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Structural analysis of the *Trichodesmium* nitrogenase iron protein: implications for aerobic nitrogen fixation activity

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Abstract

Trichodesmium spp. are marine filamentous nitrogen-fixing cyanobacteria which play an important role in the nitrogen budget of the open ocean. *Trichodesmium* is unique in that it is nonheterocystous and fixes nitrogen during the day, while evolving oxygen through photosynthesis, even though nitrogenase is sensitive to oxygen inactivation. The sequence of the gene encoding the Fe protein component of nitrogenase from the recently cultivated isolate *Trichodesmium* sp. IMS 101 was used to construct a 3-dimensional model of the Fe protein, by comparison to the X-ray crystallographic structure of the Fe protein of the γ-proteobacterium *Azotobacter vinelandii*. The primary differences in amino acid sequences of the Fe protein from diverse organisms do not impact the critical structural features of the Fe protein. It can be concluded that aerobic nitrogen fixation in *Trichodesmium* spp. is not facilitated by unique structural features of *Trichodesmium* Fe protein.

Keywords: *Trichodesmium*; Nitrogenase; Fe protein; Cyanobacteria; Fe protein structure

1. Introduction

The fixation of atmospheric nitrogen to ammonium is catalyzed by the enzyme nitrogenase, which is extremely sensitive to inactivation by oxygen. Nitrogenase is a complex of two multi-subunit proteins termed the molybdenum-iron (MoFe) protein and the iron (Fe) protein. Diverse prokaryotic organisms can fix nitrogen, including numerous cyanobacterial species [1]. Cyanobacteria evolve oxygen through photosynthesis, which at first appeared inconsistent with the oxygen sensitivity of nitrogenase. This paradox of simultaneous nitrogen fixation and oxygen evolution was resolved when it was discovered that cyanobacterial species that fixed nitrogen did so in specialized cells with reduced photosystem II activity, called heterocysts [2,3]. Subsequently, nitrogen-fixing filamentous nonheterocystous and unicellular cyanobacterial species were identified [4], but these species primarily fixed nitrogen under microaerophilic conditions or during the dark phase of a light-dark cycle [5–7]. In 1961, Dugdale and co-authors [8] first reported nitrogen fixation associated with aggregates of the filamentous nonheterocystous cyanobacteria *Trichodesmium* spp. in the Sargasso Sea. Contrary to the dogma that cyanobacteria either spatially or temporally segregate nitrogen fixation from the oxygen evolved through photosynthesis, nitrogen fixation in *Trichodesmium* spp. occurs only during the day [9]. However, *Trichodesmium* spp. were largely ignored, as they had not been iso-

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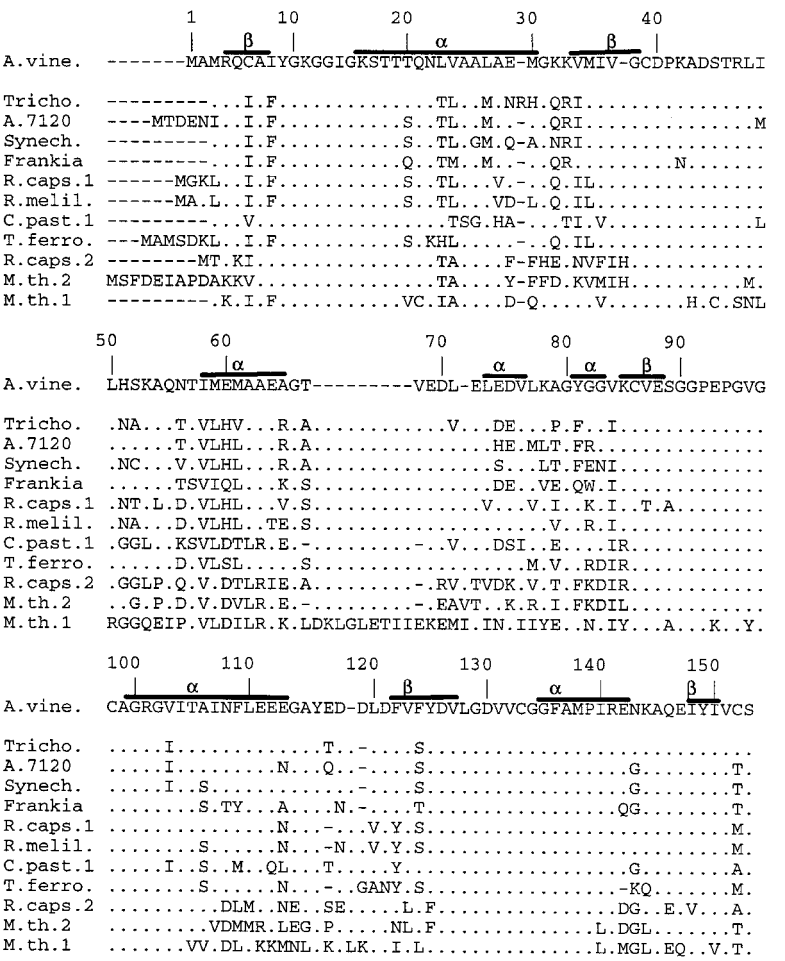

