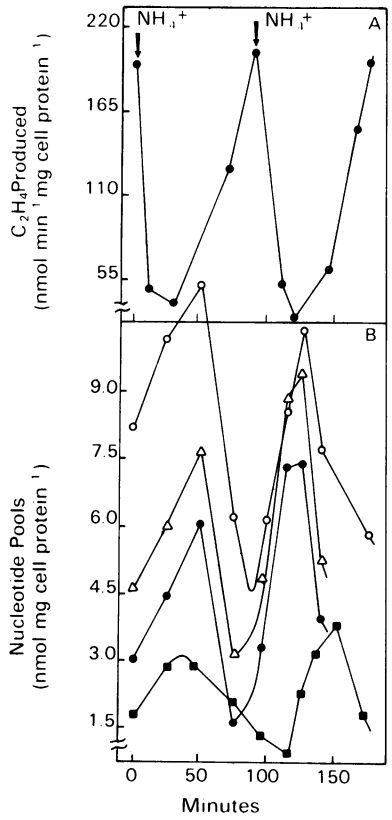

FIG. 3. Relationship between the rate of nitrogenase activity and intracellular pools of selected nucleotides during two switch-off and switch-on cycles. (A) Whole cell nitrogenase activity of 3-day-old glutamate-grown culture. NH_4Cl (final concentration, 150 μM) was added at the times indicated by the arrows. (B) Nucleotide pools. Symbols: △, NAD; ●, AMP; ■, nicotinamide; ○, ADP (UMP and ADP-ribose had the same retention times on our high-pressure liquid chromatograph). Concentrations were calculated from extinction coefficients at 260 nm.

nucleotide that met this criterion. It was clear, however, that nucleotide pools were rapidly replenished by NH_4^+ in N-starved cultures of *R. rubrum* and were depleted just as rapidly when starvation was resumed.

NH_4^+-limited cells. There have been differences of opinion as to whether phototrophs grown on NH_4^+-limited medium are capable of NH^{4+} switch-off. Switch-off was reported under these conditions in *R. rubrum* (4, 6, 17, 29, 30), but others have not been able to reproduce this effect in either this organism or other phototrophs (1, 2, 5, 10, 21, 23, 24, 25), with the exception of *Rhodopseudomonas capsulata* (2). This difference also appears to be related to the age of the culture because nitrogenase activity in NH_4^+-limited cultures was rapidly switched off when they were quite young (1 day old) and not yet fully derepressed, but by day 2, NH_4^+ switch-off was minimal and remained that way (Fig. 4). It must be emphasized that culture age is relative and depends on inoculum size (10% was used here). With a smaller inoculum cultures develop more slowly, and it takes longer to utilize the limiting NH_4^+ and to reach a state of full N starvation.

Analysis of amino acid pools of NH_4^+-limited cultures appears to provide an explanation as to why there was no switch-off in 2-day-old cultures. First, glutamate levels (before NH_4^+ shock) were seen to be lower on day 2 than on

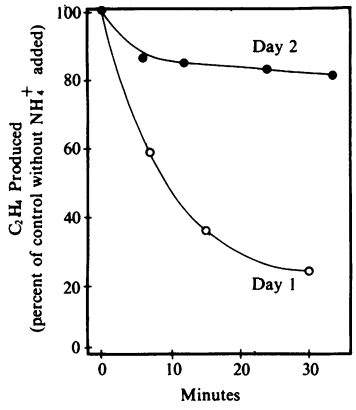

FIG. 4. Effect of culture age on switch-off of nitrogenase activity in NH_4^+-limited cells. At time zero 10 mM NH_4^+ was added anaerobically. Rates of nitrogenase activity without added NH_4^+ (100% controls) by 1- and 2-day-old cultures were 10 and 23 nmol of C_2H_4 produced ml cells^{-1} min^{-1}, respectively. An inoculum size of 10% was used to get NH_4^+ depletion and nitrogenase expression in 1 day.

day 1 (41 versus 80 nmol 10 ml of cells^{-1}); and second, intracellular glutamine concentrations that were produced after NH_4^+ was added were only half those measured on day 1 (data not shown). Furthermore, the glutamine pool in 2-day-old cultures decreased with a half-life of 3.5 versus 9 min in 1-day-old cultures. These observations suggest that nitrogenase from NH_4^+-limited cultures may be switched off, provided that N starvation has not depleted the glutamate pool to the point at which insufficient glutamine can be made. The possibility also exists that the half-life of the glutamine pool must be sufficiently long to activate the switch-off process. In cells grown on 5 mM glutamate these conditions are satisfied for up to 3 or 4 days.

