Biochem. J. (1984) **224**, 961–969
Printed in Great Britain

Adenine nucleotide levels in *Rhodospirillum rubrum* during switch-off of whole-cell nitrogenase activity

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(*Received 20 June 1984/Accepted 5 September 1984*)

Adenine nucleotide pools were measured in *Rhodospirillum rubrum* cultures that contained nitrogenase. The average energy charge {([ATP]+½[ADP])/([ATP]+[ADP]+[AMP])} was found to be 0.66 and 0.62 in glutamate-grown and N-limited cultures respectively. Treatment of glutamate-grown cells with darkness, ammonia, glutamine, carbonyl cyanide *m*-chlorophenylhydrazone, or phenazine methosulphate resulted in perturbations in the adenine nucleotide pools, and led to loss of whole-cell nitrogenase activity and modification *in vivo* of the Fe protein. Treatment of N-limited cells resulted in similar changes in adenine nucleotide pools but not enzyme modification. No correlations were found between changes in adenine nucleotide pools or ratios of these pools and switch-off of nitrogenase activity by Fe protein modification *in vivo*. Phenazine methosulphate inhibited whole-cell activity at low concentrations. The effect on nitrogenase activity was apparently independent of Fe protein modification.

Biological nitrogen fixation is an energetically expensive process, and N-fixing organisms have evolved mechanisms to conserve energy by regulating the synthesis and activity of the nitrogenase enzyme complex. At the transcriptional level, synthesis of the enzymes is controlled by repression in the presence of fixed nitrogen or O_2 (Brill, 1980). The photosynthetic bacterium *Rhodospirillum rubrum* exhibits a second type of regulation at the enzymic level. Nitrogenase activity *in vivo* is rapidly and reversibly inhibited by exposure to fixed nitrogen in the form of NH_4^+, glutamine or asparagine (Gest *et al.*, 1950; Neilson & Nordlund, 1975; Schick, 1971). This loss of whole-cell activity, termed 'switch-off' (Zumft & Castillo, 1978), has been correlated with the appearance of an inactive form of the purified enzyme complex that results from covalent modification of the *Rhsp. rubrum* Fe protein (Gotto & Yoch, 1982; Kanemoto & Ludden, 1984; Ludden *et al.*, 1982). The modifying group consists of pentose, phosphate, adenine and unidentified components (Ludden & Burris, 1978; Ludden *et al.*, 1982). Demodification of Fe protein and recovery of activity is observed *in vitro* upon addition of MgATP, free bivalent metal, and a chromatophore-associated fraction containing an activating enzyme for the Fe protein (Ludden & Burris, 1976, 1979; Nordlund *et al.*, 1977). Switch-off has also been reported in other photosynthetic bacteria (Alef & Kleiner, 1982; Bognar *et al.*, 1982; Haaker *et al.*, 1982; Hallenbeck *et al.*, 1982; Hillmer & Gest, 1977; Howard *et al.*, 1983; Jones & Monty, 1979; Zumft & Castillo, 1978).

The 'signal' for switch-off of nitrogenase has not yet been identified. Glutamine, NH_4^+ and asparagine have little effect on the activity of the nitrogenase complex *in vitro* at concentrations that cause switch-off *in vivo* (Neilson & Nordlund, 1975). The effect must therefore be indirect. In *Rhsp. rubrum*, Yoch & Gotto (1982) have suggested that the availability of ATP influences the regulatory effect of NH_4^+ on whole-cell activity. It is therefore of interest to quantify intracellular adenine nucleotide pools during switch-off to determine whether correlations exist with the loss of whole-cell nitrogenase activity and enzyme modification.

Abbreviations used: Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; SDS, sodium dodecyl sulphate; PMS, phenazine methosulphate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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Rapid switch-off is observed in cells that have been grown with N_2 or glutamate as the N-source but not under conditions of nitrogen starvation (Sweet & Burris, 1981). In the present paper, changes in adenine nucleotide pools, loss of whole-cell nitrogenase activity, and modification *in vivo* of the Fe protein are measured in *Rhsp. rubrum* under both growth conditions.

Materials and methods

Growth of bacteria

Rhodospirillum rubrum (A.T.C.C. 11170) was grown on Ormerod's medium (Ormerod *et al.*, 1961) with the modifications described by Kanemoto & Ludden (1984). For cells grown with limiting N, L-glutamate was replaced with 1.5mM-NH_4Cl.