Fig. 1. Deduced amino acid sequences (obtained from SwissProt unless otherwise noted) of the Fe protein of nitrogenase from *Trichodesmium* sp. IMS 101 (designated Tricho), and other cyanobacterial, bacterial and archaeal nitrogen-fixing species. Exact amino acid residue matches are indicated by periods. Regions of α-helices and β-sheets are shown by bars above the *A. vinelandii* sequence. Sequences are grouped phylogenetically below the *Trichodesmium* sequence, starting with sequences from other cyanobacteria (*Anabaena* (*Nostoc*) sp. PCC 7120, *Synechococcus* sp. RF-1) followed by representative bacterial conventional nitrogenases (*Frankia* sp. ArI3, *R. capsulatus, R. meliloti*, *C. pasteurianum*, and *T. ferrooxidans*). Alternative nitrogenases are represented by *R. capsulatus anfH*. Archaeal nitrogenases are represented by two *nifH* sequences from *M. thermolithotrophicus*. M. th.1 = *Methanococcus thermolithotrophicus nifH* (P25767); M. th. 2 = *Methanococcus thermolithotrophicus nifH* (P08625); R. caps. 2 = *Rhodobacter capsulatus anfH* (Q07942); C. past. 1 = *Clostridium pasteurianum nifH1* (P00456); A. vine. = *Azotobacter vinelandii* (GenBank M20568); R. melil. = *Rhizobium meliloti* (P00460); R. caps. 1 = *Rhodobacter capsulatus nifH* (GenBank X63352); T. ferro. = *Thiobacillus ferrooxidans* (P06661); Frankia = *Frankia* ArI3 (GenBank M21132); Synech. = *Synechococcus* sp. RF-1 PCC 8801 (GenBank U22146); A. 7120 = *Anabaena* (*Nostoc*) sp. PCC 7120 (P00457); and Tricho. = *Trichodesmium* sp. IMS 101 (U90952).

lated in culture and it was not clear that the nitrogen fixation was indeed due to the cyanobacterium, and not to associated bacteria [10].

More recently genetic [11] and immunological [12,13] studies have provided evidence that *Trichodesmium* spp. are indeed diazotrophic. Once strains of *Trichodesmium* spp. were isolated [14,15] they were clearly shown to fix nitrogen only during the light phase [16], apparently under the control of a circadian rhythm [17].

Attempts to elucidate the mechanism(s) that facilitate simultaneous nitrogen fixation and photosyn-

