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Nitrogenase Activity and Amounts of Nitrogenase Proteins in a *Frankia-Alnus incana* Symbiosis Subjected to Darkness[1]

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ABSTRACT

Effects of prolonged darkness on nitrogenase activity *in vivo*, nitrogenase activity *in vitro*, and the amounts of nitrogenase proteins were studied in symbiotic *Frankia*. Plants of *Alnus incana* (L.) Moench in symbiosis with a local source of *Frankia* were grown for 9 to 10 weeks in an 18/6 hour light/darkness cycle. After 12 hours of a light period, the plants were exposed to darkness for up to 40 hours. Nitrogenase activity (acetylene reduction activity) of intact plants was measured repeatedly. *Frankia* vesicle clusters were prepared from the nodules with an anaerobic homogenization and filtration technique and were used for measurements of *in vitro* nitrogenase activity and for measurements of the amounts of nitrogenase proteins on Western blots. Antisera made against dinitrogenase reductase (Fe-protein) of *Rhodospirillum rubrum* and against dinitrogenase (MoFe-protein) of *Azotobacter vinelandii* were used. Western blots were made transparent and nitrogenase proteins were quantified spectrophotometrically. Nitrogenase activity both *in vivo* and *in vitro* decreased after about 23 hours of darkness and continued to decrease to about 25% and 16% of initial activity, respectively, after 40 hours. The amount of Fe-protein and MoFe-protein in *Frankia* of the same plants decreased to 60% and 35%, respectively, after 40 hours of darkness. Loss of nitrogenase activity thus appeared to be largely explained by loss of MoFe-protein.

Symbiotic *Frankia* is dependent on its host for the carbon compounds necessary for growth processes and nitrogen fixation. Photoassimilates produced in the shoot are transported to the root nodules. Stress conditions applied to the plant reduce nitrogenase activity in the root nodules. This was the case for *Frankia-Alnus* symbioses after addition of ammonium (8, 11), drought stress (22), darkening and defoliation (10, 25), and decreased temperature (12). In some of these studies, nitrogenase activity of intact plants (*in vivo*) and nitrogenase activity *in vitro* were measured (8, 10, 22). *In vitro* activity was measured on root nodule homogenates supplied with Mg-ATP and the electron donor dithionite. The *in vitro* activity was fairly stable when expressed as the ratio *in vitro* activity/*in vivo* activity of the same plant. By comparing this *in vitro*/*in vivo* ratio between control plants and treated plants, it was found for all types of stress studied that the reduced nitrogenase activity *in vivo* could be ascribed to loss of active nitrogenase rather than a limitation of energy for nitrogen fixation.

A reduced amount of active nitrogenase can be due to inactivation or degradation not compensated for by synthesis or a combination of these possibilities. To distinguish among them, it is necessary to determine the amounts of nitrogenase protein during a stress treatment. Since nitrogenase consists of two proteins, dinitrogenase reductase (Fe-protein) and dinitrogenase (MoFe-protein), both necessary for activity, the amounts of both proteins have to be determined. In this paper, we describe immunological techniques used to quantify the Fe-protein and the MoFe-protein of *Frankia* in symbiosis with *Alnus incana* (L.) Moench exposed to prolonged darkness.

MATERIAL AND METHODS

Plant Material, *Frankia* and Growth Conditions

A clone of gray alder, *Alnus incana* (L.) Moench, was used (9). One-leaf internode cuttings were rooted, inoculated with a water suspension of crushed root nodules containing a local source of *Frankia* (phenotypically spore (+), hydrogen uptake activity (−) in symbiosis; [11, 21]), planted into pots with gravel, and grown in a climate chamber as described previously (9) but with the following modifications. The concentration of NH_4NO_3 was reduced to 0.036 mM, and, after 3 weeks, nitrogen was omitted from the nutrient solution. The plants received a continuous supply of the nutrient solution, fourfold diluted, distributed to the plants with an air-lift system. Twelve plants shared 20 L of the recirculated solution which was renewed twice a week. After 4 weeks, the plants were transferred to a climate chamber with higher photosynthetic photon flux density (600 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at plant level) and an 18/6 h cycle of light and temperature (25°C/15°C) with a 1-h transition period from day to night conditions and vice versa. Plants were used 9 to 10 weeks after inoculation and had an average leaf area of 730 (range 330–1120) cm^2 and an average height of 66 (range 53–84) cm.

