JOURNAL OF BACTERIOLOGY, Feb. 1996, p. 606–610 Vol. 178, No. 3
0021-9193/96/$04.00+0

Proteolytic Degradation of Dinitrogenase Reductase from *Anabaena variabilis* (ATCC 29413) as a Consequence of ATP Depletion and Impact of Oxygen

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Received 27 July 1995/Accepted 14 November 1995

Both components of nitrogenase, dinitrogenase and dinitrogenase reductase, are rapidly inactivated by oxygen. To investigate the proteolytic degradation of dintrogenase reductase irreversibly destroyed by high oxygen concentrations, we carried out in vitro experiments with heterocyst extracts from *Anabaena variabilis* ATCC 29413. The results indicate a direct dependence of degradation on the applied oxygen concentration. Although the degrees of degradation were similar for both the modified and unmodified subunits of dinitrogenase reductase, there was a significant difference with respect to the cleavage products observed. The pattern of effective protease inhibitors suggests the involvement of serine proteases with chymotrypsin- and trypsin-like specificity. A protective effect was obtained by saturation of the nucleotide binding sites of dinitrogenase reductase with either ATP or ADP. As shown by gel filtration experiments, the adenylates prevented the nitrogenase subunits from extensive noncovalent aggregation, which is usually considered evidence for a denaturing process. The in vitro degradation of dinitrogenase reductase is discussed in connection with previous reports on degradation of nitrogenase in cyanobacteria under oxygen stress and/or starvation.

Biological nitrogen fixation is catalyzed by nitrogenase, a multienzyme complex, which is extremely O_2 sensitive, regardless of source (for reviews, see references 12 and 13). Since O_2 production accompanies cyanobacterial photosynthesis, this lability presents a unique problem (22). The different strategies to cope with O_2 are either a temporal or a spatial separation of oxygenic photosynthesis and nitrogen fixation. Under nitrogen-fixing conditions, many cyanobacteria differentiate highly specialized cells called heterocysts with biochemical and structural features which provide substantial protection of nitrogenase (16). It is assumed that high respiration rates and lack of photosynthetic O_2 evolution enable these cells to fix nitrogen aerobically. Heterocysts do not catalyze CO_2 fixation and are dependent on adjacent photosynthetic vegetative cells for a supply of fixed carbon.

Nitrogenase is composed of two different proteins, dinitrogenase (MoFe protein) and dinitrogenase reductase (Fe protein). The Fe-protein is particularly sensitive to O_2; its half-life in air is only 30 s to 2 min (12). In cyanobacteria, moderate O_2 treatment of whole cells leads to reversible inactivation probably accompanied by a posttranslational modification of the Fe protein (3, 26), which does not involve an ADP ribosylation (9) as described for the purple bacterium *Rhodospirillum rubrum* (4). Nonetheless, it is well documented that O_2 not only destroys the integrity of the Fe-S and Mo-Fe cofactors (12, 13) but irreversibly affects cyanobacterial nitrogenase subunits themselves under harsh in vivo conditions with respect to O_2 tension or C starvation (i.e., a shortage of respiratory substrates and reductants [13, 21]). O_2 not only inactivates and leads to destruction of nitrogenase but also suppresses nitrogenase synthesis. However, although many structural *nif* genes are coregulated by O_2, repression of *nifHDK* in *Anabaena* sp. strain PCC 7120 requires a concentration of O_2 greater than that needed to destroy nitrogenase activity (10). Similar results were obtained earlier for *Azotobacter* spp., in which the minimum pO_2 to repress nitrogenase synthesis is 20 times higher than that which inactivates nitrogenase (23). Experiments with *Gloeothece* sp. and *Oscillatoria limosa* using chloramphenicol (which inhibits protein synthesis in cyanobacteria) supported the idea of a continuous synthesis of nitrogenase even under O_2 (13, 28). In *Synechococcus* sp., a circadian rhythm of nitrogen fixation during a light-dark cycle is due to de novo synthesis of the Fe protein in the dark and degradation in the light (5). Thus far, studies on the interrelations between O_2 and nitrogen fixation have been mainly focused either on in vitro oxidation (or destruction) of the catalytical metal clusters of the nitrogenase subunits or on the capacity of various organisms to cope with exogenous O_2, i.e., temporary inactivation of nitrogenase and transcriptional control of the *nif* genes. In this study, we report on the proteolytic degradation of the Fe protein of *Anabaena variabilis*. We first provide strong evidence that O_2 not only destroys nitrogenase activity but triggers proteolysis of nitrogenase subunits. We also show that saturation of the nucleotide binding sites protects the Fe protein from degradation. The physiological significance of these results is discussed.