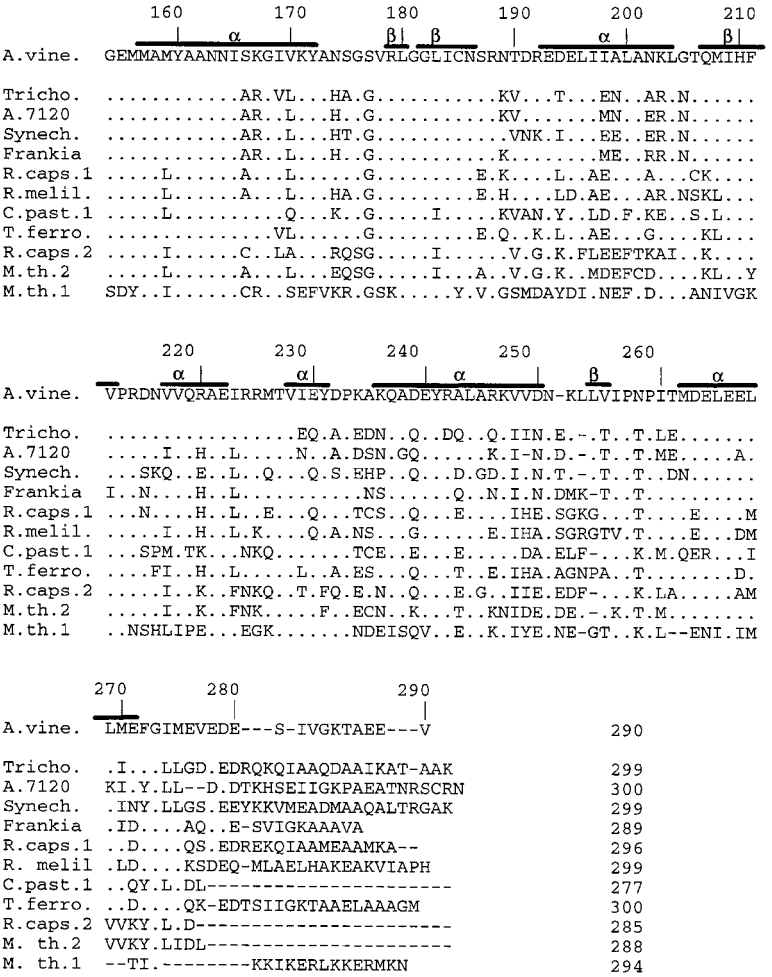

Fig. 1 (*continued*).

thesis in *Trichodesmium* spp. have largely failed. A major impediment to studies of nitrogen fixation in *Trichodesmium* has been the lack of cultures. Even in culture, *Trichodesmium* grows slowly and to low densities, preventing the isolation of pure nitrogenase and in vitro biochemical studies. In other organisms, both the nitrogenase MoFe protein and the Fe protein are rapidly inactivated by oxygen. The *Trichodesmium* nitrogenase could be less sensitive to oxygen inactivation than the nitrogenase of other species [18]. This hypothesis has never been addressed because of the lack of cultures, and inability to successfully obtain in vitro nitrogenase activity from extracts obtained from slow-growing *Trichodesmium* cultures.

The Fe protein gene sequences (*nifH*) of *Trichodesmium* spp. are most closely related to sequences from other cyanobacteria [18], but phylogenetic analysis indicated that *Trichodesmium* spp. *nifH* genes are one of the most distantly related groups of *nifH* sequences within the cyanobacterial cluster [19]. This suggested that there could be unique features of *Trichodesmium* spp. nitrogenase Fe protein. In this paper, the sequence of *nifH* from the recently obtained *Trichodesmium* sp. IMS 101 culture [15] was used to construct a 3-dimensional model of the Fe protein to determine if there are unique features of the nitrogenase protein in *Trichodesmium* sp. that could contribute to oxygen tolerance.

2. Methods

A genomic library was constructed using *Trichodesmium* sp. IMS 101 genomic DNA. The *Sau*3AI partially digested DNA was cloned into Lambda GEM-11 vector (Promega) using a *Xho*I half-site strategy. The library was screened with a radiolabelled 359 bp *nifH* DNA fragment cloned from *Trichodesmium* sp. [11]. A clone containing a 13 kb insert was identified by hybridization and a 1.2 kb fragment of this insert subcloned into pUC18. The 1.2 kb insert containing the *nifH* gene was completely sequenced (by primer walking) on both strands by the University of Maine sequencing facility. The Fe protein sequences of *Azotobacter vinelandii*, *Clostridium pasteurianum* (*nifH1*), *Frankia* sp. ArI3, *Rhizobium meliloti*, *Rhodobacter capsulatus* (*nifH* and *anfH*), *Thiobacillus ferrooxidans*, *Synechococcus* sp. RF-1, *Anabaena* (*Nostoc*) sp. PCC 7120, and *Methanococcus thermolithotrophicus* were obtained from SwissProt or GenBank.