For studies of specificity of antisera, a nonnitrogen-fixing culture of *Frankia* CpI1 (catalog No. HFP070101) was used. Cells, kindly provided by Dr. D. R. Benson, University of Connecticut, Storrs, CT, were grown on a succinate medium with NH_4^+ as nitrogen source at 30°C (17).

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Dark Treatment of Plants

Dark treatments were carried out in the climate chamber by placing the plants under a stand covered with black cloth. The PPFD was thereby reduced to <1 $\mu mol \cdot m^{-2} \cdot s^{-1}$, but temperature was not affected. The dark treatments started after 12 h of light in the 18/6 h light/dark cycle and persisted for up to 40 h. Control plants were left untreated.

Nitrogenase Activity *in Vivo*

ARA[2] was measured repeatedly on intact plants by enclosing the potted root system in a gas-tight chamber (6) and adding C_2H_2 to 10% (v/v) in air. These incubations were always done at noon (after 8 h of light) or in the morning (after about 2 h of light). The plants were kept in light (controls) or in darkness (treated plants) in the climate chamber during incubations. Gas samples were taken at 10-min intervals during 25 to 55 min after addition of C_2H_2 and immediately analyzed for C_2H_4 by gas chromatography as described earlier (10) but with a column length of 1 m.

Nitrogenase Activity *in Vitro*

ARA *in vitro* was measured on three aliquots of each vesicle cluster preparation, prepared anaerobically as described below. An aliquot (0.5 mL) was added to a 13-mL anaerobic glass vial sealed with a rubber membrane stopper (Suba Seal, William Freeman & Co Ltd, U.K.). The vials contained Hepes (Sigma), $Na_2S_2O_4$, Na_2-ATP, and $MgCl_2$, which, after addition of the vesicle cluster preparation had a final concentration of 50, (pH 8.0), 100, 40, and 20 mM, respectively. The reaction, at 25°C, was started by addition of C_2H_2 to 10% (v/v) of the Ar gas phase. Gas samples of 0.2 mL were taken at 5-min intervals during 15 to 30 min after addition of C_2H_2 and immediately analyzed for C_2H_4 as described above.

Preparation of Vesicle Clusters

Within 1 to 3 h after each measurement of nitrogenase activity *in vivo*, all root nodules were collected directly into liquid nitrogen and stored there for up to 6 weeks until used. The nodules ($\bar{x}$ = 2.31 g, range 1.30–3.11 g fresh weight) from each plant were homogenized anaerobically in 15 mL ice-cold anaerobic homogenization buffer containing 50 mM Hepes (pH 8.0), 0.2 M sucrose, 4% (w/v) PVP (K25; Roth), 2 mM Na_2-EDTA (Merck), 5 mM DTT (Boehringer), and 10 mM $Na_2S_2O_4$. The homogenization lasted for 2 s, and, when the foam had settled, a second homogenization (2 s) followed. The homogenate was immediately passed through a 100 μm nylon filter; the residue was resuspended in 15 mL of buffer; and homogenization of the nodule pieces was repeated. The remaining residue was washed with 10 mL of buffer.

To accomplish this homogenization rapidly under anaerobic conditions, the cutting end of the homogenizer (Ultra-Turrax TP 18–10, Janke & Kunkel KG) was mounted in a gas-tight way directly into a Sartorius SM 16510 filter apparatus. In addition, a continuous flow of Ar was passed through the filter apparatus.

To obtain a preparation of *Frankia* vesicle clusters, the filtrate was anaerobically transferred to a second filter apparatus and filtered anaerobically through a 20 μm nylon filter. The 20 μm filter residue was washed with the homogenization buffer (8 × 5 mL) and an anaerobic washing buffer (5 × 5 mL) containing 50 mM Hepes (pH 7.8), 4% (w/v) PVP, 2 mM Na_2-EDTA, and 2 mM $Na_2S_2O_4$ and finally collected into about 8 mL of the washing buffer. From this suspension, three aliquots were immediately used for measurements of nitrogenase activity *in vitro* as described above. The remaining suspension was centrifuged anaerobically for 10 min at 5°C and 2300*g*. The supernatant was discarded except for 1 mL which together with the pellet (the *Frankia* vesicle clusters) was stored in liquid nitrogen. In vesicle cluster preparations made in a similar way, less than 2% of the particle volume was plant material (23).