MATERIALS AND METHODS

Preparation of heterocysts. Nitrogen-fixing *A. variabilis* (ATCC 29413) was cultivated in minimal medium without combined nitrogen at 30°C in a thermostatically controlled Kniese-Edwards apparatus as described previously (9). For heterocyst isolation, a 24-h culture of *A. variabilis* was bubbled with H_2 for 5 min and subsequently harvested (4,000 × *g*, 5 min). Chlorophyll *a* content of the cell suspension was adjusted to 200 to 250 μg ml^{-1} with H/P buffer [30 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 30 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 1 mM $MgCl_2$ (pH 7.2)]; 10 mM neutralized EDTA and 1 mg of lysozyme ml^{-1} were added, and the mixture was stirred for 30 min at 30°C. To eliminate the lysozyme, the suspension was centrifuged at 1,400 × *g* for 5 min, and the pellet was resuspended in H/P buffer. After sonication for 8 to 12 min in a sonic cleaning bath (Sonorex PK 102; 240 W; Bandelin, Berlin, Germany), the heterocysts were sedimented at 1,400 × *g* for 5 min. After three washes in H/P buffer, the cells were centrifuged three times for

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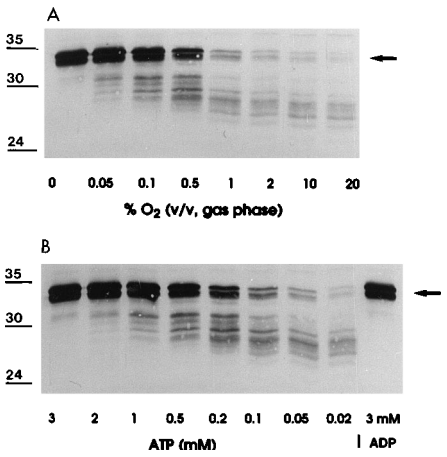

FIG. 1. Proteolytic degradation of cyanobacterial Fe protein of nitrogenase. (A) Proteolysis dependent on the oxygen concentration. Soluble heterocyst extracts were incubated at 24°C for 45 min under argon with the indicated amounts of oxygen. Prior to addition of the protein (130 μg), the incubation medium (50 mM Tris-HCl [pH 7.8], 1 mM dithiothreitol; final volume of 50 μl) was equilibrated against the gas phase. The samples (6.5 μg of protein) were analyzed by SDS-PAGE (18% gel) containing 3 M urea and subsequent Western blotting with Fe protein antibodies. For detection, the alkaline phosphatase method was applied. The arrow at the right points to the homodimer of the Fe protein (33 and 34 kDa) of nitrogenase, with one subunit probably posttranslationally modified (9, 24). Positions of molecular weight markers are given at the left in kilodaltons. (B) Protection by ATP and ADP against degradation. Aerobic incubation was for 45 min in the presence of the indicated concentrations of ATP and ADP as described in the text. SDS-PAGE and Western blotting were performed as described above.