Inhibitor treatment

Glass centrifuge bottles (200ml) were capped with rubber stoppers containing a sampling tube and inlet and outlet lines for gases. The bottles were evacuated and flushed with helium three times before use. When *Rhsp. rubrum* nitrogenase activity reached at least 400nmol of acetylene reduced·h^{-1}·ml^{-1}, samples of cells were removed from the 500ml fermenters and placed in the prepared centrifuge bottles. These cultures were then placed in the water bath of a Gilson respirometer, where they were illuminated from below. The cultures were gently shaken in the 30°C water bath, and filter-sterilized helium was blown over the cultures throughout the experiments. All additions were handled anaerobically via the sampling tubes, and the cultures were vigorously shaken to disperse the inhibitors. The cultures were then returned to a gentle-shaking mode in the illuminated respirometer. At the beginning of dark treatment, culture bottles were wrapped in two or three layers of aluminium foil to exclude all light and then returned to the respirometer.

Adenine nucleotide extraction and assay

Adenine nucleotides were determined by the firefly-luciferin–luciferase assay (Strehler, 1968) after extraction of samples with 0.3M-$HClO_4$ at 0°C with constant agitation. After 30min, the extracts were neutralized with KOH to which 0.8M-Tricine was added. The final concentration of Tricine was 100mM and the pH was 7.4. Precipitated $KClO_4$ was removed by centrifugation. All extractions were performed in duplicate. ADP and AMP were determined as described by Kimmich *et al.* (1975), except that neutralization was to the Metacresol Purple end point at pH7.8. An Aminco Chem-Glow photometer with integrator was used to monitor the bioluminescence. Two to five portions of each sample were analysed, and a standard curve for ATP was run for each experiment. Culture medium that had been filtered to remove cells was also tested for adenine nucleotides. No significant amount of nucleotide was found in the medium before or after treatment with darkness or inhibitors. Recoveries of ATP, ADP, and AMP during the extraction procedure were determined by using internal standards and were 99%, 85% and 94% respectively. Energy charge was calculated as:

$$\frac{[ATP]+\frac{1}{2}[ADP]}{[ATP]+[ADP]+[AMP]}$$

Nitrogenase assay in vivo

Nitrogenase activity was monitored by the whole-cell acetylene-reduction method (Koch & Evans, 1966; Stewart *et al.*, 1967) as described by Kanemoto & Ludden (1984).

Rapid extraction of Fe protein

The extraction and immunoprecipitation procedure of Nordlund & Ludden (1983) was used with modifications suggested by Kanemoto & Ludden (1984), except that deoxyribonuclease and ribonuclease were not included.

SDS/polyacrylamide-gel electrophoresis

The SDS/polyacrylamide-gel electrophoresis system of Laemmli (1970) was used, except that the *NN'*-methylenebisacrylamide cross-linker concentration was decreased to 0.129% (w/v) in a 10% (w/v) acrylamide gel to improve resolution of the Fe protein subunits. The samples from rapid extraction of *Rhsp. rubrum* cells were heated with SDS-gel 'cocktail' (Laemmli, 1970) in a boiling-water bath for 1.5min to facilitate solubilization of the protein pellets before electrophoresis. Protein subunits were stained with Coomassie Brilliant Blue. After destaining, Fe protein subunits were quantified by scanning the gel with a Zeineh soft laser densitometer with integrator function (Biomed Instruments). The percentage of inactive Fe protein was calculated as described previously (Kanemoto & Ludden, 1984) by dividing the amount of upper subunit by the average of the amounts of upper and lower subunits and multiplying by 100.

Uptake of PMS

Uptake of PMS by *Rhsp. rubrum* and *K. pneumoniae* was calculated on the basis of ΔA_{387} (Zaugg, 1964). PMS of known concentration was added anaerobically to cultures of *Rhsp. rubrum* and *K. pneumoniae*. After 5min the yellow colour had disappeared and the cultures were centrifuged at 10000*g* for 10min to pellet the cells. Samples of

Table 1. *Adenine nucleotide pools in Rhsp. rubrum cells that contain active nitrogenase*
Values reported for pool sizes represent the ranges observed in extracts from 14 different glutamate-grown cultures or five different NH_4^+-limited cultures.

Growth conditions (N source)	Adenine nucleotide pools (nmol/mg of protein) ATP	ADP	AMP	Total	ATP/ADP ratio*	Energy charge*
Glutamate (27mM)	3.7±1.8	2.85±1.17	0.93±0.32	7.44±2.49	1.17	0.67
Limiting NH_4^+ (1.5mM)	4.7±1.42	3.6±0.37	2.3±1.47	10.6±1.38	1.32†	0.61†

* Calculated from ΣATP, ΣADP and ΣAMP values as described in the Materials and methods section.
† Average of individual determinations from five different cultures.

the media were analysed for A_{387} after samples were shaken vigorously in air to re-oxidize all of the PMS. Medium without cells was treated with PMS in an identical manner for comparison.

Bacteriochlorophyll assay

Bacteriochlorophyll was determined in whole cells by using the method of Clayton (1968).