Protein Extraction

Each stored vesicle cluster preparation was thawed and pelleted at 14,000*g*. The pellet was resuspended to 0.1 mg pellet per μL of buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, and 2.5% [w/v] SDS). The suspension was kept at 100°C for 5 plus 5 min with vortexing in between and then sonicated (Branson sonifier B15) with a microtip for 60 s at output 3 using the 50% pulsed duty cycle. The sonicated preparation was centrifuged for 10 min at room temperature and 14,000*g*, and the supernatant (protein extract) was stored at −20°C.

Protein extraction of cultured *Frankia* CpI1 was done as described for vesicle clusters. A protein extract of *A. incana* roots was obtained by homogenization with an Ultra-Turrax in anaerobic buffer.

Protein Determination

Before protein determination of the vesicle cluster samples used for *in vitro* nitrogenase activity measurements, the samples were washed twice in dithionite-free washing buffer. In a separate experiment, this did not reduce the protein concentration of the samples significantly (*t* test, $P < 0.05$) but prevented the interference of dithionite in the protein assay.

NaOH was added to vesicle clusters from the *in vitro* measurements and to samples of the protein extracts to a final concentration of 1 N and 0.05 N, respectively. After 15 min at 90°C, the protein concentration was determined with the bicinchoninic acid protein assay according to the manufacturer (Pierce; the 37°C-protocol) with BSA (Fraction V, Sigma) as standard.

Western Blot Analysis

The protein extracts were all diluted to the same protein concentration, and β-mercaptoethanol, glycerol, bromphenol blue, and SDS were added to a final concentration of 5% (v/v), 10% (w/v), 0.02% (w/v), and 2.5% (w/v), respectively. After 10 min at 100°C, the extracts were centrifuged at 14,000*g* for 5 min. SDS-PAGE was performed according to the procedure of Laemmli (14). Samples (8 μL) of supernatants containing 1.8 μg protein were loaded onto a Mini Protean II Dual Slab Cell (Bio-Rad). The gel had a 10-mm-long stacking zone and a 50-mm-long separating zone and

[2] Abbreviation: ARA, acetylene reduction activity (C_2H_2-dependent C_2H_4-production); TST, Tris-NaCl-Tween buffer.

was 80 mm wide and 0.75 mm thick. Total acrylamide concentration was 4% in the stacking zone and 15% in the separating zone. Electrophoresis was performed for 45 min at 200 V, constant voltage, at room temperature. Two gels were run in parallel, and each gel contained samples from both treated and control plants. A dilution series of one of the control samples was included in each gel.

The gel, nitrocellulose filter (0.45 μm pore size; Schleicher & Schuell), and Whatman filter paper were equilibrated in blotting buffer (10 mM $NaHCO_3$, 3 mM Na_2CO_3 [pH 9.9], 20% methanol; [3]) for 15 min. The polypeptides were electroblotted onto nitrocellulose filters for 60 min at 18°C and 252 mA constant current in a Mini TransBlot (Bio-Rad) transfer cell. The voltage was about 55 V. Electroblotting for 30 min resulted in lower transfer of polypeptides than after 60 min, while blotting times longer than 60 min did not improve the result (data not shown).

Immunochemical detection of the nitrogenase proteins was performed essentially as recommended by Dakopatts (Stockholm, Sweden), modified from Blake *et al.* (1). The nitrocellulose filters were incubated for 30 min in a blocking solution containing 4% (w/v) BSA in TST (50 mM Tris-HCl [pH 10.3], 0.5 M NaCl, 0.5% Tween 20). The filters were incubated for 16 h with primary antisera diluted 1:150 (antidinitrogenase reductase) and 1:750 (antidinitrogenase) with 0.2% (w/v) BSA in TST. Both antisera were used in the same incubation. However, in tests of recognition and monospecificity, the two primary antisera were used separately.