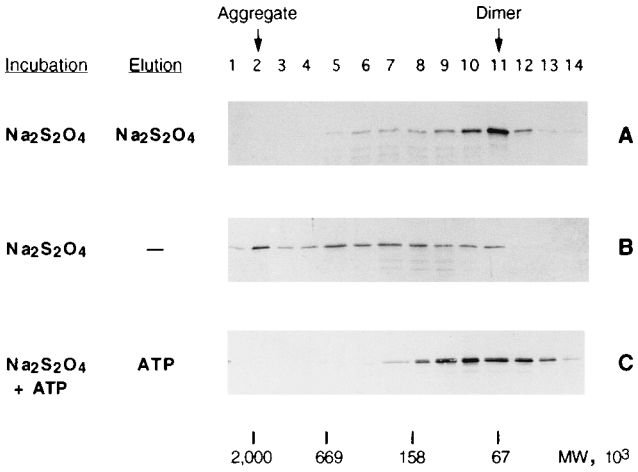

FIG. 2. Aggregation of the Fe protein dependent on ATP and oxygen. Heterocyst extracts (1 mg of protein) were incubated at 24°C for 1 h in 50 mM morpholinethanesulfonic acid (MES)-NaOH (pH 6.8) (pretreatment) and then separated on a fast protein liquid chromatograph-coupled Superose 6 column (flowrate, 0.2 ml/min). $Na_2S_2O_4$ (2 mM) and ATP (1 mM) were added as indicated. The fraction size was 1 ml. After concentration with a vacuum centrifuge, the fractions were analyzed by SDS-PAGE and immunoblotting. Molecular weight (MW) markers were blue dextran (~2,000), thyroglobulin (669), aldolase (158), and bovine serum albumin (67).

5 min each time at 1,000 × *g*, 650 × *g*, and 500 × *g*, respectively, resuspended in 2 ml of H/P buffer, and stored in liquid nitrogen. Cell extracts were prepared anaerobically as described previously (2).

Purification of Fe protein from *A. variabilis*. Heterocyst extracts from *A. variabilis* containing either active or inactive nitrogenase (obtained by an 8-h dark incubation as described previously [2]) were applied to a Mono Q anion-exchange column (HR 10/10; Pharmacia, Freiburg, Germany) that had been equilibrated with 50 mM Tris-HCl (pH 7.5). Proteins were eluted with a linear gradient of $MgCl_2$ (120 ml, 80 to 200 mM). The Fe protein eluted at 100 mM. The chromatography was carried out under argon.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting, and quantification of blots. The technique used has been described elsewhere (8); the polyacrylamide concentration was 12.5% (12.5% total monomer concentration, 0.82% cross-linker concentration) for the separating gel and 5% (5% total monomer concentration, 2.7% cross-linker concentration) for the stacking gel. The gels were stained with Coomassie brilliant blue R 250 (Pierce, Freiburg, Germany) or subjected to immunoblotting. Proteins were transferred to Immobilon-P (Millipore, Eschborn, Germany) in 25 mM Tris–192 mM glycine–20% (vol/vol) methanol. Proteins recognized by anti-Fe protein antibody (1:20,000; the antibody from rabbits is described in references 8 and 24) were detected by using alkaline phosphatase-conjugated secondary antibodies and nitroblue tetrazolium-bromochloroindolyl phosphate.

After Coomassie blue staining or immunoblotting, gels and blots were scanned with a laser densitometer at 633 nm (Ultroscan 2202; LKB/Pharmacia). The scanned profiles (four for each lane) were analyzed with the GELSCAN 2190 program (USCD Apple). Alternatively, a video-based imaging system (Bioprofil; Vilber Lourmat) was used. With a high-resolution charge-coupled device camera and BIO-1D software, a scan density of 50 μm in both dimensions was achieved.