Protein assay

The cells from 10ml samples of cultures were collected by filtration on to Whatman GF/C glass-fibre filters. After decolorization by ethanol washing, the filters were placed in 3ml of 1M-NaOH and heated for 5–10min in a boiling-water bath. Whole-cell protein was measured by the microbiuret method (Goa, 1953), with bovine serum albumin as a standard.

Modification of Fe protein in vivo

A change in the subunit composition of *Rhsp. rubrum* Fe protein on SDS/polyacrylamide-gel electrophoresis has been correlated with modification of the enzyme *in vivo* (Preston & Ludden, 1982; Kanemoto & Ludden, 1984). This subunit conversion was used as a basis to quantify the modification of Fe protein during treatment of whole cells with inhibitors of nitrogenase activity *in vivo*.

Reagents

L-Glutamate, DL-malate and Mops were from United States Biochemicals or Sigma. Carborundum (150 grit powder) used in rapid extractions was from Fischer Scientific Co. Firefly extract (FLE-50) was obtained from Sigma. Dithionite was from J. T Baker Co. Acrylamide, *NN'*-methylenebisacrylamide, SDS and Coomassie Brilliant Blue were from Bio-Rad Laboratories. All other chemicals were obtained from Sigma. Acetylene was generated by adding CaC_2 to water.

Results

Adenine nucleotide pools and energy charge in Rhsp. rubrum

ATP, ADP and AMP were measured in extracts of cells grown on NH_4^+ or glutamate. Cell samples were taken when cells were growing in exponential phase in the light (Table 1). The total adenine nucleotide content generally varied by less than 20% during the course of an experiment. The pools measured were well within a range of values reported in other N-fixing bacteria (Upchurch & Mortenson, 1980). Total adenine nucleotide pool measurements agreed with the values reported by Schön (1969) for *Rhsp. rubrum* grown with high concentrations of NH_4^+. The values for ATP pools in cells that have active nitrogenase (Table 1) are about 40% lower than values for ATP pools in cells grown on NH_4^+ (Table 1; Schön, 1969). Similarly, the ATP/ADP ratio and energy charge were also significantly lower in glutamate-grown or NH_4^+-limited cells (about 60 and 15% respectively). Changes in the ATP/ADP ratio with various treatments of whole cells generally mirrored changes in the ATP pool. Energy charge also reflected changes in the ATP pool, but varied to a much smaller extent (results not shown).

Effects of treatment with an uncoupler of photophosphorylation

CCCP has been reported to uncouple photophosphorylation in *Rhsp. rubrum* chromatophores at a concentration of 20μM (reviewed by Baltscheffsky, 1978). The addition of CCCP to glutamate-grown cells (Figs. 1*a* and 1*b*) resulted in a rapid drastic decrease in the ATP pool. Adenine nucleotide pools were not quantified by the bioluminescence assay in NH_4^+-limited cells treated with CCCP. However, radiolabelling of nucleotide pools indicates a similar rapid loss (T. D. Paul & P. W. Ludden, unpublished work). Regardless of the culture N source during growth, CCCP inhibited whole-cell nitrogenase activity more than 90%