The secondary antibody, swine anti-rabbit immunoglobulin G (IgG) conjugated to alkaline phosphatase (Dakopatts, Stockholm, Sweden), was diluted 1:1000 with 1% BSA in TST and incubated with filters for 2 h. All incubations were done at 23°C, and between each incubation the filters were rinsed with TST for 3 × 5 min. The filters were stained for alkaline phosphatase activity with a freshly prepared staining solution containing 0.06 $mg \cdot mL^{-1}$ 5-bromo-4-chloro-3-indolylphosphate, 0.1 $mg \cdot mL^{-1}$ nitro blue tetrazolium, 4 mM $MgCl_2$, and 0.6 mM ethanolamine (pH 9.6).

The primary antiserum raised against purified dinitrogenase reductase (Fe-protein, Rr2) from *Rhodospirillum rubrum* was a kind gift from Dr. S. Nordlund, University of Stockholm, Sweden. The primary antiserum against purified dinitrogenase (MoFe-protein, Av1) from *Azotobacter vinelandii* was a kind gift from Dr. R. C. van den Bos, The Agricultural University, Wageningen, The Netherlands.

For molecular mass determinations, low molecular mass markers (14.4–94 kD; Pharmacia) were included in the gel. After electroblotting, the part of the nitrocellulose filter containing the markers was incubated in a solution of 0.001% trinitrobenzene sulfonic acid in 0.25 M $NaHCO_3$ for 20 min to obtain trinitrophenyl groups on the markers. After washing in 0.25 M $NaHCO_3$ the filter was incubated for 2 h in antidinitrophenyl antiserum (Dakopatts, Stockholm, Sweden) diluted 1:500 and then detected as described above.

Dry, stained blots were placed on a glass plate and were made transparent by addition of immersion oil for microscopy. The blots were scanned at the absorbance maximum 568 nm with an integrating gel scanner (Beckman DU 8 Spectrophotometer) to quantify the relative amounts of the Fe-protein and the MoFe-protein.

RESULTS

Nitrogenase Activity *in Vivo* and *in Vitro*

The *in vivo* nitrogenase activity of the control plants kept under normal light/dark conditions was essentially constant except for a slight decrease at the end of the experimental period (Fig. 1A). In contrast, nitrogenase activity was markedly reduced in response to the dark treatment (Fig. 1A). After 13 h in darkness, the treated plants still had about the same activity as the control plants. The main loss of activity occurred during the following 7 h of darkness, and the activity continued to decrease to about 25% of initial activity after 38 h of darkness.

Figure 1B shows the nitrogenase activity *in vitro* of control and dark treated plants. In general, the recorded response of nitrogenase activity to dark treatment was similar *in vivo* and *in vitro*. After 16 h of darkness, nitrogenase activity *in vitro* was rather similar to the activity in control plants. After 23 h, the *in vitro* activity of dark treated plants decreased and, at