RESULTS

Oxygen-induced degradation of the Fe protein. It has been shown for several species of both unicellular and filamentous cyanobacteria, e.g., *Gloeothece* sp., *Synechococcus* sp., and *Anabaena cylindrica*, that the amount of detectable Fe protein drastically decreases during light or O_2 treatment (5, 7, 13, 21). The same holds true for *A. variabilis*. To examine this event, we established an in vitro system. Figure 1 shows that an efficiently working proteolytic machinery is present in the extracts prepared from heterocysts. The apparent molecular mass of the modified subunit appeared slightly greater (34 kDa) than that of the unmodified one (33 kDa). The visible cleavage products were rapidly processed. Even with the use of a streptavidin-biotin-conjugated secondary antibody, peptides smaller than 10 kDa could not be detected (data not shown). This may be due to the rapid processing of the medium-size peptides resulting in free amino acids or to the specificity of the polyclonal Fe protein antibody. Corresponding experiments with respect to a turnover of the MoFe protein have been hampered by the poor specificity of the available antibody for degradation products (data not shown). In vitro degradation of the Fe protein started immediately upon addition of O_2 (Fig. 1A). In a sample treated with 0.5% O_2, the Fe protein was completely degraded after 150 min, while in the absence of O_2, the protein was stable for several hours. Neither O_2^- nor H_2O_2 showed efficient induction of the degradation of the Fe protein. In case of 0.1 mM H_2O_2, there was substantial proteolytic activity, although this concentration seems to have no physiological relevance. Addition of catalase (4 U) suppressed the effect of added H_2O_2, but there was only weak protection against O_2-dependent degradation (not shown). It should be noted, however, that in previous work the specific activity of active nitrogenase was significantly reduced by reactive oxygen species (1).

Influence of ATP and ADP on proteolytic degradation. Next we examined the influence of adenylates. As summarized by Gallon (13) and Fay (12), starvation (e.g., darkness and subsequent decrease of carbohydrates in case of filamentous species) is assumed to inhibit both O_2-consuming respiration and ATP generation, resulting in a parallel increase of cellular O_2 and a strong decrease of the energy charge. As shown in Fig. 1B, addition of ATP resulted in protection of the Fe protein. Because of the presence of 0.015 to 0.02 mM ATP and ADP in the protein extract, the lowest concentration assayed was not zero but 0.02 mM. Note that these experiments were done in the presence of air instead of an adjusted content of O_2. In control experiments (20% O_2 rather than air, incubation in the presence of 0.02 mM ATP for 45 min), we did not see any significant difference (data not shown). Complete depletion of

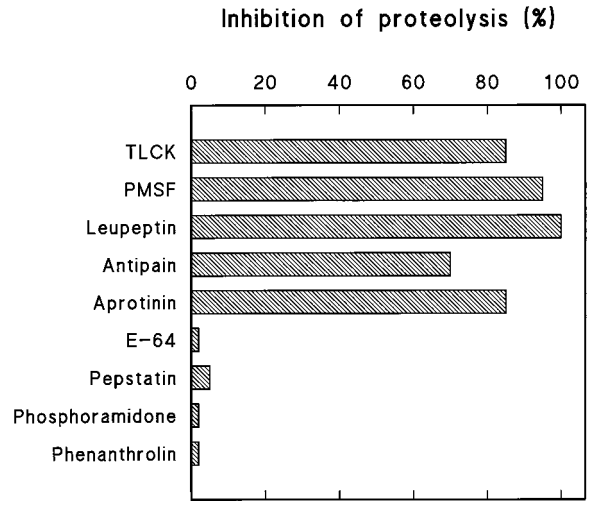

FIG. 3. Efficiency of protease inhibitors. Nitrogenase-containing heterocyst extracts were aerobically incubated (2 h) in the presence of 100 μM L-1-chloro-3-(4-tosylamino)-7-amino-2-heptanone hydrochloride (TLCK), 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 μM leupeptin, 20 μM antipain, and 150 μM aprotinin. Inhibition of proteolysis was measured by densitometric scanning of the remaining full-length protein (both subunits). In the cases of *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), pepstatin, phosphoramidone, and phenanthroline (each at a final concentration of 250 μM), there was no significant inhibition of degradation.

