Biochimica et Biophysica Acta, 1057 (1991) 97–101

ADONIS 000527289100081A

BBABIO 43347

Rhodobacter capsulatus nitrogenase reduction by natural in vivo electron carriers: Reactivity with FdI reduced by chloroplasts

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(Received 10 September 1990)

Key words: Nitrogenase reduction; Electron donor; Ferredoxin; (*R. capsulatus*)

To date, most in vitro studies of nitrogenase action have used sodium dithionite. It is of interest to know whether various aspects of nitrogenase function are the same when it is reduced by a natural electron carrier. However, in very few cases is the in vivo electron carrier to nitrogenase known with certainty. Here it is shown using immunoblotting that *Rhodobacter capsulatus* ferredoxin I is probably *nif* specific since its synthesis is under the control of the *nif* regulatory gene *nifR1* and is repressed by oxygen and ammonia, factors known to repress *nif* gene expression. Chloroplast-reduced ferredoxin I was used to investigate the effects of the substitution of a biological reductant for dithionite on some aspects of nitrogenase function. Both the effect of the dilution of total nitrogenase and the effect of titration with excess Mo-Fe protein on nitrogenase activity were unchanged. These results strongly suggest, therefore, that under the conditions used in the present study, nitrogenase components appear to freely associate and dissociate during enzyme turnover driven by ferredoxin I.

Introduction

A detailed kinetic model of nitrogenase action has been developed using sodium dithionite as the electron donor to nitrogenase (reviewed in Ref. 1). In addition, dithionite has been used in most studies of substrate reduction by nitrogenase [2]. The present model of nitrogenase turnover [1] predicts that: (1) there is an obligatory dissociation, supported by data from cross-linking studies [3], of the Fe protein and the Mo-Fe protein, since only free Mo-Fe protein binds substrates and releases product; and (2) the dissociation of oxidized Fe protein from reduced Mo-Fe protein is the rate-limiting step in nitrogenase catalysis. Thus this model successfully predicts: (1) the lag observed in H_2 evolution at high Mo-Fe:Fe protein ratios [4]; (2) inhibition of substrate reduction as the Mo-Fe: Fe protein ratio is increased [4,5]; and (3) the decrease in nitrogenase activity when it is highly diluted [5,6]. However, dithionite is known to either weaken or suppress interaction between Fe protein and Mo-Fe protein [7–9]. Thus, it is not known to what extent models of nitrogenase action based on chemical reduction by dithionite can be extrapolated to in vitro reduction by biological electron carriers or to in vivo nitrogen-fixing conditions. Indeed, it has been suggested that the present model of nitrogenase action may not pertain in vivo since: (1) Fe protein specific activity in vivo may be 5- to 10-fold higher than in vitro [10,11] and (2) inhibition of electron transport to nitrogenase in intact bacteroids has been reported to have no effect on the electron allocation coefficient [12].

Relatively little is at present known about the identity of natural in vivo electron donors for nitrogenase. The role of low-potential electron carriers in nitrogen fixation has been confusing. In *Klebsiella pneumoniae*, although several investigators had proposed potential in vivo electron carriers to nitrogenase on the basis of in vitro studies, it was not until recently that the true carrier was identified through biochemical studies of specific *nif*$^-$ mutants. The protein products of two genes, *nifF* and *nifJ*, have been shown to constitute a specific electron transport system from pyruvate to nitrogenase in *K. pneumoniae* [13–16]. The *nifF* gene product has been shown to be a flavodoxin [13,14,16]. *Azotobacter vinelandii* contains a gene homologous to *nifF* [17], but even strains mutated in both flavodoxin (*nifF*) and ferredoxin I (*fdxA*) are capable of diazotrophic growth [18]. However, *fdxN*, which codes for