The structure of the Fe protein of *Azotobacter vinelandii*, determined by X-ray crystallography [20] was obtained from the Brookhaven Protein Database and the sequence extracted using Insight II and Homology programs (Biosym Inc.). The amino acid sequence for *Trichodesmium* sp. IMS 101 *nifH* (GenBank U90952) was manually aligned to the *A. vinelandii nifH* sequence (GenBank M20568), and a model for *nifH* created using Insight II and Homology (Biosym Inc.) programs. The model of *nifH* was thermodynamically relaxed using the Discover program (Biosym Inc.).

3. Results and discussion

The deduced amino acid sequence of the *Trichodesmium* sp. Fe protein is 74% identical to that of *A. vinelandii* over a 147 amino acid residue region (Fig. 1). The Fe protein is a dimer of identical subunits that coordinate an FeS cluster. Important features are nucleotide binding regions (Walker's binding motifs A and B), salt bridges between the identical subunits, and 4–5 conserved cysteines, which are necessary to coordinate the FeS cluster. The regions surrounding the two cysteines which are absolutely necessary to coordinate the FeS cluster (*A. vinelandii* residues 98 and 133) are highly conserved in all of the sequences. The regions around two other conserved cysteines (*A. vinelandii* residues 39 and 86) are also highly conserved, even though these cysteines have been shown not to be necessary for active protein [21].

The FeS cluster is located near the interface where the Fe protein interacts with the MoFe protein (Fig. 2). A loop (*A. vinelandii* residues 92–97) from one of the subunits fits into a notch (*A. vinelandii* 133–168) of the other subunit, and the whole complex forms a lung-shaped structure [20]. The loop and notch regions are highly conserved, even in the alternative (non-Mo-containing nitrogenases) and archaeal proteins as well as the *Trichodesmium* sp. protein (Fig. 1). Arginine and glutamate residues (*A. vinelandii* residues 101 and 113 in the *A. vinelandii* deduced protein sequence, respectively) are important for interaction with the MoFe protein [20]. The arginine is the site of post-translational ADP-ribosylation in some organisms [22], and also has been demonstrated to be important for active protein by mutant analysis [21]. The cyanobacterial Fe protein sequences have valine replaced by isoleucine in the sequence GRGVIT, but otherwise this site too is found in all of the Fe protein sequences. The *Trichodesmium* sp. Fe protein is thus very similar in primary sequence to the Fe proteins of conventional and alternative nitrogenases from other organisms [11,23].

The solvent exposure of the FeS center of the Fe protein may contribute to oxygen sensitivity [20]. If the *Trichodesmium* sp. Fe protein tertiary structure provided protection of the FeS cluster, the protein could potentially be more tolerant of oxygen. Examination of the Fe protein structure in the regions surrounding the FeS cluster and at the Fe protein-MoFe protein interface show that the nitrogenase proteins are particularly well conserved at these sites (Fig. 2). The loops surrounding the FeS cluster in the *Trichodesmium* sp. and *Azotobacter* structures are virtually identical (FeS cluster shown in red and gold in Fig. 2). The helices spanning the region between Arg-101 and Glu-113 are equally well conserved (shown in orange in Fig. 2), which are at the interface between the Fe protein and MoFe protein. However, this analysis was performed on the model of Fe protein with ADP bound. It is possible, although unlikely, that differences in oxygen sensitiv-