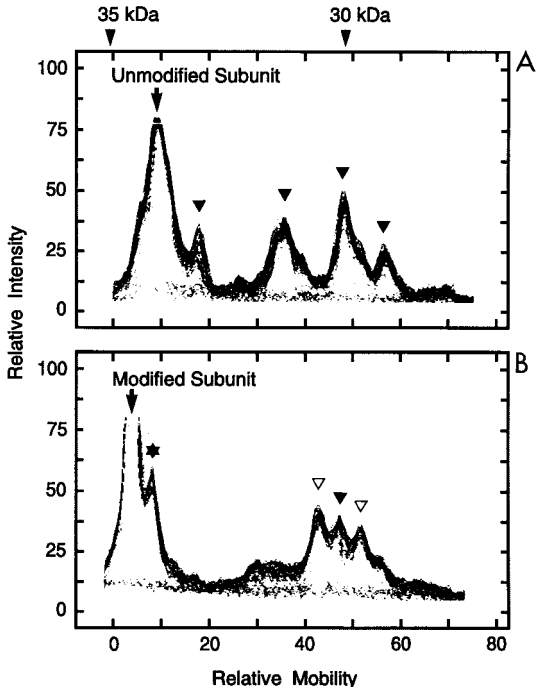

FIG. 4. Comparison of the first degradation products of the modified and unmodified subunits. Purified Fe protein (5 μg; purification was as described earlier [9]) from either nitrogen-fixing or dark-inactivated *A. variabilis* (2) was incubated for 5 min in the presence of a heterocyst extract without Fe protein (which was obtained by aerobic preincubation of the extract for 2 h, leading to complete degradation of the Fe protein). After separation by SDS-PAGE and immunoblotting, the banding pattern obtained was scanned densitometrically. The relative band intensity is plotted against the relative migration distance. The positions of molecular weight standards (35 and 30 kDa) are indicated. (A) Active Fe protein (unmodified subunit). The arrowheads point to degradation products. (B) Inactive Fe protein (modified subunit). The open arrowheads point to degradation products. The asterisk and solid arrowhead indicate the presence of a small amount of unmodified Fe protein also in this sample and its major degradation product at position 49, respectively.

nucleotides by anaerobic desalting or hexokinase treatment resulted in extracts in which a substantial amount of the Fe protein had already been proteolytically processed. Interestingly, an almost identical effect was observed when ADP (in presence of ATP-depleting hexokinase) but not AMP (3 mM; data not shown) was provided. CTP, GTP, UTP, or ITP had no effect at any concentration.

Aggregation of Fe protein subunits prior to degradation. Among the signals leading to proteolytic degradation of a substrate, changes in the tertiary or quaternary structure play an important role. Figure 2 shows the chromatographic behavior of the Fe protein dependent on ATP. After gel filtration (Superose 6) of samples pretreated as indicated, the fractions were analyzed by SDS-PAGE and immunoblotting. A sample treated anaerobically throughout extraction and chromatography yielded the expected appearance of the dimer with an apparent M_r of 66,000 (Fig. 2A). The faint degradation products visible in fractions 5 to 10 were not present immediately after the chromatographic procedure; they appeared after the (aerobic) concentration of the fractions in a vacuum centrifuge. In contrast to the anaerobic treatment, the omission of $Na_2S_2O_4$ from the column resulted in strong shift of the elution profile, with a distinct peak at an M_r of 1,900,000 and with a marked disappearance of the dimer (Fig. 2B). As shown with a specific antibody, there was no coelution, i.e., no association with the dinitrogenase tetramer (MoFe protein). Fractions 1 to 3 consisted almost exclusively of Fe protein. The omission of $Na_2S_2O_4$ from both preincubation and elution buffers resulted in the complete disappearance of the Fe protein. This event could be partially prevented by the addition of ATP (data not shown). Significant repression of aggregates with high molecular weights was obtained by an anaerobic preincubation with subsequent aerobic chromatography in the presence of ATP (Fig. 2C). Note that the slightly different positions of the dimer either in fractions 10 and 11 was due to incomplete synchronization of fractions. Similar data were obtained for the addition of ADP (not shown). A comparison of samples in Fig. 2B and C (treated identically, except that ATP was added to the sample shown in Fig. 2C) suggests that the saturation of nucleotide binding sites prevented the Fe protein from aggregating even in presence of O_2.