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a low-molecular-weight bacterial ferredoxin in *Rhizobium meliloti*, has been shown to be essential for symbiotic nitrogen-fixation [19]. Two soluble ferredoxins have previously been isolated from *Rhodospirillum rubrum* [20] and *Rhodobacter capsulatus* [21,22]. Ferredoxin I (*R. capsulatus*), which is effective in electron donation to nitrogenase, appeared to be more abundantly synthesized during diazotrophic photoheterotrophic growth [21,22]. Further recent evidence [23] for a role for ferredoxin I in nitrogen fixation is provided by the localization of its structural gene (*fdxN*) within a region known to contain *nif* genes [24,25] and by the fact that transcription of *fdxN* is repressed by ammonia. N-terminal sequence data [23] as well as the recent complete sequencing of FdI [26] have convincingly demonstrated that it is encoded for by *fdxN*. Additional sequence data indicate the adjacent presence of a 2Fe-2S ferredoxin gene [27] and a putative flavodoxin [28].

Here it is shown, using immunoblotting, that synthesis of ferredoxin I, like other *nif* genes in this organism, is repressed by ammonia and oxygen, and more importantly, is under the control of the *nif*-specific [29] regulatory gene, *nifR1*. In addition, some of the characteristics of nitrogenase activity when reduced by ferredoxin were determined. The results presented here suggest, at least with FdI photochemically reduced by chloroplast fragments, that the general features of nitrogenase reactivity remain the same when reduced ferredoxin I is substituted for dithionite.

Materials and Methods

Preparation of proteins. Ferredoxin I was isolated as previously described [22]. A nitrogenase complex was reconstituted with Mo-Fe protein and Fe protein, purified as previously described [30].

Nitrogenase assays. Nitrogenase activity was measured essentially as previously described [30,31]. Assays with ferredoxin I reduced by chloroplast fragments contained, in either a total volume of 0.5 ml (6.4 ml reaction vessel) or 0.2 ml (2 ml reaction vessel); sodium ascorbate, 8 mM; dichlorophenol indophenol, 50 μM; dichlorodimethyl urea, 20 μM; heated chloroplast fragments (61 μg chlorophyll or 24 μg chlorophyll respectively). The concentrations of ferredoxin, Fe protein and Mo-Fe protein are indicated in the text.

Analytical methods. Protein concentrations were determined as described by Lowry [32]. Ferredoxin I concentrations were determined spectrophotometrically using the following extinction coefficient: 26.1 mM^{-1} cm^{-1} [22]. For immunoblots, protein fractions, as indicated in the text with RCVG = RCV base [33] plus 7 mM glutamate, RCV = RCV base plus 7 mM $(NH_4)_2SO_4$, were subjected to discontinuous SDS-PAGE [34] with 15% acrylamide using a mini-Protean II gel electrophoresis apparatus (Bio-Rad). The gel was then electroblotted onto nitrocellulose using a mini-transblot transfer cell and the blot developed using rabbit-anti-*R. capsulatus* FdI (prepared as previously described [35]) and horseradish peroxidase conjugated to goat anti-rabbit IgG using an Express Blot kit and procedures (Bio-Rad).

Results and Discussion

Immunoblotting analysis (Fig. 1) clearly demonstrates that ferredoxin I protein is present only under conditions that are permissive for *nif* expression [36,37], the absence of oxygen and a low N/C ratio. The antibody used cross-reacts with Fe protein as well as other undefined proteins (Fig. 1). At present it is unclear whether this cross-reactivity is inherent to the anti-FdI antibody or whether very minor amounts of contaminants in the FdI preparation gave an immune response equal to that of the poorly immunogenic FdI. A previous report concluded that ferredoxin I synthesis was light dependent [21]. However, the dark-grown cultures used in those experiments were micro-aerobic, possibly accounting for that effect. Indeed it has previously been shown [20] that ferredoxin I is not synthesized in aerobically grown *Rhodospirillum rubrum*. More