Additional evidence to support a unifying concept of switch-off in NH_4^+-limited and glutamate-grown cultures is seen in Fig. 5 and 6. If the nitrogenase in older NH_4^+-limited cultures of *R. rubrum* is insensitive to NH_4^+ inhibition simply because of the low pool sizes of glutamate, then the addition of glutamate to the culture should render it susceptible to NH_4^+ switch-off. This is exactly what happened in a 3-day-old, NH_4^+-limited culture (Fig. 5A). Glutamate pool levels became very high, and when NH_4^+ was added the glutamine pool rose rapidly (Fig. 5B) to a level that was sufficient to initiate switch-off. Controls without added glutamate showed no switch-off.

Ammonia-insensitive nitrogenase from older NH_4^+-limited cultures can even be switched off in the absence of

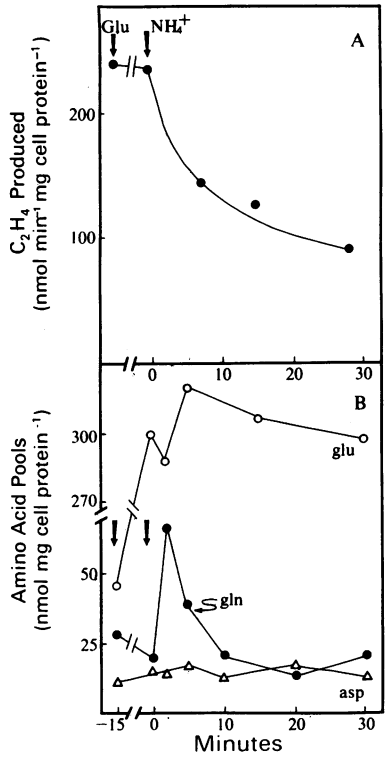

FIG. 5. Effect of added glutamate on NH_4^+ switch-off of nitrogenase and amino acid pools in NH_4^+-limited cells. Glutamate (5 mM) was added anaerobically to a 3-day-old culture (A_{600} = 1.32) in a lollipop flask 15 min before NH_4^+ (10 mM) was added, as indicated by the arrows. Cell samples were taken at the times indicated for whole cell nitrogenase activity (A) and analysis of amino acid pools (B). The arrows at −15 and 0 min indicate the additions of glutamate and NH_4^+, respectively.