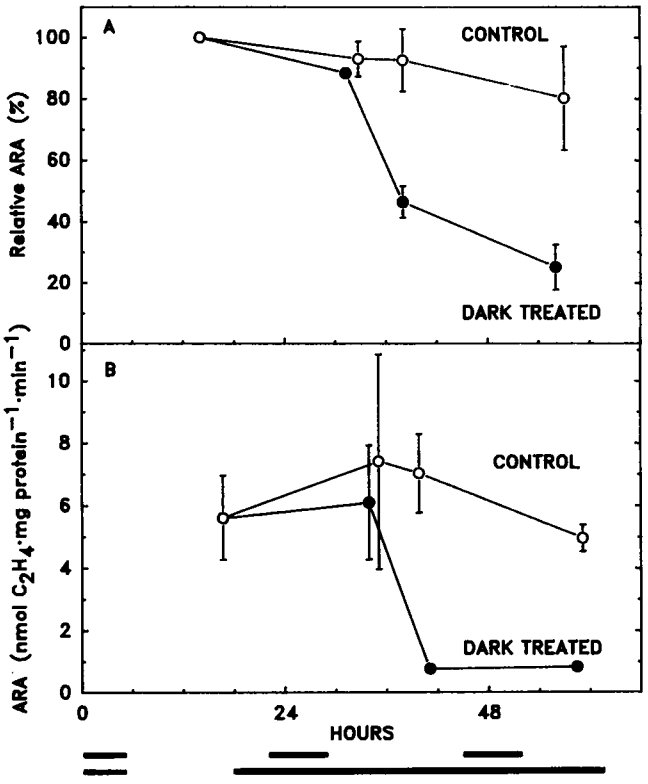

Figure 1. A, *In vivo* nitrogenase activity (ARA) of intact *A. incana* plants expressed as percentage of initial activity. Initial activity was 91.6 ± 5.41 μmol $C_2H_4 \cdot plant^{-1} \cdot h^{-1}$ ($\bar{x}$ ± SE). Each point represents $\bar{x}$ ± SE for n = 7 and 9 (31–32 h), n = 5 and 6 (38 h), and n = 3 and 3 (56–57 h) control and dark treated plants, respectively. (○), Control plants; (●), dark treated plants. B, *In vitro* nitrogenase activity (ARA) of anaerobically prepared *Frankia* vesicle clusters. Each point represents $\bar{x}$ ± SE for n = 3 plants, except for after 35 and 40 h when two control plants were studied. Error bars are smaller than the size of the symbol when not shown. Symbols as in (A.) The normal light/dark cycle for control plants and the darkness period for dark treated plants are indicated by bars below *x* axis.

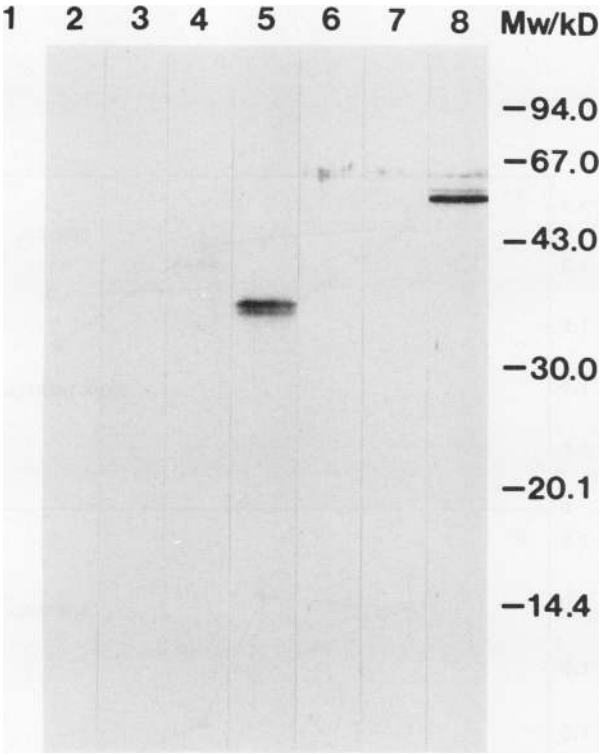

Figure 2. Western blots performed to test recognition and specificity of antisera against nitrogenase proteins. Lane 1, *Frankia* vesicle cluster protein, primary and secondary antisera excluded; lane 2, *Frankia* vesicle cluster protein, primary antiserum excluded; lanes 3 to 5 incubated with antiserum against the Fe-protein as the primary antiserum; lane 3, protein of ammonium-grown culture of *Frankia* (strain CpI1); lane 4, *A. incana* root protein; lane 5, *Frankia* vesicle cluster protein; lanes 6 to 8, as lanes 3 to 5 but with antiserum against MoFe-protein as primary antiserum.

the end of the experiment, was only about 16% of the activity in control plants (Fig. 1B).

Recognition and Specificity of Antisera

The recognition and monospecificity of antisera made against the *R. rubrum* Fe-protein and the *A. vinelandii* MoFe-protein to the *Frankia* vesicle cluster protein are shown in Figure 2. A major immunoreactive band with a relative molecular mass of about 38 kD and a minor band with a slightly lower molecular mass were obtained in the Western blots when the antiserum against the *R. rubrum* Fe-protein was used. This is comparable to the Fe-protein from other nitrogen-fixing organisms which consists of two identical subunits of about 33 to 36 kD (4, 13, 19).

