Studies on the Regulatory Mechanism of Nitrogenase Gene Expression by Transcriptional Regulator VnfA from *Azotobacter vinelandii*

(*Azotobacter vinelandii* 由来転写調節因子 VnfA による窒素固定酵素の発現制御機構に関する研究)

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Chapter I

General Introduction

1. Nitrogen, an essential element of all life

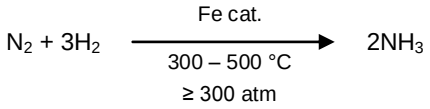
Nitrogen atom is a key element for the biological molecules and an important component of fertilizers and medicaments (1,2). Nitrogen is also crucial for non-biological systems such as dyes, explosives, and resins. Synthesis of all these materials requires ammonia as a source of nitrogen atom.

The Haber-Bosch process developed in early 1900's is the most notable abiological source of ammonia (Fig. 1 (A)) (1). Since ammonia is a limited nutrient in agriculture, the production of ammonia at the industrial scale by the Haber-Bosch process has enabled the enormous increase in food production.

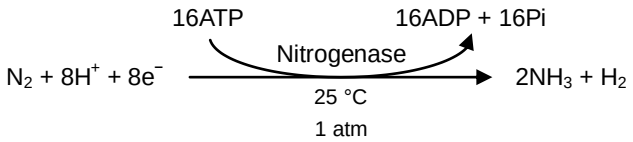
Lightning and biological N_2 fixation are known as natural processes, which convert unreactive N_2 to nitrogen compounds (Fig. 1 (B) and (C)) (3,4). Since the amount of nitrogen compound produced by the biological N_2 fixation is much larger than that by the lightning, the biological N_2 fixation is relatively important as a source of nitrogen compounds. The biological N_2 fixation has been evolved by only a limited number of species of bacteria and archaea. Rhizobium bacteria are one group of well-resolved bacteria having N_2 fixation ability. The bacteria stimulate leguminous plants to develop root nodules and inhabits after infection (5,6). There are two organisms in symbiosis; plants reduce carbon dioxide into sugars by the photosynthesis and transport them to the root where the bacteria use them as a fuel, and the bacteria reduce (fix) N_2 into ammonia which is provided to the plant for assimilation. Application of N_2-fixation legumes to agriculture has been ongoing for many centuries because some of the fixed nitrogen remains in unharvested parts of the crop and is released to the soil to improve soil fertility (2).

In addition to symbiotic bacteria, there are free-living bacteria capable of fixing N_2 such as *Klebsiella pneumoniae* and *Azotobacter* species. *K. pneumoniae* is an enteric diazotroph related to *Escherichia coli* and *Salmonella typhimurium*. Since both bacteria have been extensively studied at

(A) Haber-Bosch process



(B) Biological N_2 fixation



(C) Lightning

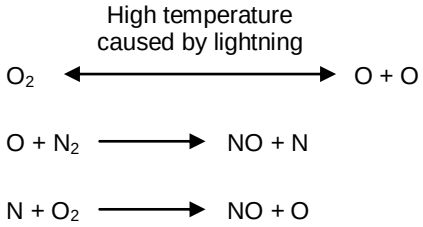


Fig. 1. Industrial and natural processes for producing nitrogen compounds.

a genetic level, the genetic analysis of *K. pneumoniae* as well as its biochemical and physiological studies is well progressed (7-9). Consequently, genes related to the N_2 fixation including nitrogenase, an enzyme catalyzing the N_2 fixation, were already identified. Similarly, genetics of the N_2 fixation in *Azotobacter* species have been studied, extensively. A major discovery in the studies on *Azotobacter* species is that *Azotobacter vinelandii* contains additional types of nitrogenase (10-16). Due to a unique feature in the N_2 fixation, *A. vinelandii* has been regarded as one of the model organisms for studying the N_2 fixation system.