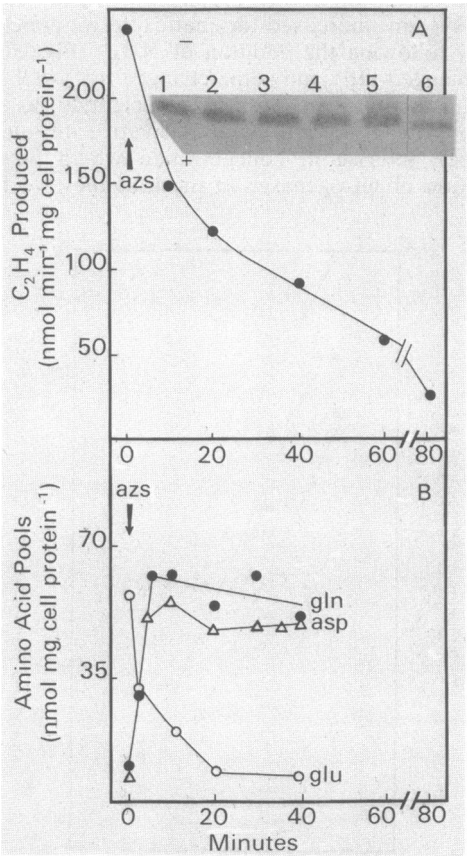

FIG. 6. Effect of azaserine on nitrogenase activity and the amino acid pools in NH_4^+-limited cells. At time zero 200 μM azaserine was added to a 2-day-old culture. (A) Whole cell nitrogenase activity. (Inset to panel A) Immunoblots with antiserum against *R. rubrum* Fe protein of SDS-extracts separated by SDS-polyacrylamide gel electrophoresis. Lane 1, cells taken before azaserine was added to the culture; lanes 2 to 5, cell samples taken at 10, 20, 40, and 60 min, respectively, after the addition of azaserine; lane 6, purified inactive Fe protein control. (B) Amino acid pools of cells extracted after the addition of azaserine (azs).

NH_4^+, if a way is provided to increase the size of the glutamine pool of the cell. GOGAT activity was blocked with azaserine to achieve this purpose. The addition of 200 μM azaserine at time zero caused switch-off in vivo (Fig. 6A) because of modification (and inactivation) of the Fe protein (6), as seen by the appearance of a second, slower migrating subunit (Fig. 6, inset). Azaserine also caused a rapid and sustained rise in the glutamine pool (Fig. 6B). The rise in glutamine levels was always accompanied by a parallel increase in the aspartate pool and a concomitant decrease in intracellular glutamate levels. The observation of switch-off in cells with a depleted glutamate pool was good evidence that glutamate itself is not directly involved in the activation of this process when glutamine is present.

Testing molecules derived from glutamine as the switch-off signal. Because glutamine is the precursor of 12 to 15 different nitrogen-containing molecules, it is possible that one of these molecules, rather than glutamine itself, is directly involved in switch-off. When tested, several classes of these derivative compounds could indeed initiate switch-off, but all activity was abolished in the presence of MSX (Table 1). While MSX inhibits many enzymes, all the results could be explained if these molecules were first deaminated and the amino group was used for the synthesis of glutamine by GS. Thus, the inhibition of GS by MSX would prevent switch-off by amino acids (represented by histidine), pyrimidines, and purines. The inability of nicotinamide to switch off nitrogenase was unexpected because *R. rubrum* can use it as an N source, which suggests that *R. rubrum* has the ability to deaminate this molecule (D. C. Yoch, unpublished data).

Dark switch-off. When dark switch-off of nitrogenase activity was examined in *R. rubrum*, we found essentially the same effects of culture age and nitrogen nutrition that were so readily apparent for NH_4^+ switch-off. Young cultures grown on 5 mM glutamate were switched off more rapidly in the dark than were older cells, and we confirmed the observation of Paul and Ludden (21) that NH_4^+-limited cultures are not susceptible to dark switch-off (Fig. 7A).

To determine if glutamine had a role in dark switch-off, cells were exposed to MSX before they were placed in the dark (Fig. 7B). While this GS inhibitor abolishes NH_4^+ switch-off, it had no effect (under a variety of conditions tested) on dark switch-off and switch-on. In addition, the amino acid pools remained essentially unchanged (Fig. 8A) while switch-off took place in the dark. Results of both lines of experimentation indicated that glutamine is not required for dark switch-off of nitrogenase activity. The appearance of the slower migrating subunit (Fig. 7B, inset) after the cells are placed in the dark (Fig. 7B, lane 2) and its subsequent disappearance (Fig. 7B, lane 3) in the light confirms that inhibition of activity in the dark is due to the modification of an Fe protein subunit.

Finally, we examined a large number of intracellular nucleotides, some of which could not be identified; but the concentration of none of them changed rapidly enough after a shift from light to dark to serve as a switch-off signal. The change in the pool sizes of some of the major nucleotides are shown in Fig. 8B and are indicative of a large number of minor components.