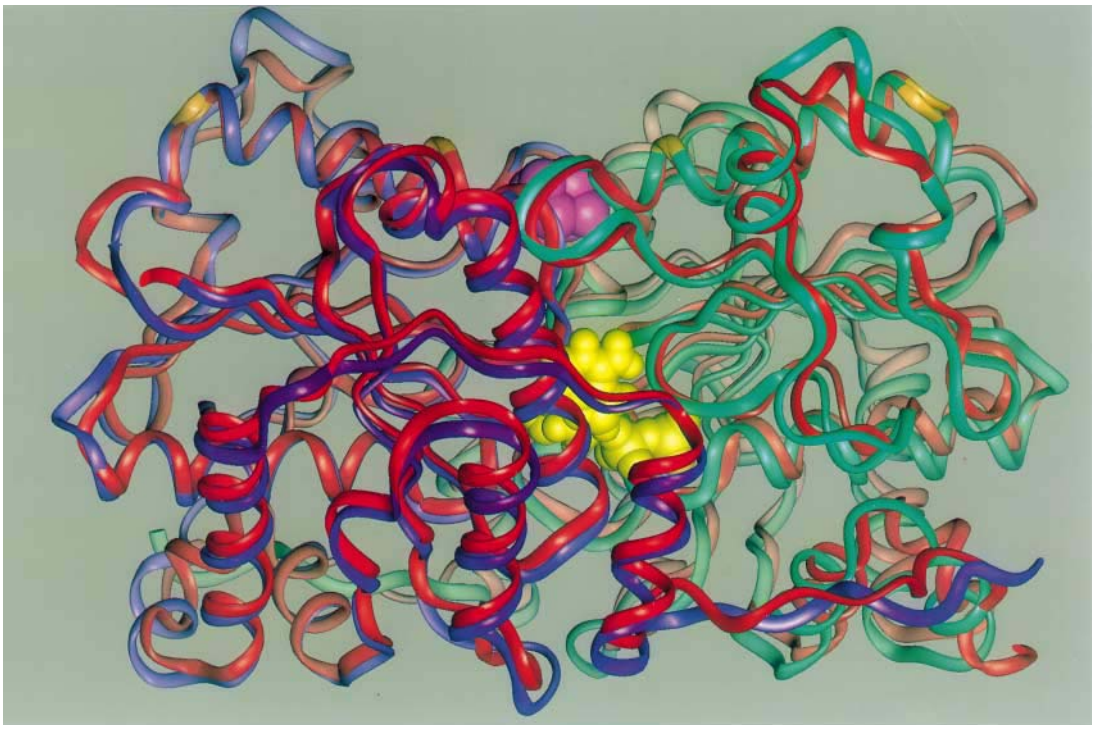

Fig. 2. Predicted structure of the Fe protein of nitrogenase. *A. vinelandii* subunits are shown in red, with the two identical subunits of the *Trichodesmium thiebautii* protein shown in purple and green. The FeS cluster is shown in pink, and ADP shown in gold. Arginine and glutamate residues (corresponding to *A. vinelandii* positions 101 and 113) are shown in orange.

ity of the FeS clusters could arise when ATP is bound to the nucleotide binding site.

There are regions where the *Trichodesmium* sp. sequence differs from the *A. vinelandii* sequence (*A. vinelandii* 23–35, 57–62, 199–204, 230–237, 275–280), but these regions are conserved at least within the cyanobacteria (*Anabaena* sp. 7120 and *Synechococcus* sp. RF-1; Fig. 1). The largest differences between the *Trichodesmium* sp. and *A. vinelandii* structures are at the carboxy-terminus and in loop regions between the major features of the protein (Fig. 2). The *Trichodesmium* sp. Fe protein sequence extends a few residues beyond the terminus of the *Azotobacter* Fe protein, as do the other cyanobacterial sequences (Fig. 1). Interestingly, the cyanobacterial sequences are not highly conserved in the carboxy-terminus region (Fig. 1). The tertiary structure of this region of the *A. vinelandii* protein was not observed by X-ray crystallography [20] and the two sequences differ in total length by only 6 amino acid residues (Fig. 1).

The majority of structural differences between the Fe proteins are on exterior surfaces remote from the FeS cluster, the nucleotide binding site, and the binding site for the MoFe protein. His-29 (*A. vinelandii* numbering) in the *Trichodesmium* sp. Fe protein, site of a single residue insertion, lies on an exterior α-β turn. A poorly conserved area around *A. vinelandii* residue 52 corresponds to an external random coil. Residues 56–76 form a surface helical hairpin, and the largest internal insertion in the set of proteins examined (11 residues in *M. thermolithotrophicus* 2 compared to *M. thermolithotrophicus* 1, Fig. 1), occurs at the loop joining the two short helices. The majority of the other proteins have just one additional residue in this loop.