In the Western blots where *Frankia* vesicle cluster protein was tested with antiserum against both types of subunits of the MoFe-protein from *A. vinelandii,* a major band with a relative molecular mass of about 58 kD and a minor band of slightly larger relative molecular mass were detected. The MoFe-protein of other nitrogen-fixing organisms consists of two pairs of nonidentical subunits with molecular masses of about 56 and 59 to 60 kD (13, 19). The weaker band of larger molecular mass most likely corresponded to the larger type of subunit of the MoFe-protein. A Western blot with a shorter blocking time before incubation with antiserum showed that the two bands for MoFe-protein covered the same area as if they were present in equal amounts, but with less intensity of the band for the larger polypeptide (data not shown). This may indicate less cross-reactivity of the polyclonal antiserum to the larger subunit or that the larger subunit bound less efficiently than the smaller subunit to the nitrocellulose.

No bands were detected on blots where the primary antiserum or both the primary and the secondary antisera were omitted (Fig. 2). The *Frankia* vesicle cluster protein was thus free from alkaline phosphatase activity that could interfere with the immunodetection of nitrogenase proteins. A few faint bands, none of the same molecular mass as either of the nitrogenase proteins, were detected when a root extract or when a protein extract from a nonfixing *Frankia* culture grown on ammonium was used as sample (Fig. 2).

Sequences of the structural genes for the Fe-protein and the MoFe-protein are highly conserved among nitrogen-fixing organisms (2, 5, 18). In addition, the antisera tested here cross-reacted only with proteins from nitrogen-fixing vesicle clusters with molecular masses in good accordance with the nitrogenase proteins from other nitrogen-fixing organisms. Therefore, we consider these antisera useful to measure relative amounts of *Frankia* nitrogenase proteins. The 38 kD band and the 58 kD band were used to quantify the Fe-protein and the MoFe-protein, respectively.

Quantification of Nitrogenase Proteins

The values of absorbance area measured on transparent Western blots were found to increase linearly with increasing amounts of *Frankia* vesicle cluster protein (Fig. 3). Since a dilution series was included in each blot, the relative amounts of nitrogenase proteins could be calculated.

Western Blot Analyses

The amount of the Fe-protein in extracts of *Frankia* from nodules of control plants varied slightly during the experimental period, but no consistent decrease or increase was

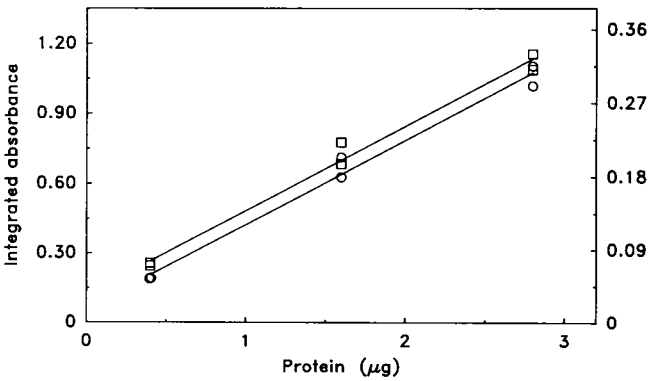

Figure 3. Absorbance values for Fe-protein (○) and MoFe-protein (□) on a Western blot of a dilution series of *Frankia* vesicle cluster protein. Left scale for Fe-protein and right scale for MoFe-protein. Each point represents a single determination.

observed (Fig. 4A). In dark treated plants, the amount of Fe-protein remained fairly constant during the first 23 h in darkness and similar to the initial amount of Fe-protein from control plants (Fig. 4A). After 40 h of darkness, the amount of Fe-protein was about 40% lower, but there was overlap with the values obtained for the control plants.

The amount of the MoFe-protein (Fig. 4B) appeared to be fairly constant in nodules of control plants. After 23 h of darkness, the amount of the MoFe-protein had decreased and after 40 h of darkness the amount was about 35% of the control amount (Fig. 4B).