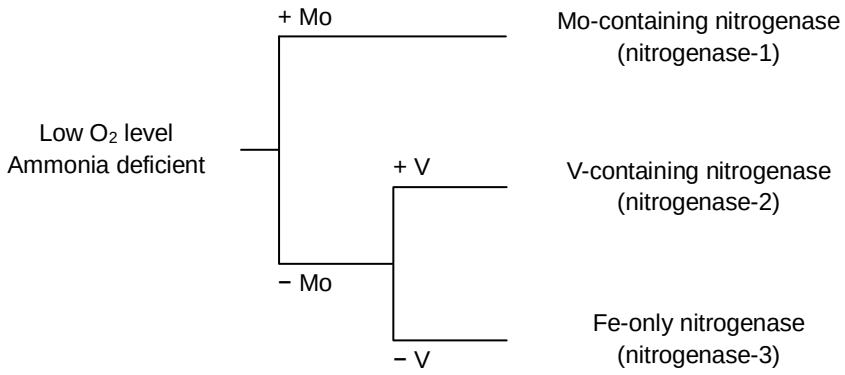


Fig. 2. Expression of nitrogenase dependent on cellular conditions in the diazotroph *A. vinelandii*.

2. Nitrogen fixation in diazotroph *Azotobacter vinelandii*

The diazotroph *Azotobacter vinelandii* discovered in 1903 was an obligately aerobic bacterium which utilizes NO_3^- and N_2 as nitrogen sources to obtain ammonia (17-19). The obtained ammonia is assimilated into glutamine and then glutamate, which are source materials of nucleic acids and amino acids, via glutamine synthetase (GS) and glutamate synthase (GOGAT) pathways (20).

The N_2 fixation system in *A. vinelandii* has been studied for many years due to the unique feature of genetically three distinct nitrogenases (Fig. 2) (10-16). Among them, a classical molybdenum (Mo)-containing nitrogenase (nitrogenase-1) encoded by *nif* operon is most characterized, while further studies are required for other Mo-independent nitrogenases; vanadium (V)-containing nitrogenase (nitrogenase-2) encoded by *vnf* operon and Fe-only nitrogenase (nitrogenase-3) encoded by *anf* operon. The three nitrogenases are expressed in response to provision of Mo and V. In the presence of Mo, nitrogenase-1 is expressed and nitrogenase-2 and -3 are repressed. Nitrogenase-2 is expressed under Mo-deficient conditions in the presence of V, whereas both nitrogenase-1 and -3 are repressed. Nitrogenase-3 is expressed when both Mo and V