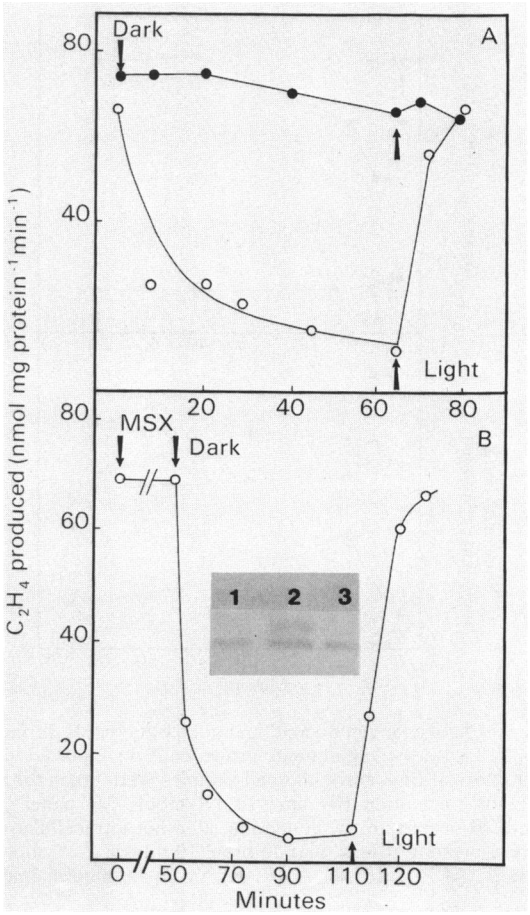

FIG. 7. Dark switch-off of nitrogenase activity. (A) Comparison of the effects of darkness on 2-day-old *R. rubrum* cultures grown on limiting NH_4^+ (●) or glutamate as a nitrogen source (○). (B) Effect of MSX. Cells were grown on 25 mM glutamate as a nitrogen source (A_{600} = 0.8). MSX (300 μM) was added, and the culture was placed in the dark as indicated by the arrows. (Inset to panel B) Immunoblots with antiserum against *R. rubrum* Fe protein of SDS extracts separated by SDS-polyacrylamide gel electrophoresis. Fe protein is from cells taken from a lollipop vessel after 50 min in full light plus MSX (lane 1), after 20 min in dark (lane 2), and after cells were returned to light for 12 min (lane 3).

TABLE 1. Survey of nitrogen-containing molecules for their ability to switch off nitrogenase activity[a]

Inhibitor	% switch-off[b]	
	−MSX	+MSX
NH_4^+	90 (3)	0 (3)
Glutamine	77.5 (2)	96.4 (2)
Asparagine	100 (2)	76.5 (2)
Histidine	85.4 (6)	0 (2)
Adenine	83.7 (10)	0 (10)
Cytosine	76.0 (3)	0 (3)
p-Aminobenzoate	0 (6)	0 (6)
Nicotinamide	0 (6)	0 (6)
Nicotinic acid	0 (6)	0 (6)

[a] *R. rubrum* was grown on N_2 for 2 days to a turbidity (Klett reading) of approximately 100. The inhibitor molecules (final concentration, 2.5 mM) were added 30 min after acetylene (4.5%) was added, while MSX (final concentration, 50 μM) was added 10 min before acetylene was added.

[b] The activity of the controls (no addition) ranged from 30 to 50 μmol of ethylene produced 1.5 ml culture^{-1} min^{-1}. Percent switch-off was based on the average rate of C_2H_2 reduction 0 to 30 min after the addition of the inhibitor. Values represent the mean; numbers in parentheses represent the number of replica samples.

DISCUSSION

Observations that glutamine pools increase rapidly in size prior to NH_4^+ switch-off suggest that this molecule could