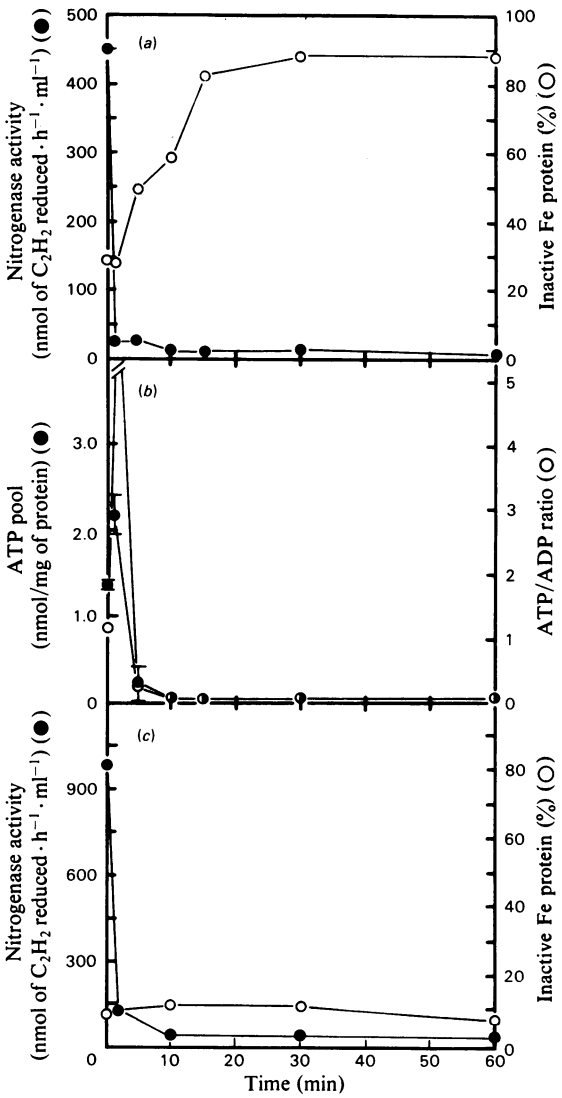

Fig. 1. *Effects of CCCP treatment on nitrogenase and adenine nucleotide pools*

At zero time, 20 μM-CCCP was added anaerobically to illuminated cultures of *Rhsp. rubrum* that had been grown with either glutamate or limiting NH_4^+ as the N source. (*a*) Glutamate-grown cells: ●, whole-cell nitrogenase activity; ○, degree of modification of Fe protein *in vivo*. (*b*) Glutamate-grown cells: ●, ATP pool size; ○, ATP/ADP ratio. (*c*) NH_4^+-limited cells: ●, whole-cell nitrogenase activity; ○, degree of modification of Fe protein *in vivo*. The total adenine nucleotide pool and bacteriochlorophyll content were 5.3 nmol/mg of protein and 22.3 nmol/mg of protein respectively in the glutamate-grown culture. The bacteriochlorophyll content of the NH_4^+-limited culture was 25.1 nmol/mg of protein.

within 1 min (Figs. 1*a* and 1*c*). CCCP did not inhibit the acetylene-reduction activity of nitrogenase *in vitro*. The effect of CCCP on modification of the Fe protein was significantly different under the two growth conditions (Figs. 1*a* and 1*c*). The proportion of inactive Fe protein increased to about 90% in cells that were grown with glutamate. This modification of the Fe protein was significantly slower than the loss of whole-cell nitrogenase activity. Kanemoto & Ludden (1984) estimated a $t_{\frac{1}{2}}$ of 12 min for incorporation of ^{32}P into the upper subunit of Fe protein in PMS-treated cells. No increase in the proportion of inactive Fe protein was observed in NH_4^+-limited cells (Fig. 1*c*).

In control experiments, *Rhsp. rubrum* grown with glutamate as the N source was illuminated and received no addition of inhibitor. Whole-cell nitrogenase activity remained high, and little change was observed in the pools of adenine nucleotides. These cells exhibited a low proportion of inactive (modified) Fe protein that did not change with time. Control experiments with NH_4^+-limited cells gave essentially identical results (not shown).

Dark treatment

Nitrogenase requires MgATP and reducing equivalents for activity (Ljones, 1979). Photophosphorylation has been shown to provide sufficient ATP to support nitrogenase activity in *Rhsp. rubrum* (Yoch & Arnon, 1970). Even dim light can support significant synthesis of ATP (Ramirez & Smith, 1968), so precautions were taken in sampling dark cultures to minimize photophosphorylation. Fig. 2 illustrates the changes that occurred in glutamate-grown and NH_4^+-limited cells on dark treatment. A slight (10%) transient decrease in the ATP pool was noted in both cultures immediately after they had been placed in total darkness (results not shown). Recovery was rapid, and within 30 s the ATP pool then increased significantly above the starting value (Fig. 2*b*). Fluctuations continued to occur for at least 30 min (Fig. 2*b* and 2*d*). However, the ATP pool remained high for the duration of the experiment in both glutamate-grown and NH_4^+-limited cells. Because illumination is required for whole-cell nitrogenase activity in *Rhsp. rubrum* under anaerobic conditions when malate is the carbon source (Ludden & Burris, 1981; Schick, 1971), assay times were short (2.5 min) to minimize re-activation of dark-inactivated nitrogenase (Kanemoto & Ludden, 1984). Whole-cell activity in glutamate-grown cells decreased by about 80% over 60 min, with a concomitant increase in the proportion of inactive Fe protein to about 73% (Fig. 2*a*). Whole-cell activity and inactive Fe protein remained constant