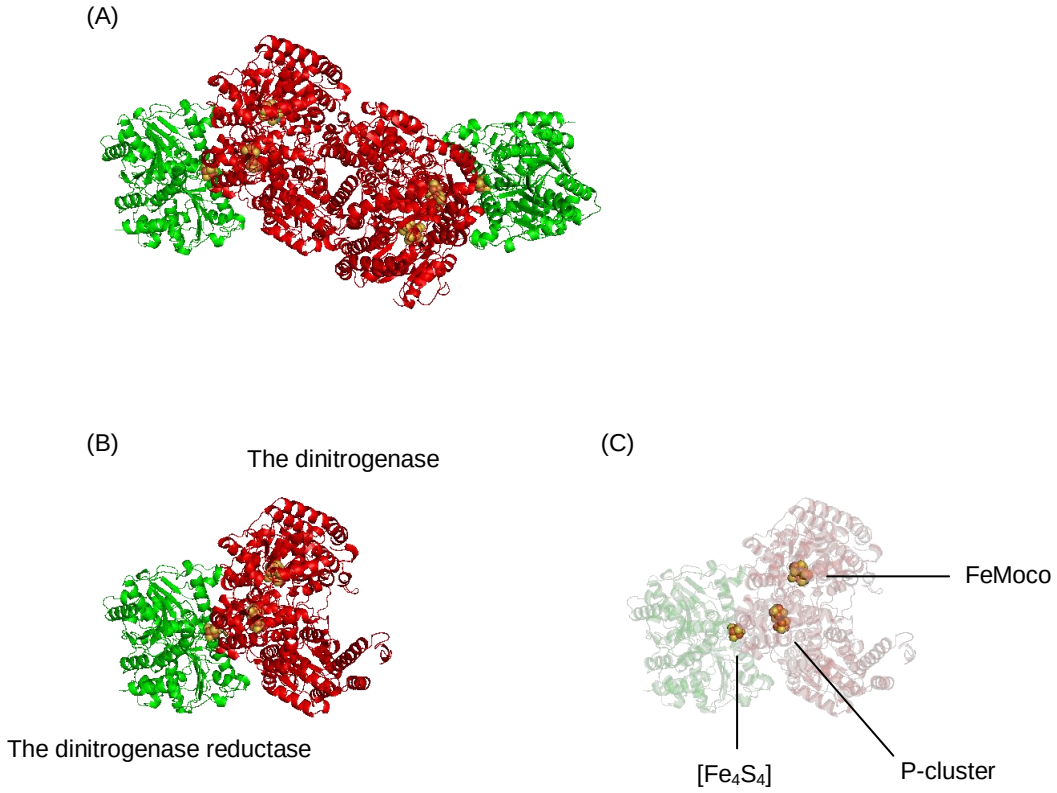


Fig. 3. Overall structure of nitrogenase-1 from *A. vinelandii* (PDB ID: 1N2C). (A) The entire complex with dinitrogenase reductase and dinitrogenase. (B) One half-complex. (C) One half-complex with transparent backbone. Dinitrogenase reductase and dinitrogenase are depicted in green and red, respectively. The clusters and the cofactor are shown in ball-and-stick representation. Atoms are color-coded with Fe in orange, S in yellow and Mo in cyan.

are deficient. In spite of the phenomenalistic understanding of the nitrogenase expression, there are many problems to be solved on the regulatory mechanisms of both nitrogenase-2 and -3 by Mo and V.

The three nitrogenases are O_2-labile Fe-S proteins comprised of two separable components, dinitrogenase reductase and dinitrogenase (Fig. 3) (21-25). The dinitrogenase reductase harboring 4Fe-4S type cluster ($[Fe_4S_4]$) serves as an obligate electron donor to dinitrogenase in the MgATP-

and reductant-dependent process (26-28). In the dinitrogenase component of nitrogenase-1, electrons are transferred by the 8Fe-7S type cluster (P-cluster) to the Fe- and Mo-containing cofactor (FeMoco) which provides the active site for the reduction of N_2 to ammonia. Although extensive studies on nitrogenase-1 and model compounds of both P-cluster and FeMoco have been carried out (29-37), the whole catalytic mechanism of nitrogenase-1 has not been deciphered, so far.

The cofactor corresponding to FeMoco in nitrogenase-2 is designated FeVco which contains Fe and V (23,38-42). Spectroscopic analyses revealed that the FeVco extracted from nitrogenase-2 is similar to but yet distinct from FeMoco in electronic properties and structural topology. The N_2 reduction activity of nitrogenase-2 was reported to be less than that of nitrogenase-1. While nitrogenase-3 has been actively examined, there are still a lot of problems including the properties of cofactor to be solved.

The Mo-independent nitrogenases exert their advantage in the diazotrophic growth under Mo limitation conditions (17). Local depletion of Mo is induced such as in acid soil with high iron oxide contents, where MoO_4^{2-} is tightly bound to the iron oxide and, thus, biologically unavailable. Moreover, Mo uptake by plants could result in the local depletion of Mo in the rhizosphere. Due to the Mo-independent nitrogenases, *A. vinelandii* may survive under these severe conditions from other diazotrophs.

3. Transcriptional regulation of *nitrogenase* genes by the σ^N-dependent transcriptional regulators

Transcription is the key regulatory point for numerous cellular activities (43). Bacteria regulate levels of gene expression by using transcriptional factors. The factors modulate the recruitment of σ-RNA polymerase to its specific promoter in DNA. The σ subunit is required for

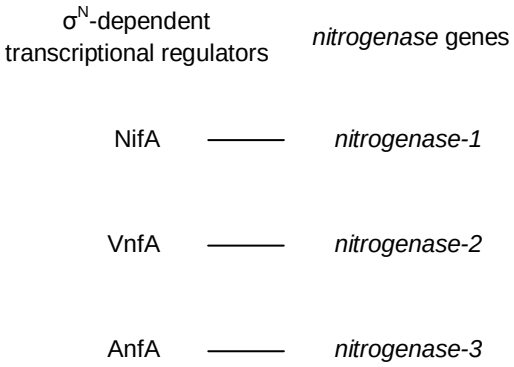


Fig. 4. σ^N-dependent transcriptional regulators responsible for gene transcription of *nitrogenase-1, -2* and *-3* in *A. vinelandii*.

promoter recognition and initiation of transcription. Many bacteria also regulate gene expression by using a second class of transcriptional factors that obtain energy from nucleotide hydrolysis to promote transcription initiation.