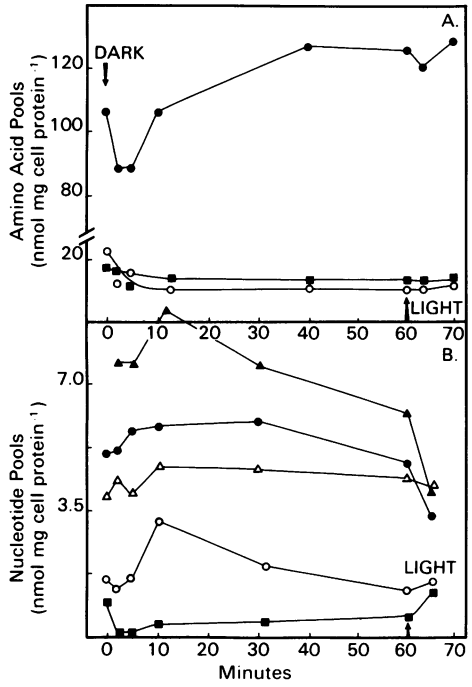

FIG. 8. Changes in amino acid and nucleotide pools during dark switch-off. Two-day-old glutamate-grown cells were placed in complete darkness at time zero, and cell samples were taken for amino acid (A) or nucleotide (B) analysis. Symbols for panel A: ●, glutamate; ■, aspartate; ○, glutamine; all other intracellular amino acids were present at less than 10 nmol 10 ml cell^{-1}. Symbols for panel B: △, ADP-UMP; ●, AMP; ▲, NAD; ○, nicotinamide; ■, ATP.

serve as the signal for this process. In contrast, however, dark-induced Fe protein inactivation is not related to or dependent on amino acid pool changes. The role of glutamine in the initiation of switch-off remains unknown, but several possibilities exist. Glutamine (or a derivative) could serve as an effector to activate the Fe protein-inactivating enzyme or it could be required for the synthesis of NAD, which is used as a substrate for ADP ribosylation (and inactivation) of the Fe protein (12). Our kinetic data (Fig. 3) did not show a fast enough rise in NAD levels (compared with the rate of inhibition) to suggest that glutamine initiates switch-off simply by raising intracellular NAD levels. Furthermore, no evidence was found that any of the other nucleotides we could separate on an isocratic HPLC system changed concentration fast enough after the addition of NH_4^+ to serve as the signal for the switch-off (Fig. 3B). Finally, the same changes in nucleotide concentration were observed in NH_4^+-limited cells, in which switch-off did not readily occur (data not shown).

The correlation of NH_4^+ switch-off with culture age, nitrogen nutrition of the cells, and amino acid pool sizes has led to a unifying concept of what is required for this process to occur. Equally important as the glutamine pool is the pool size of glutamate, which is the precursor of glutamine. We found that when cultures grown on 5 mM glutamate got older, the rate of NH_4^+ switch-off decreased because in the early stages of N starvation the sizes of the glutamate pools fell dramatically. Because NH_4^+-limited cultures are by definition N starved, the sizes of the glutamate pools are always too low (except in very young cultures) to lead to glutamine pools sufficiently high (following the NH_4^+ shock) to trigger the switch-off mechanism. The absence of an adequate glutamate pool in NH_4^+-limited cells may explain the need that Preston and Ludden (23) found for adding α-oxoglutarate along with NH_4^+ to initiate nitrogenase switch-off. Presumably, the reason that nitrogenase in N_2-grown cells is susceptible to NH_4^+ inhibition (17) is that glutamate pools are always sufficiently high to produce adequate levels of glutamine when NH_4^+ is added to the cell environment.

Dark switch-off of nitrogenase activity (in contrast to NH_4^+ switch-off) appears to be regulated by a different set of effectors, as glutamine levels are essentially unaffected by darkness. This observation was reinforced by the fact that MSX did not block dark-dependent switch-off. This is in contrast with the results of report by Kanemoto and Ludden (10), who showed that MSX decreased the rate of switch-off by threefold. To ensure that our culture conditions were similar to theirs, we increased the glutamate content of the media from 5 to 25 mM, but MSX remained ineffective in blocking dark switch-off. The uptake of MSX did not appear to be a problem because it inhibited GS activity in vivo. Our data suggest that glutamine does not play a role as an effector in triggering dark-induced switch-off of nitrogenase activity.

In the event that the change in concentration of a nucleotide or a nucleotidelike molecule might be the signal for dark switch-off, the concentrations were monitored after the culture was darkened. While only six nucleotides are shown in Fig. 8B, there was none with a rate of change in pool size that differed from those shown in Fig. 8. No correlation seems to exist between nucleotide pool sizes and nitrogenase switch-off, a finding that is consistent with those of previous investigators (19, 21, 29). Because the nitrogen nutrition of *R. rubrum* is an important factor in determining whether it is capable of dark switch-off, there may be a combination of a nitrogen-containing molecule and a dark-generated (or depleted) effector, that together initiate switch-off.

ACKNOWLEDGMENTS

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