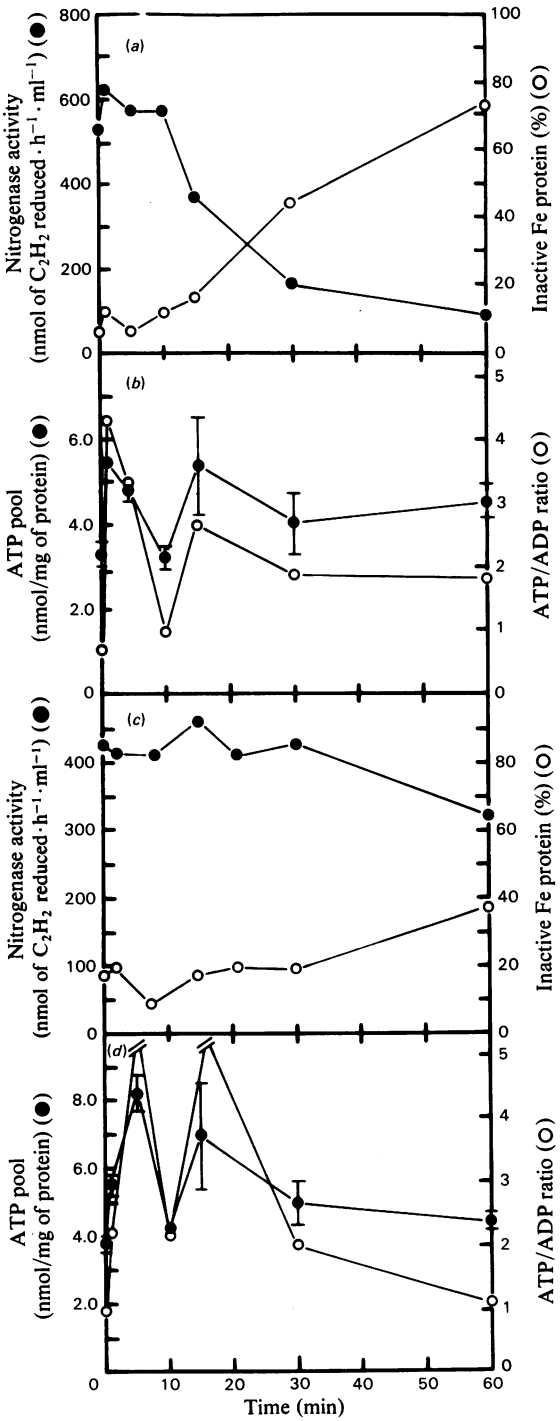

Fig. 2. *Effects of darkness on nitrogenase and adenine nucleotide pools*

At zero time, glutamate- or NH_4^+-limited cultures of *Rhsp. rubrum* were placed in total darkness. (*a*) Glutamate-grown cells: ●, whole-cell nitrogenase activity; ○, degree of modification of Fe protein *in vivo*. (*b*) Glutamate-grown cells: ●, ATP pool size; ○, ATP/ADP ratio. (*c*) NH_4^+-limited cells: ●, whole-cell nitrogenase activity; ○, degree of modification of Fe protein *in vivo*. (*d*) NH_4^+-limited cells: ●, ATP pool size; ○, ATP/ADP ratio. The total adenine nucleotide pool and bacteriochlorophyll content were 8.1 nmol/mg of protein and 29.1 nmol/mg of protein respectively in the glutamate-grown culture and 12.0 nmol/mg of protein and 30.0 nmol/mg of protein respectively in the NH_4^+-limited culture.

for at least 30 min in NH_4^+-limited cells (Fig. 2*c*). After 60 min, a slight decrease in whole-cell activity was observed, with a modest increase in the proportion of inactive Fe protein.

Effects of NH_4^+ or glutamine shock

Switch-off of whole-cell nitrogenase activity in glutamate- or N_2-grown *Rhsp. rubrum* by NH_4^+ or glutamine is well known (Gest *et al.*, 1950; Neilson & Nordlund, 1975; Ormerod *et al.*, 1961; Schick, 1971; Sweet & Burris, 1981). This loss of whole-cell activity occurs at low concentrations of NH_4^+ and glutamine, which have no effect on the nitrogenase activity *in vitro* and which are insufficient to uncouple photophosphorylation in chromatophores (Neilson & Nordlund, 1975). Switch-off does not occur in *Rhsp. rubrum* that has been starved for nitrogen (Sweet & Burris, 1981; Yoch & Cantu, 1980). We monitored intracellular adenine nucleotide pools during NH_4^+ or glutamine shock in both glutamate-grown and NH_4^+-limited cells for comparison with nitrogenase activity and Fe protein modification. The effects of NH_4^+ shock are illustrated in Fig. 3, and glutamine shock gave essentially identical results. We observed a 45% decrease in whole-cell activity over a 60 min period in glutamate-grown cells (Fig. 3*a*). The proportion of inactive Fe protein increased to about 50% over the same period. The rate of switch-off was rather slow in comparison with N_2-grown cells (Gest *et al.*, 1950; Neilson & Nordlund, 1975; Schick, 1971). The ATP pool decreased by up to 50% in the first 30 s of treatment (data point not shown), but recovered rapidly and returned to a high value (Fig. 3*b*). NH_4^+-limited cells did not switch off, and the proportion of inactive Fe protein *in vivo* remained low (Fig. 3*d*). The ATP pool decreased transiently about 15% (data point not shown), but recovered within 1 min; it gradually decreased by about 35% over a 60 min period (Fig. 3*d*).