The gene transcription of *nitrogenase-1, -2* and *-3* was reported to be regulated by NifA, VnfA and AnfA, respectively, which belong to the σ^N-dependent transcriptional regulator family (Fig. 4) (17,44). The σ^N subunit (also known as RpoN, NtrA and σ^{54}) is a class of the σ subunits and recognizes −24/−12 promoter, TGGCAC-N5-TTGC underlined at conserved −24 GG and −12 GC motifs (45,46). Since the σ^N-RNA polymerase forms a transcriptionally inactive closed complex with its specific promoter, the σ^N-dependent transcriptional regulator is required for remodeling the complex into a transcriptionally competent open complex in which the DNA strands in the region of the transcription start site are locally denatured (Fig. 5) (43,47-51). In the remodeling process, the σ^N-dependent transcriptional regulator binds to its specific binding site (known as upstream activator sequences, UAS) near the −24/−12 promoter, and then contacts with the σ^N-RNA polymerase by a DNA-looping mechanism (52). The looping event is often facilitated by the binding of Integration

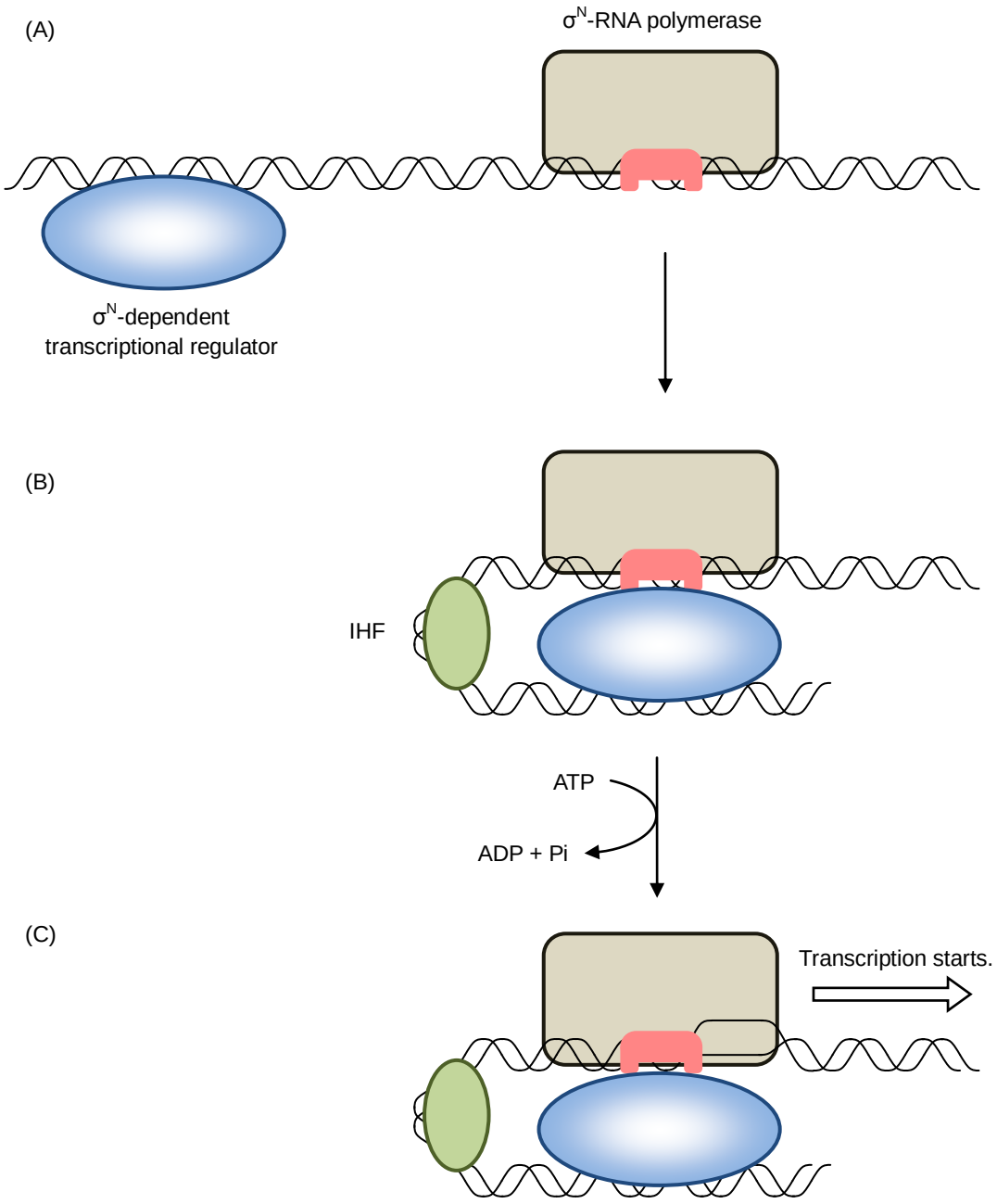


Fig. 5. Transcriptional activation by the σ^N-dependent transcriptional regulator. (A) The σ^N subunit binds to its specific promoter at positions −24/−12 relative to the transcription start site, where it forms a transcriptionally inactive closed complex with core RNA polymerase. The σ^N-dependent transcriptional regulator also binds to its specific binding site. (B) The σ^N-dependent transcriptional regulator contacts with the σ^N-RNA polymerase complex via DNA looping which is assisted by the integration host factor (IHF). (C) ATP hydrolysis catalyzed by the σ^N-dependent transcriptional regulator is coupled with the open complex formation, resulting in the initiation of the transcription.