Inhibition of degradation by protease inhibitors. Figure 3 shows the pattern of effective protease inhibitors. Proteolysis was strongly inhibited by the trypsin inhibitors L-1-chloro-3-(4-tosylamino-7-amino-2-heptanone hydrochloride and aprotinin. Furthermore, inhibitors of serine proteases (leupeptin, antipain, and phenylmethylsulfonyl fluoride) significantly reduced degradation of the Fe protein. These results are indicative of the involvement of serine proteases with chymotrypsin- and trypsin-like specificity. The addition of $CaCl_2$ or EGTA (an inhibitor of Ca^{2+}-dependent proteases) did not have any influence on proteolysis (not shown).

Peptide patterns of the modified and unmodified subunits. As shown in Fig. 1, in vitro degradation was almost the same for the two forms. To examine whether there are any differences at all, we digested purified subunits by a nitrogenase-free heterocyst extract (Fig. 4). The cleavage products after a 5-min incubation were analyzed by Western blotting (immunoblotting) and laser densitometry. The densitogram clearly indicates significant variations of the peptide patterns. Although the modified fraction (Fig. 4B) contained some unmodified Fe protein which resulted in the presence of the corresponding cleavage products also in this lane (there is a prominent one at

position 49; the degradation products at positions 33 to 35 and 56 become apparent as a broad peak and as a distinct shoulder, respectively), the appearance of different cleavage products is obvious.

DISCUSSION

Proteolysis of the Fe protein in vitro obviously depends on the concentration of applied O_2 (Fig. 1). This result is in line with in vivo studies on O_2 treatment of *A. cylindrica* and *Gloeothece* sp. (13, 21) and with reports on light treatment (i.e., elevated levels of intracellular O_2) of *Synechococcus* sp. and *Gloeothece* sp. (5, 7). Assuming complete gas exchange, 0.05% (vol/vol) O_2 in the gas phase corresponds to 0.6 μM dissolved oxygen concentration, which is tolerated by nitrogenase in vivo and in vitro (10). On the other hand, it has been shown that a dissolved O_2 concentration of 0.55 μM is inhibitory to the in vivo nitrogenase activity of *Klebsiella pneumoniae* (20). Until now there has been no conclusive evidence for the activation of a protease by O_2 or reactive oxygen species. Although this might be the case for a protease in heterocyst extracts (25), we assume that the O_2-dependent proteolysis reflects the inherent O_2 sensitivity of the Fe protein. It remains unclear whether a reactive oxygen species triggers the destruction and, if so, which one does so. For *Gloeothece* sp., a decrease of the Fe protein by treatment with O_2^- or H_2O_2 has been reported (7). However, even for the well-known Fenton mechanism (Fe^{2+} and H_2O_2), previously unknown species of biologically relevant intermediates have been described (32). Rather than the species added (1, 7), a nitrogenase-catalyzed peroxide (31) may destroy the enzyme, as reported for other self-catalyzed oxidations in presence of iron (27).

It appears unlikely that the proteases involved are inhibited either by ATP and ADP. Thus far, only ATP-dependent systems have been described. A more convincing explanation may be drawn from the nucleotide-induced dramatic changes in Fe-protein conformation similar to those found for actomyosin and the viral GTP-binding $p21^{ras}$ protein (18, 30). It is widely accepted that both ATP and ADP bind to the Fe protein, probably at the same two sites, but AMP does not. The calculated dissociation constants reported vary considerably. For the oxidized Fe protein from *Azotobacter vinelandii*, the K_m values (ATP) are between 49 and 290 μM; the corresponding values determined for the reduced protein vary between 220 and 1,710 μM. Saturation of binding sites is achieved between 0.5 and 1 mM (6). Considering the effective nucleotide concentration range shown in Fig. 1, we assume that protection against proteolysis was due to binding of ATP and ADP to nitrogenase; i.e., proteolysis follows the conformation change reported upon nucleotide depletion. This assumption is consistent with the starvation situation in vivo, in which case the decrease of the ATP pool is paralleled by an increase not of ADP but of AMP as a result of a myokinase present (11).