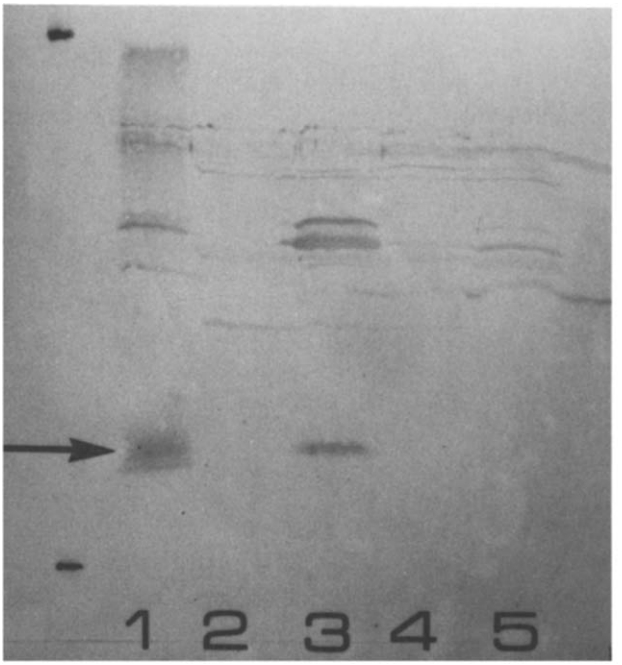

Fig. 1. The presence of ferredoxin I in strains of *R. capsulatus* grown under various conditions. The presence or absence of FdI in various cultures was assessed using immunoelectrophoresis as described under Materials and Methods. Lane 1, *R. capsulatus* FdI; lane 2, wild-type (SB1003) grown aerobically on RCVG (65 μg); lane 3, wild-type grown anaerobically on RCVG (53 μg); lane 4, strain J61 grown anaerobically on RCVG (67 μg); lane 5, wild-type grown anaerobically on RCV (52 μg).

significantly, here it is shown that no ferredoxin I synthesis is apparent under permissive conditions with the *nif*$^-$ strain J61. Strain J61 was previously shown to contain a pleiotrophic *nif* regulatory mutation [38] and in fact is mutated in the *ntrC*-like *nifRI* [29]. A previous study [23] suggested that *fdxN* was a *nif* gene since it was found near a cluster of *nif* genes and since its transcription was ammonia-sensitive. However, no convincing evidence for a corresponding *nif* promotor or requirement for *nif* regulatory factors was presented. *NifR1* is absolutely required for *nif* expression [29] and is thought to be *nif*-specific, since strains mutated in this gene are still capable of growth on glutamine, proline or arginine [38]. Taken together, these results strongly suggest that *R. capsulatus* ferredoxin I is *nif*-specific. The definitive demonstration of the in vivo role of FdI will require the chromosomal disruption of *fdxN*. Experiments to this end are currently in progress. The photosynthetic bacteria are capable of carrying out nitrogen fixation under at least five different growth modes [39]. However, at present virtually nothing is known under any circumstances about how ferredoxin is reduced. Therefore several methods for reducing ferredoxin were investigated.

Previously [31], *R. capsulatus* nitrogenase activity was shown to give essentially the same response to variation in redox potential when reduced by *Clostridium pasteurianum* ferredoxin coupled to *C. pasteurianum* hydrogenase as that obtained with dithionite/bisulfite. This system has several advantages: the redox potential can be simply and accurately poised, and it is composed of defined, well-characterized components. However, when the ability of ferredoxin I to function with *C. pasteurianum* hydrogenase was tested and compared with *C. pasteurianum* ferredoxin and spinach ferredoxin, it was found to be relatively ineffective. Taking the relative effectiveness of *C. pasteurianum* as 100%, *R. capsulatus* and spinach ferredoxins were equally poor in mediating electron transfer, giving 7% the rate of nitrogenase activity on a molar basis (results not shown).