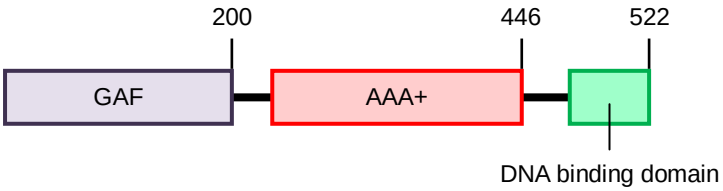


Fig. 6. Domain structure of NifA from *A. vinelandii*. The amino-terminal (GAF), central (AAA+) and C-terminal DNA-binding domains are shown as purple, red and green rectangles, respectively. The linker regions are shown as solid bars. The number corresponds to the residue number at the end of each domain.

Host Factor (IHF) to the region between the UAS and the −24/−12 promoter (53). Formation of the open complex and subsequent transcription initiation are coupled with the ATP hydrolysis catalyzed by the σ^N-dependent transcriptional regulator.

3.1 The NifA protein

Among the σ^N-dependent transcriptional regulators of the nitrogenase gene expression in *A. vinelandii*, the most characterized regulator is NifA (17,54-58). The NifA protein regulates the transcription of *nif* operon including the structural gene of nitrogenase-1 and is composed of three distinct domains (Fig. 6); an N-terminal GAF domain (i.e. cGMP-specific and -stimulated phosphodiesterases, *Anabaena* adenylate cyclases and *Escherichia coli* FhlA), a central domain that is characteristic of all of σ^N-dependent transcriptional regulators and belongs to AAA+ (ATPase associated with a variety of cellular activities) domain, and a C-terminal DNA binding domain.

The GAF domains which are known to be ubiquitous signaling modules found in all kingdoms of life frequently exert regulatory effects on adjacent catalytic domain (59,60). In the case of NifA, the GAF domain regulates the activity of the AAA+ domain in response to 2-oxoglutarate which