Considering the results shown in Fig. 1 (degradation of the Fe protein in the presence of O_2 and the highly efficient protection by ATP or ADP) and Fig. 2 (aggregation of the Fe protein in presence of O_2 and absence of ATP or ADP), the noncovalent aggregated Fe protein seems to be the target for proteases. A similar situation has been reported for other proteins, e.g., the degradation of H_2O_2-modified hemoglobin, in that the formation of extensive noncovalent aggregation is a prerequisite for proteolysis via the proteasome (14). We could not prove any interaction of the Fe protein with other protecting proteins. There is no evidence for the existence in cyanobacteria of the Shethna FeS protein, which forms an O_2-tolerant complex with nitrogenase from *A. vinelandii* (12, 13). To assay the previously discussed GroEL/ES association of the Fe protein (14) which may be responsible for enabling the Fe protein to escape proteolysis, cross-linking experiments with *Escherichia coli* GroEL/ES were carried out but did not prove any physical interaction (data not shown). We assume that the formation of higher-order polymers of Fe subunits reflects the higher hydrophobicity of partially unfolded proteins.

Most recently it has been shown that the modification and degradation of the Fe protein from *Synechococcus* sp. resembles the oxidative marking and subsequent proteolysis of glutamine synthetase and RubisCo (5). In our in vitro system, both forms are degraded. However, the difference of the first cleavage products (Fig. 4) indeed indicates a difference in the initial steps of degradation which may play an important role during in vivo proteolysis of the Fe protein. In cyanobacteria, the nature of the posttranslational modification is still unknown. Previously, we described the in vitro activation of the Fe protein from *A. variabilis* by membrane fractions from *A. variabilis* or *R. rubrum* (2). This activation was most probably due to reconstitution of the nitrogenase complex with MoFe subunits associated with the membranes. A posttranslational modification by ADP ribosylation could be excluded (9). However, rather than a specific modification of target amino acids, the differences may reflect a change in the tertiary structure, i.e., a different accessibility for proteases. Peptide mapping with specific endoproteases is under way.

Summarizing our data, we suggest a model for the degradation of the Fe protein of nitrogenase. Starting point is starvation of the cell, i.e., loss of ATP with a parallel increase of intracellular dissolved O_2. The ATP/ADP-modulated conformation change (step I) clearly is a reversible process and depends on the concentration of these nucleotides and therefore on the energy charge of the cell. On the other hand, prolonged exposure to O_2 might result in an irreversible destruction of the Fe-S cluster and perhaps in an irreversible change of the Fe protein tertiary structure (step II). As shown in Fig. 1, both events are necessary to degrade the Fe protein. It is likely that irreversible damage by O_2 is the point from which subsequent proteolysis will start. It is well known that the exposure of enzymes to oxygen increases their degradation in extracts of *E. coli*, liver, and heart mitochondria (27). More recently, we found that the Fe protein from *A. variabilis* is a target for ubiquitin conjugation (8). However, ubiquitination might not be an exclusive pathway for degradation as reported for other proteins (for reviews on the ubiquitin system, see references 17 and 19). Among the cyanobacterial proteases, a recently discovered Ca^{2+}-independent serine protease from *A. variabilis* (29) corresponds well with the efficiency of protease inhibitors shown in Fig. 3. At present we do not know whether one of the previously described cyanobacterial proteases (29, 33) is involved in the proteolysis of the Fe protein.

ACKNOWLEDGMENTS

We thank R. Grimm for skillful technical assistance.

This study was supported by the Deutsche Forschungsgemeinschaft through its Sonderforschungsbereich 248 "Stoffhaushalt des Bodensees."

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