Effects of treatment with PMS

PMS is an electron-transfer agent that has been reported to accelerate photophosphorylation in chromatophores from *Rhsp. rubrum* (Baltscheffsky, 1978). It has also been used as an oxidizing agent to oxidize the purified Fe protein reversibly. Activity is recovered on reduction of the enzyme (Ludden & Burris, 1979). PMS is reduced by dithionite

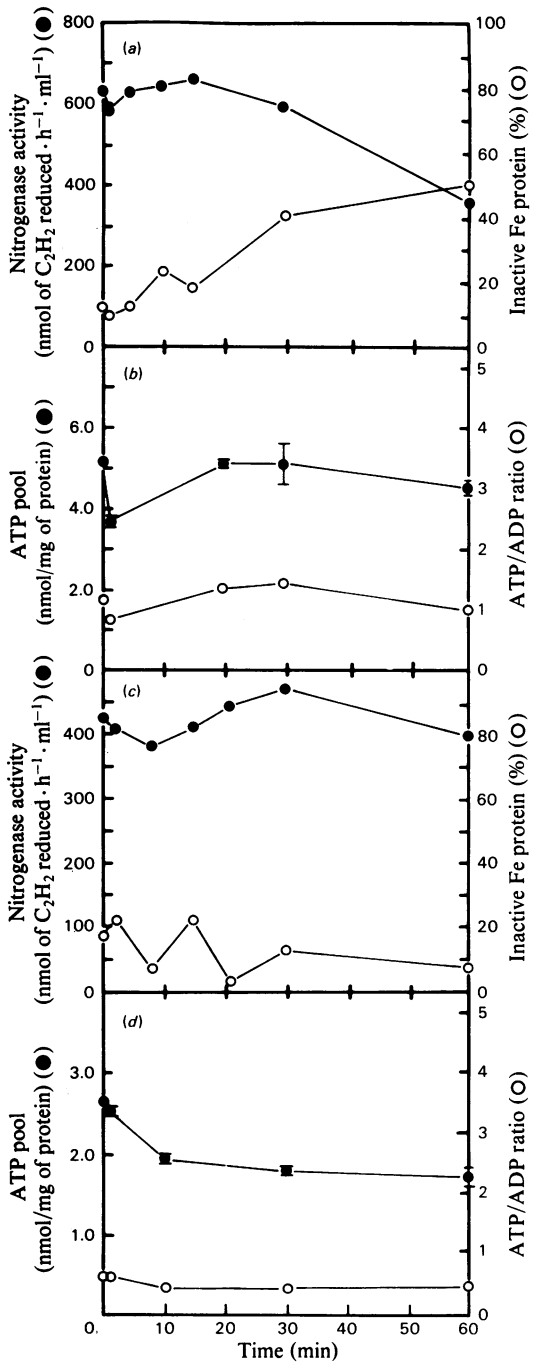

Fig. 3. *Effects of ammonia shock on nitrogenase and adenine nucleotide pools*

At zero time, 2mM-NH_4Cl and 2mM-α-oxoglutarate were added anaerobically to illuminated cultures of *Rhsp. rubrum* that had been grown with either glutamate or limiting NH_4^+ as the N source. [α-Oxoglutarate has been reported to stimulate NH_4^+ switch-off in *Rhsp. rubrum* (Ramirez & Smith, 1968).] (*a*) Glutamate-grown cells: ●, whole-cell nitrogenase activity; ○, degree of modification of Fe protein *in vivo*. (*b*) Glutamate-grown cells: ●, ATP pool size; ○, ATP/ADP ratio. (*c*) NH_4^+-limited cells: ●, whole-cell nitrogenase activity; ○, degree of modification of Fe protein *in vivo*. (*d*) NH_4^+-limited cells: ●, ATP pool size; ○, ATP/ADP ratio. The total adenine nucleotide pool and bacteriochlorophyll content were 10.7nmol/mg of protein and 22.2nmol/mg of protein respectively in the glutamate-grown culture and 12.2nmol/mg of protein and 28.9nmol/mg of protein respectively in the NH_4^+-limited culture.

(Zaugg, 1964), and we found that the prereduced compound at 100 μM had no effect on nitrogenase activity *in vitro*. However, both oxidized and reduced PMS (100 μM) inhibited whole-cell activity rapidly and completely. Fig. 4 shows the effects of PMS addition. In *Rhsp. rubrum* cultures grown with glutamate as the N source, a rapid loss of whole-cell activity was observed for PMS-treated cultures (Fig. 4*a*). The proportion of inactive Fe protein increased to nearly 100% in this experiment; however, modification occurred at a much slower rate than loss of whole-cell activity. Loss of whole-cell activity also occurred in NH_4^+-limited cultures, but was not accompanied by modification of the Fe protein (Fig. 4*c*). ATP pools responded to PMS addition with a rapid transient decrease (data point not shown) and recovered to varying extents. In three experiments, the ATP pool in glutamate-grown cells reached a new value that was 40% lower than the starting value (Fig. 4*b*). In two experiments using NH_4^+-limited cells, the ATP pool decreased by 46 and 48% respectively (Fig. 4*d*).