It is obvious, given the poor reactivity of ferredoxin I in this system due to its lack of effective interaction with *C. pasteurianum* hydrogenase, that this does not present a suitable method for studying the details of nitrogenase action when reduced by ferredoxin I. Illuminated chloroplast fragments provide a strong reductant source capable of reducing many ferredoxins. Previously, this system was used with proteins from *Rhodospirillum rubrum* to demonstrate that ferredoxin I was three times as effective at reducing nitrogenase as ferredoxin II [40]. Indeed under the appropriate conditions, *R. capsulatus* ferredoxin I reduced by illuminated chloroplasts is able to drive nitrogenase-mediated acetylene reduction at twice the rate obtained with dithionite alone as reductant (Fig. 2, see also Ref. 21). Under the conditions used here, two different concentrations of ferredoxin I (0.94 μM and 3.76 μM) gave optimum nitrogenase activity with chloroplast fragments equivalent to 25 μg chlorophyll per ml (final concentration) which was maintained up to 125 μg chlorophyll per ml (results not shown). Concentrations in excess of this amount were slightly inhibitory. This suggests that ferredoxin I reduction was not rate limiting at the proper chloroplast concentrations.

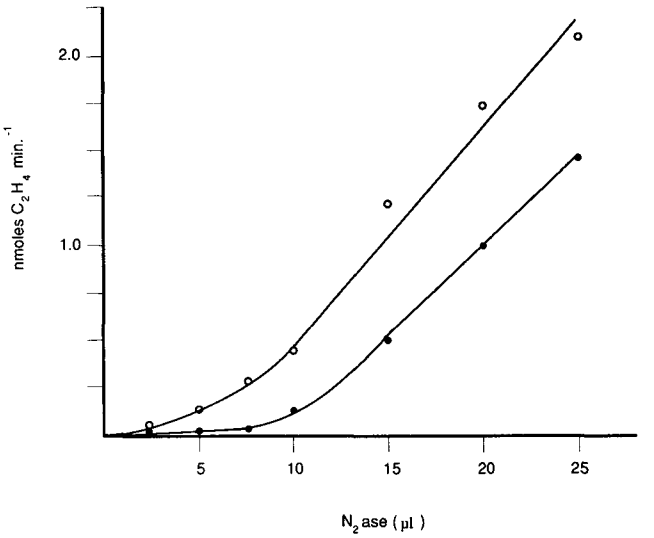

Fig. 2. Nitrogenase dilution effect with chloroplast-reduced ferredoxin I as electron donor. Reactions were carried out in 2 ml reaction vessels as described under Materials and Methods. Fe protein and Mo-Fe protein were added together to give a solution that was 5 μM Fe protein, 16 μM Mo-Fe protein. The indicated volumes were then assayed (in a total reaction volume of 0.2 ml) for nitrogenase activity (acetylene reduction) with either chloroplast-reduced ferredoxin I (9.4 μM final concentration) (○) or dithionite (5 mM final concentration) (●) as electron donor.

When nitrogenase was assayed at various concentrations with chloroplast-reduced ferredoxin I, activity was found to vary non-linearily with protein concentration at low protein concentrations (Fig. 2). Essentially the same effect was found for dithionite-reduced nitrogenase; except, as noted above, for the lower activity observed with dithionite. Both reactions became linear at the same nitrogenase concentration. Since this dilution effect is considered to be due to mass-action effects on the association between Fe protein and Mo-Fe protein [5–7], one can conclude that the equilibrium between free and complexed nitrogenase components is not altered by substituting reduced ferredoxin I for dithionite.

The effect of increasing concentrations of Mo-Fe protein on nitrogenase activity with chloroplast-reduced ferredoxin I as electron donor was examined (Fig. 3). Under these conditions, excess Mo-Fe protein was inhibitory to ferredoxin I driven nitrogenase activity throughout the range tested. In fact, the effect seen was essentially that found for reduction of nitrogenase by dithionite. The shapes of the curves obtained are typical

of those commonly observed in titrations of Fe proteins with Mo-Fe proteins. The inhibition observed at high [Mo-Fe]/[Fe] has been rationalized as due to the back reaction of oxidized Fe protein with Mo-Fe protein effectively competing with its reduction [6]. In accordance with this notion is the observation [5] made with *Azotobacter vinelandii* that a mixture of flavodoxin and dithionite can relieve Mo-Fe protein inhibition during such titrations presumably due to more effective reduction of the Fe protein. However, in this study, the presence of reduced ferredoxin I alone was not sufficient to overcome Mo-Fe protein inhibition, suggesting that maximal activity was limited by Fe protein reduction.