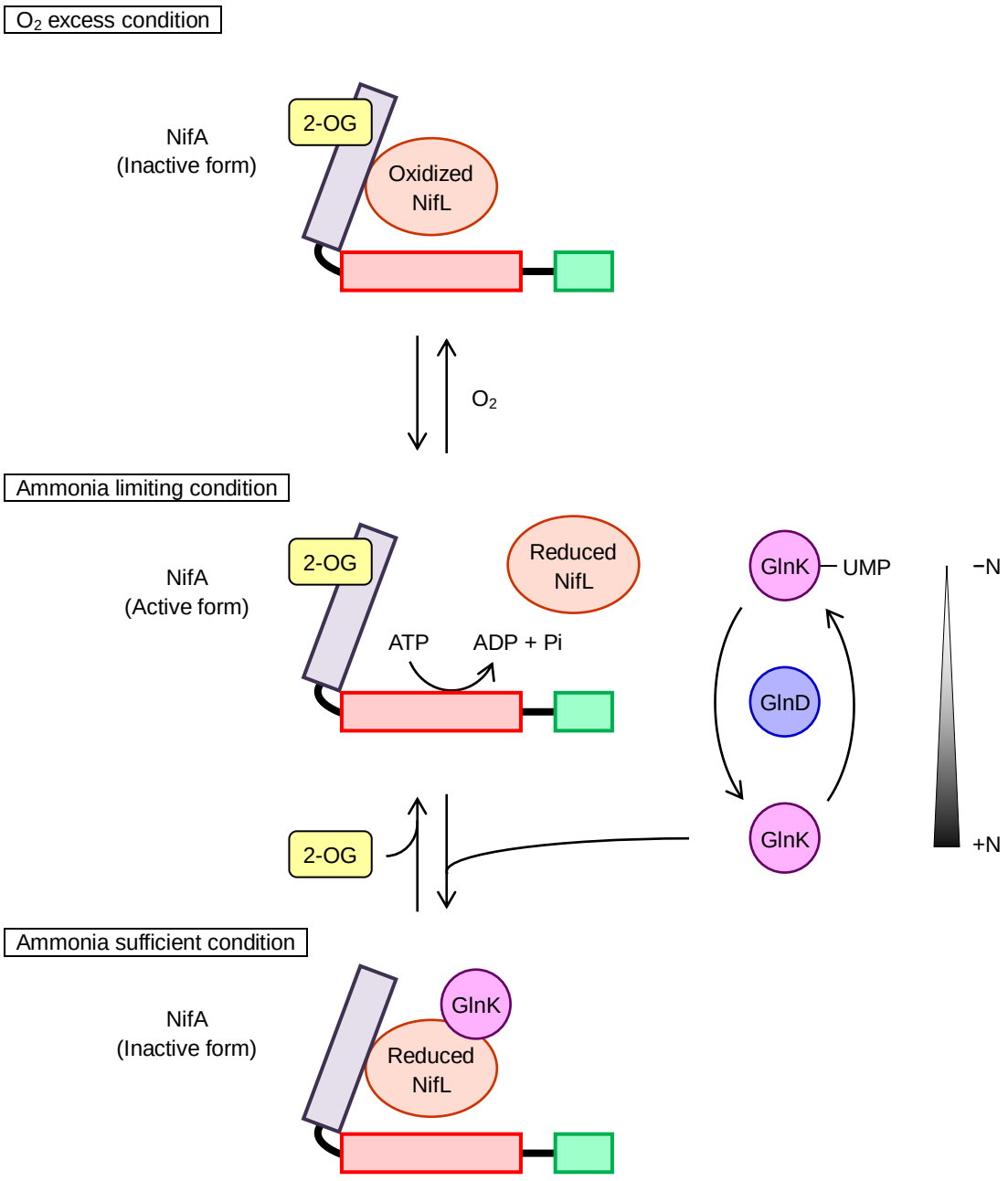


Fig. 7. Model showing the regulatory mechanism of *nif* gene transcription by NifA in *A. vinelandii*. The three domains of NifA are shown as purple, red and green rectangles, respectively. Under conditions of ammonia limitation, GlnK is mainly urydylylated and not competent to interact with NifL. Binding of 2-oxoglutarate (2-OG) to the GAF domain of NifA relieves inhibition by NifL, freeing NifA to catalyze ATP hydrolysis. However, when NifL is oxidized, the NifL-NifA complex is formed. Formation of the complex sequesters NifA, preventing transcriptional activation. Under conditions of ammonia sufficiency, GlnD deuridylylates GlnK. The GlnK interacts with NifL to promote formation of the NifL-NifA complex.

is a key metabolic signal of the carbon status, and/or the antiactivator protein NifL which senses signal of both O_2 and ammonia excess (Fig. 7) (55,57). The NifL senses high O_2 level via a redox reaction of its flavin cofactor. The O_2 level sensing is essential to protect the O_2-labile nitrogenases from inactivation. The cytosolic ammonia is also sensed by NifL cooperatively with GlnD and GlnK. The GlnK deurydylylated by GlnD in the presence of ammonia binds to NifL. The bound GlnK facilitates NifL to interact with NifA.