Whole-cell nitrogenase activity was sensitive to very low concentrations of PMS; complete inhibition was consistently observed at values as low as 2 μM.

Discussion

It is evident that changes in intracellular adenine nucleotide contents are not consistently correlated with switch-off and modification of Fe protein. We observed a transient decrease in the ATP pool in *Rhsp. rubrum* on addition of NH_4^+, but recovery of the pool was rapid, and no significant decrease in nitrogenase activity or modification occurred until after that time. In addition, treatment with glutamine gave essentially identical results. The ATP pool actually increased during dark treatment, and inactivation of the Fe protein still occurred. Ramirez & Smith (1968) have reported that ATP decreases very slowly under dark, anaerobic, conditions in *Rhsp. rubrum* grown with malate as the carbon source unless they have been pretreated to deplete the

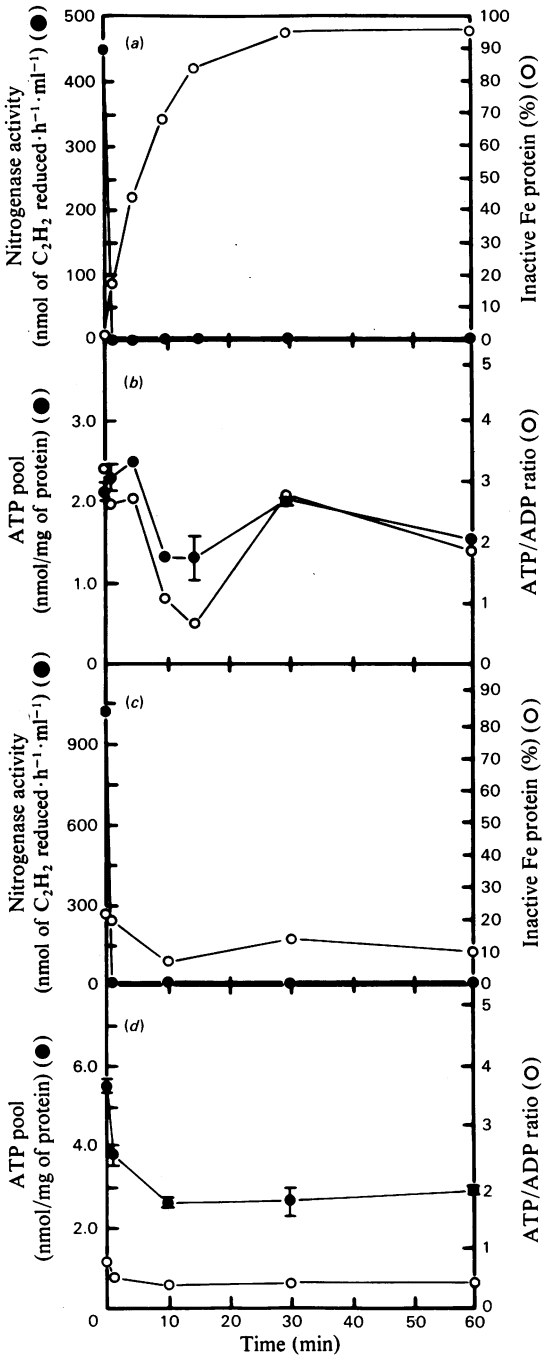

Fig. 4. *Effects of PMS treatment on nitrogenase and adenine nucleotide pools*

At zero time, 100 μM-PMS was added anaerobically to illuminated cultures of *Rhsp. rubrum* that had been grown with either glutamate or limiting NH_4^+ as the N source. (*a*) Glutamate-grown cells: ●, whole-cell nitrogenase activity; ○, degree of modification of Fe protein *in vivo*. (*b*) Glutamate-grown cells: ●, ATP pool size; ○, ATP/ADP ratio. (*c*) NH_4^+-limited cells: ●, whole-cell nitrogenase activity; ○, degree of modification of Fe protein *in vivo*. (*d*) NH_4^+-limited cells: ●, ATP pool size; ○, ATP/ADP ratio. The total adenine nucleotide pool and bacteriochlorophyll content was 3.7 nmol/mg of protein and 25.3 nmol/mg of protein respectively in the glutamate-grown culture and 9.5 nmol/mg of protein and 21.6 nmol/mg of protein respectively in the NH_4^+-limited culture.