The ratio MoFe-protein to Fe-protein (Fig. 4C) was calculated to minimize variation between individual plants. This ratio was constant for the control plants during the experimental period. For the dark treated plants, the ratio MoFe-protein/Fe-protein was the same as for controls after 16 h of darkness. However, after 23 h of darkness, at approximately the same time as the major loss of *in vivo* nitrogenase activity had occurred (Fig. 1), the ratio of MoFe-protein/Fe-protein was 46% lower than before treatment (Fig. 4C). After 40 h of darkness, the ratio was 57% lower for the dark treated plants than for the control plants. The results in Figure 4 taken together thus show that the amount of the proteins decreased in response to the dark treatment and that the MoFe-protein decreased faster than the Fe-protein.

DISCUSSION

The loss of nitrogenase activity both *in vivo* and *in vitro* (Fig. 1) corroborates earlier studies which indicated that the loss of *in vivo* activity in response to plant stress was due to loss of active nitrogenase rather than shortage of reductant and ATP (8, 10, 22). Loss of active nitrogenase was concluded since the nitrogenase activity of nodule homogenates, supplemented with dithionite and Mg-ATP, from treated plants were inhibited compared with control plants. In the earlier studies, measurements of *in vitro* nitrogenase activity were made on total nodule homogenates rather than on *Frankia* vesicle cluster preparations. The use of vesicle cluster preparations in the present study facilitated comparisons between nitrogenase activity *in vitro* and amounts of nitrogenase proteins.

During a period of prolonged darkness of up to 40 h, the nitrogenase activity *in vivo* decreased by 75% and nitrogenase activity *in vitro* by about 84% (Fig. 1). The sequence of events leading to loss of nitrogenase activity is not fully understood. Keeping plants in darkness most likely causes carbon stress in the nodules which, in turn, causes a reduced respiration rate. The consequences for nitrogenase activity may then be a reduced ATP production, but also an increased oxygen concentration which could inactivate nitrogenase and inhibit its synthesis. A reduced ATP production would give low nitrogenase activity *in vivo,* but not *in vitro,* since ATP and dithionite (reductant) were provided in the *in vitro* assay. However, we found decreased activity also *in vitro* which indicates lack of active nitrogenase. The parallel decrease of activity *in vivo* and *in vitro* in the *Frankia-Alnus*-symbiosis is thus different from dark treated soybeans (20). In soybean plants exposed to darkness for 8 d, nitrogenase activity *in vivo* decreased faster than nitrogenase activity of prepared bacter-