To examine this possibility further, the nitrogenase complex was titrated with excess Fe protein, with reduced ferredoxin I or dithionite as electron donor as shown in Fig. 4. Under these conditions, apparent Mo-Fe protein specific activity was 4.5-fold higher when reduced by dithionite than when reduced by ferredoxin I. More importantly, when reduced by ferredoxin I, much lower concentrations of Fe protein were required to saturate Mo-Fe protein activity. These results can be interpreted to mean either that: (1) Mo-Fe protein was not available to excess Fe-protein because after the addition of Fe protein to a total concentration of 1.8 μM they had formed a stable 1:1 complex in the presence of ferredoxin I but not in the presence of dithionite, or (2) the amount of Fe protein effective in the assay with ferredoxin I was kinetically limited by its rate of reduction. However, since under no other circumstances with ferredoxin I as reductant, i.e., dilution of total nitrogenase (Fig. 2) or titration with excess Mo-Fe protein (Fig. 3) was any evidence obtained to suggest tight complex formation between the two nitrogenase components, it is more likely that Fe protein reduction is limiting under these circumstances. This is also suggested by the observation that with chloroplast reduced ferredoxin I as reductant total nitrogenase activity (acetylene reduction) was linear with added nitrogenase up to 0.5 μM Fe protein, 1.63 μM Mo-Fe protein and higher levels actually showed a decrease in total activity (results not shown).

Thus the results obtained here show that the substitution of reduced ferredoxin for dithionite as nitrogenase reductant does not apparently change the association of reduced (Fig. 2) or oxidized (Fig. 3) Fe protein with Mo-Fe protein. However, whether or not these effects hold at higher effective concentrations of reduced Fe protein relative to the oxidized species can-

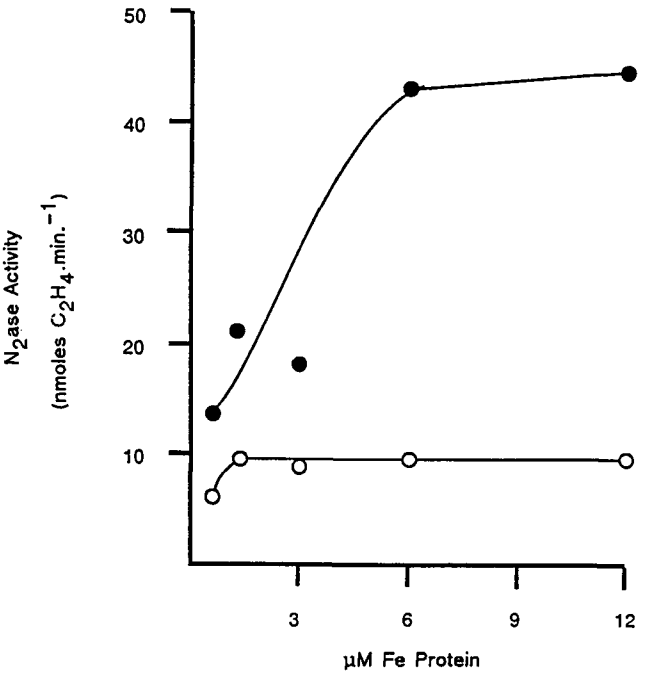

Fig. 4. Effect of Fe protein addition on nitrogenase activity with chloroplast-reduced ferredoxin I as electron donor. Reactions were carried out under the conditions described under Materials and Methods. Fe protein and Mo-Fe protein were added together to give final concentrations (in a total reaction volume of 0.5 ml) of 0.5 μM Fe protein, 1.63 μM Mo-Fe protein. Excess Fe protein was added to the final concentrations given and the reaction mixtures were assayed for nitrogenase activity with either chloroplast-reduced ferredoxin I (○), or dithionite (●) as electron donor.