ATP pool artificially. PMS treatment resulted in variable changes in the ATP pool. However, loss of nitrogenase activity was observed consistently, even at concentrations as low as 2 μM-PMS.

Comparison of the effects of various treatments on glutamate-grown and NH_4^+-limited cells also illustrates the lack of correlation between adenine nucleotide pools and switch-off of whole-cell activity or enzyme modification. Although the changes in ATP pools observed on inhibitor treatment were similar for both growth conditions, the effect on nitrogenase was quite different. Loss of whole-cell activity and modification of the Fe protein were evident in all of the treatments of glutamate-grown cells except the illuminated control. Yet we saw very little enzyme modification in any of the treatments of NH_4^+-limited cultures, and whole-cell nitrogenase activities remained high during treatment with darkness, NH_4^+ or glutamine. Yoch & Cantu (1980) reported a significant decrease in the ATP pool (36%) of NH_4^+-limited *Rhsp. rubrum* in the dark, but after a much longer incubation time. However, from their data it is apparent that less than 18% of the Fe protein became modified during that time.

Inhibition of nitrogenase activity *in vivo* by CCCP or PMS appears to be independent of Fe protein modification, and it presumably occurs by a different mechanism. The uncoupler's effect on activity can be explained by a decrease of the ATP supply to nitrogenase. In Fig. 2 the loss of activity appears to be slightly faster than the decrease in the ATP pool. This is somewhat misleading, however, because the acid extraction of nucleotides is fast, whereas the assay for acetylene reduction activity takes 2.5 min, during which time the cells continue to be exposed to CCCP.

Modification of the Fe protein lags significantly behind the loss of whole-cell activity in glutamate-grown cultures treated with CCCP or PMS and is not observed at all in NH_4^+-limited cultures. The signal for protein modification does not appear to involve ATP pools, since pool changes in glutamate-grown and NH_4^+-limited cells were similar, but the effects on Fe protein modification were opposite under the two growth conditions.

The ATP/ADP ratio has a large effect on the activity of the nitrogenase enzyme complex *in vitro*

(Davis & Orme-Johnson, 1976; Upchurch & Mortenson, 1980; Privalle & Burris, 1983). We found that, in *Rhsp. rubrum*, changes in the ATP/ADP ratio and the related parameter, energy charge, generally reflected the changes that occurred in the ATP pool (ADP and AMP pool changes were less significant). Although some fluctuations in these parameters occurred in glutamate-grown cells that exhibited nitrogenase switch-off and enzyme modification, similar changes were seen in NH_4^+-limited cells in which little loss of whole-cell activity or modification was evident. As with ATP pools, neither the ATP/ADP ratio nor the energy charge appears to correlate with switch-off of activity or modification *in vivo*.

The low energy charges observed in cells that contain nitrogenase are significant. Schön (1969) and Privalle & Burris (1983) found an average energy charge of 0.76 in cultures of *Rhsp. rubrum* that had been grown photosynthetically with concentrations of NH_4^+ high enough to repress nitrogenase synthesis. We found average energy charges of 0.66 and 0.62 in glutamate- and NH_4^+-limited cultures respectively. These values agree with other reports of energy charge in *Rhsp. rubrum* and other organisms expressing nitrogenase activity (Upchurch & Mortenson, 1980; Privalle & Burris, 1983). Nitrogenase activity *in vitro* is strongly inhibited at such low ATP/ADP ratios when an excess of Mg^{2+} is supplied (Davis & Orme-Johnson, 1976; Upchurch & Mortenson, 1980; Privalle & Burris, 1983). This raises the question of how reasonable rates of N-fixation can be maintained *in vivo*. Davis & Kotake (1980) have stressed the potentiating effect of Mg^{2+} in controlling nitrogenase activity by the ATP/ADP ratio *in vitro*.

Other possible regulators of nitrogenase activity have been proposed. These include glutamine synthetase (Hillmer & Fahlbusch, 1979; Yoch & Cantu, 1980), the glutamine pool (Falk *et al.*, 1982; Jones & Monty, 1979; Yoch & Gotto, 1982), and the cellular membrane potential (Haaker *et al.*, 1974; Hawkesford *et al.*, 1982). However, no relationship with protein modification has yet been demonstrated. It will be interesting to determine whether any correlations of these factors with switch-off and Fe protein modification can be identified in *Rhsp. rubrum* under conditions similar to the ones employed in the present investigation.

We acknowledge discussion with Mr. R. H. Kanemoto and the assistance of Mr. Scott Murrell and Mr. Tim Hoover in some aspects of this work. This work was supported by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, and by United States Department of Agriculture Competitive Research Grant no. 81-CRCR-1-0703. P. W. L. is a Steenbock Career Development Award Recipient.

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