A pattern of small insertions and deletions around *A. vinelandii* residue 118 corresponds to an external α-β turn. A few other potential insertion sites are located in external random coil sequences, in α-β turns along one edge of the β-sheet, and in an external 'strap' connecting two helices corresponding to

the region near *A. vinelandii* residue 251. Other sites of potential interest include a proline found in *Trichodesmium* sp. Fe protein, corresponding to a turn at residues 79–80 in *A. vinelandii* at the outside corner of a β-sheet. Regions of relatively poor conservation include an external helix (*A. vinelandii* residues 196–202) and an external 'strap' connecting two helices (*A. vinelandii* residues 249–261). None of these regions would be likely to affect the FeS cluster, nucleotide binding site, or interactions between monomers. It is unlikely that they reflect differences that would affect stability or catalytic activity.

It is possible that the 11 residue insert in *M. thermolithotrophicus* sequence 2 in Fig. 1) is a modification that affects electron interactions with the FeS cluster. While the site of this insertion is 20–25 Å distant from the FeS cluster, it is within 10 Å of the helix extending between *A. vinelandii* residues 101 and 113 (Figs. 1 and 2). This helix is involved in interactions with the MoFe protein and, depending on the orientation of the insertion, interactions between the MoFe and Fe proteins could be affected.

The carboxy-terminus of the deduced amino acid sequence of the Fe protein obtained from *Trichodesmium* sp. IMS 101 differs substantially from that reported by Sroga et al. [23] for *T. thiebautii*. The deduced amino acid sequence of the *Trichodesmium* sp. IMS 101 Fe protein is very similar to the carboxy-terminus of other diazotrophs, particularly the sequence from *R. capsulatus* (note the amino acid sequence KQIAQDAAIKA, Fig. 1). The sequence reported by Sroga et al. [23] is from another species (*Trichodesmium* sp. IMS 101 is believed to be most closely related to *T. erythraeum*). There are at least two nucleotide differences between the sequences near the 3′ end of *nifH*, which results in a reading frame shift with respect to the *Trichodesmium* sp. IMS 101 amino acid sequence reported here. The high similarity of the *Trichodesmium* sp. IMS 101 Fe protein sequence to the Fe protein sequences of other diazotrophs suggests that there is most likely a minor error in the nucleotide sequence reported by Sroga et al. [23] for *T. thiebautii*.

The sequence of NifH generally varies substantially in length and sequence among organisms at the carboxy-terminus [20], and the carboxy-terminus may be important in protein interactions with the MoFe protein [21]. For example, the *Clostridium pasteurianum* NifH is 13 residues shorter than that of *A. vinelandii*, and incorporation of this sequence in the *A. vinelandii* protein reduces activity of *A. vinelandii* nitrogenase [24]. The carboxy-terminus may affect the stability of the nitrogenase complex with the MoFe protein [20]. Other cyanobacterial *nifH* sequences also encode a carboxy-terminal tail, with the longest carboxy-terminal sequences in *Anabaena* (*Nostoc*) sp. PCC7120, and *Synechococcus* sp. RF-1 (Fig. 1). Thus, even though the tertiary structure of the carboxy-terminus of the *Trichodesmium* sp. Fe protein cannot be determined from these data, it is unlikely that its unique sequence is responsible for tolerance to oxygen. However, given the similarity in structure of the rest of the Fe protein, the carboxy-terminus would be the only characteristic that might confer different biochemical properties to *Trichodesmium* sp. nitrogenase.

Azotobacter spp. fix nitrogen aerobically by maintaining a high respiratory rate that maintains a low oxygen environment within the cell [25]. In contrast, *Trichodesmium* spp. and other nonheterocystous cyanobacteria fix nitrogen while evolving oxygen through photosynthesis. Thus, respiratory protection alone is not likely to provide an adequate explanation for nitrogen fixation in these photosynthetic organisms. Many hypotheses have been offered to explain the paradox of simultaneous nitrogen fixation and photosynthesis in *Trichodesmium* spp., but the question of whether the *Trichodesmium* sp. nitrogenase differed in oxygen sensitivity due to structural characteristics had previously remained an unresolved issue. Based on the predicted structure presented here, it appears certain that analysis of the structure of the nitrogenase Fe protein cannot resolve the paradox. Therefore, the mechanisms involved in aerobic nitrogen fixation in *Trichodesmium* are likely to be protective in nature, and have yet to be elucidated.

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