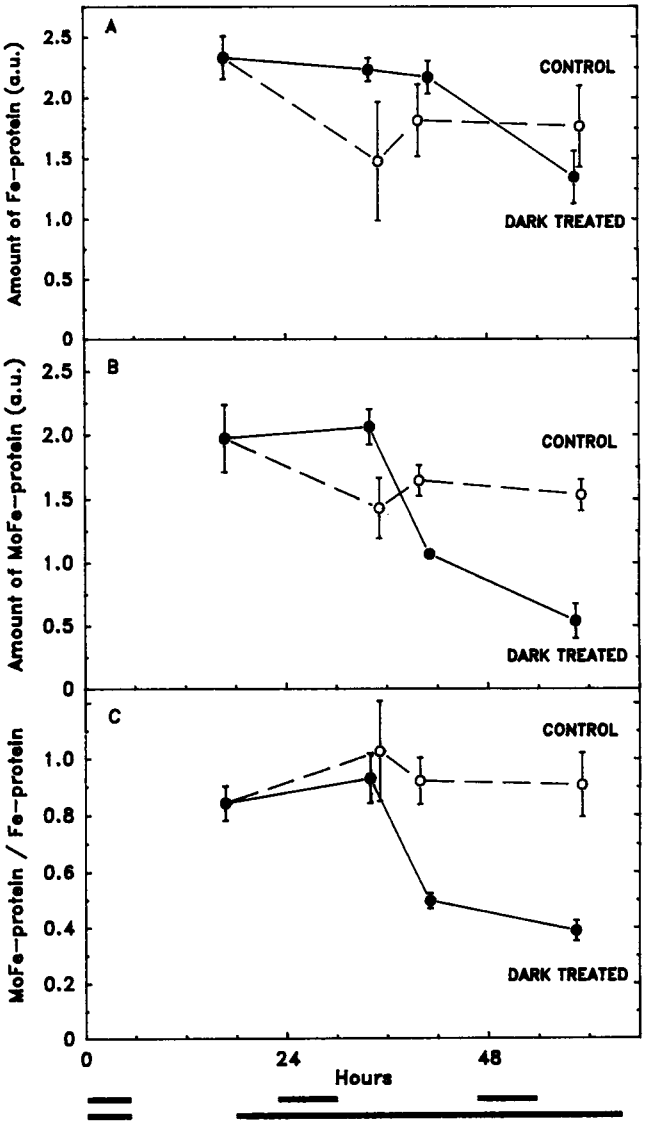

Figure 4. Relative amounts of Fe-protein (A) and MoFe-protein (B) in *Frankia* vesicle clusters as quantified from Western blots and expressed as arbitrary units (a.u.). Ratios of Fe-protein/MoFe-protein (C) were calculated for each vesicle cluster preparation. The vesicle cluster preparations were identical to the preparations used in Figure 1B. Each point represents $\bar{x} \pm SE$ for $n = 3$ plants, except for after 35 and 40 h when two control plants were studied. (○), control plants; (●), dark treated plants. The normal light/dark cycle for control plants and the darkness period for dark treated plants are indicated by bars below *x* axis.

oids supplemented with succinate as a respiratory substrate (20). A number of studies on legume symbioses exposed to stress has suggested the involvement of a variable oxygen diffusion barrier which causes a reduced oxygen concentration in the nodule and energy limitation of nitrogenase activity (15, 24).

The loss of active nitrogenase could be due to degradation of nitrogenase protein not compensated for by synthesis and/or reversible or irreversible inactivation. Western blot analyses were used to study amounts of nitrogenase proteins to be able to distinguish among these possibilities. The amounts of both nitrogenase proteins were measured, since both are necessary for nitrogenase activity. The antisera made against the *R. rubrum* Fe-protein and against the *A. vinelandii* MoFe-protein showed good cross-reactivity with two polypeptides (38 and 58 kD, Fig. 2) most likely being subunits of the *Frankia* Fe-protein and MoFe-protein, respectively. This again demonstrates the similarities between nitrogenase proteins from different organisms. The same antisera were also used to show the localization of the Fe- and MoFe-proteins in vesicles of *Frankia* in root nodules of this *Frankia-A. incana* symbiosis (7).

The use of a Western blot system which includes alkaline phosphatase conjugated secondary antibodies has previously been shown to be useful to quantify antigenic protein over a wide concentration range (1). Although we did not have the antigenic proteins purified, we could test the response to various nitrogenase concentrations in our system by making a dilution series. The integrated absorbance correlated well with the amount of protein loaded to each lane of the gel in the range tested (Fig. 3). From the dilution series used as a standard curve, relative values of the amounts of Fe-protein and MoFe-protein were obtained and could be used in comparisons of the amounts of nitrogenase protein in different plants.

According to the Western blots, there was a small decrease of Fe-protein and a large decrease of MoFe-protein (65%) after 40 h of darkness (Fig. 4). Thus, it appears that the loss of nitrogenase activity both *in vivo* and *in vitro* can be explained by a loss of nitrogenase protein, especially the MoFe-protein. The possibility that nitrogenase was inactivated may have been an earlier event, perhaps explaining the slightly more pronounced loss of activity than loss of protein. In the cyanobacterium *Anabaena cylindrica,* exposure of extracts to oxygen was necessary before degradation of the MoFe-protein by proteolytic enzymes occurred (16).

In conclusion, subjecting nitrogen-fixing plants of *A. incana* to prolonged darkness caused reduced nitrogenase activity *in vivo* and *in vitro,* which was due to loss of active nitrogenase. Immunological methods, developed to quantify Fe-protein and MoFe-protein of nitrogenase in symbiotic *Frankia,* showed that the loss of active nitrogenase was largely explained by a loss of MoFe-protein.

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