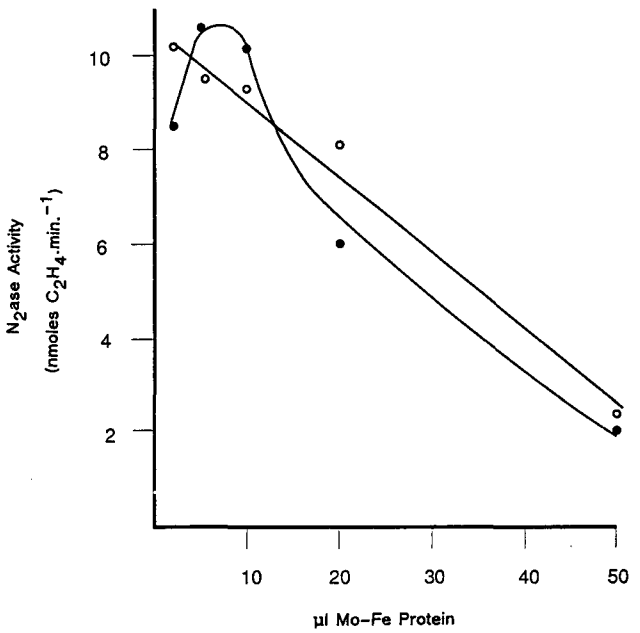

Fig. 3. Effect of Mo-Fe protein addition on nitrogenase activity with chloroplast-reduced ferredoxin I as electron donor. Reactions were carried out in 6.4 ml reaction vessels as described under Materials and Methods. Fe protein and Mo-Fe protein were added together to give final concentrations (in a total reaction volume of 0.5 mls) of 0.5 μM Fe protein, 1.63 μM Mo-Fe protein. Reactions were supplemented with Mo-Fe protein as indicated (each 10 μl of Mo-Fe protein increased the final Mo-Fe protein concentration by 2 μM) and assayed for nitrogenase activity with either: chloroplast (62 μg chlorophyll per ml final concentration) reduced ferredoxin I (4.7 μM final concentration) (○); or dithionite (5 mM) (●) as electron donor.

not be determined under the conditions used here due to apparent limitations in the rate of Fe protein reduction. Another possibility for the observed effect (Fig. 4) is that of inhibition of nitrogenase turnover by a higher ADP/ATP in the assays with chloroplast fragments due to endogenous ATPase activity. The resolution of these uncertainties will require an alternate mode of ferredoxin I reduction. Studies to this end are presently in progress using illuminated deazaflavin solutions to drive ferredoxin reduction of nitrogenase.

Conclusion

Here it has been shown, using immunoblotting, that *R. capsulatus* ferredoxin I is not synthesized in a strain mutated in *nifR1* or in the presence of ammonia or oxygen, conditions that are known to repress *nif* gene expression. Ferredoxin I is only mildly active in coupling hydrogenase (*C. pasteurianum*) with *R. capsulatus* nitrogenase. Initial studies on nitrogenase activity driven by ferredoxin I reduced by chloroplast fragments have shown that under these conditions, the substitution of reduced ferredoxin for dithionite has no apparent effect on (1) the equilibrium of association of Fe protein and Mo-Fe protein to form an active complex, and (2) the inhibitory effect of excess Mo-Fe protein on total nitrogenase activity. Thus, as is the case in the presence of dithionite, nitrogenase components appear to freely associate and dissociate during enzyme turnover in the absence of dithionite and the presence of reduced ferredoxin I. However, chloroplast reduced ferredoxin I was unable to drive Mo-Fe protein to maximum activity in the presence of excess Fe protein, suggesting a limitation in the rate of Fe protein reduction, possibly due to slow ferredoxin I reduction. Further studies will be necessary, including alternate means of ferredoxin reduction and possibly the inclusion of accessory electron transfer mediators, to fully resolve the issues raised here.

Acknowledgements

This research was supported by grant OGP0036584 from the Natural Sciences and Engineering Research Council of Canada. I thank Marie Lesage for excellent assistance in manuscript preparation.

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