The AAA+ domain is responsible for the interaction of NifA with σ^N-RNA polymerase and the ATP hydrolysis (47,61). Similar to other σ^N-dependent transcriptional regulators, the AAA+ domain in NifA shows the ATPase activity negatively regulated by the GAF domain. The domain contains highly conserved Walker A and B motifs and GAFTGA motif which appear to be essential for the ATP hydrolysis and the interaction of NifA with σ^N-RNA polymerase, respectively (48,50,62). The C-terminal DNA binding domain contains a helix-turn-helix motif which recognizes a consensus sequence TGT-N10-ACA near the −24/−12 promoter (63).

3.2 The VnfA and AnfA proteins

A *vnfA* gene encoding VnfA was found in 1989 (44) and UAS was subsequently identified as duplicated GTAC-N6-GTAC. There has been essentially no structural and functional analyses conducted *in vitro* for more than 20 years because of insolubility of the protein as well as difficulty in overexpressing the *vnf* gene using recombinant system of *E. coli*. An *anfA* gene encoding AnfA was found at the same time as the *vnfA* gene (44). Due to the same reason as VnfA, there are little reports in comparison with the research on the NifA (64).

The gene analyses suggested that the GAF domains in VnfA and AnfA contain characteristic cysteine-rich motifs (CRMs), Cys^{8}-X-Cys^{10}-XXXX-Cys^{15} and Ser^{19}-X-Cys^{21}-XXXX-Cys^{26}, respectively (44). Studies by amino-acid substitution of this motif in AnfA demonstrated that the

two cysteines are required for the AnfA activity (65). Although these motifs have been suggested to form active centers with metal atoms or clusters, the prosthetic groups of VnfA and AnfA held in these motifs were unidentified when I started the study on VnfA.

4. Fe-S clusters as prosthetic groups in transcriptional regulators

The Fe-S proteins are an ancient and important class of proteins (66). As discussed in this thesis, VnfA harbors a 3Fe-4S type cluster ($[Fe_3S_4]$) in the cysteine-rich motif (CRM) of the GAF domain. Similarly, the Fe-S clusters which were harbored in CRMs have also been found in some transcriptional regulators such as SoxR and FNR.

SoxR is the first transcriptional regulator that was shown to have an Fe-S cluster and it forms a homodimer in solution (Fig. 8 (A)) (67-70). A 2Fe-2S type cluster ($[Fe_2S_2]$) in SoxR senses O_2^- stress (71). The O_2^- stress activates SoxR to stimulate expression of the transcriptional factor SoxS, and then accumulation of SoxS leads to the activation of ~45 genes. The resultant products (e.g. superoxide dismutase) facilitate the removal of O_2^- from cells and repair of oxidative damages. SoxR in the oxidized form contains $[Fe_2S_2]^{2+}$ and is active in the *soxS* transcription, while reduction of the cluster to $[Fe_2S_2]^+$ eliminates the transcriptional activity. During the process, SoxR retains the binding ability to the promoter of *soxS*. Superoxide acts either as a reductant or as an oxidant depending on the redox partner (72,73). An *in vitro* study suggested that SoxR has $[Fe_2S_2]^+$ and is transcriptionally inactive under normal growth conditions, while exposure to the O_2^- stress oxidizes SoxR to transform it to the active form.

Fumarate and nitrate reduction regulatory proteins (FNR) originally found in *E. coli* are bacterial transcriptional regulators that coordinate the switch between aerobic and anaerobic metabolism (Fig. 8 (B)) (67,68,74-76). In the absence of O_2, FNR harbors a 4Fe-4S type cluster

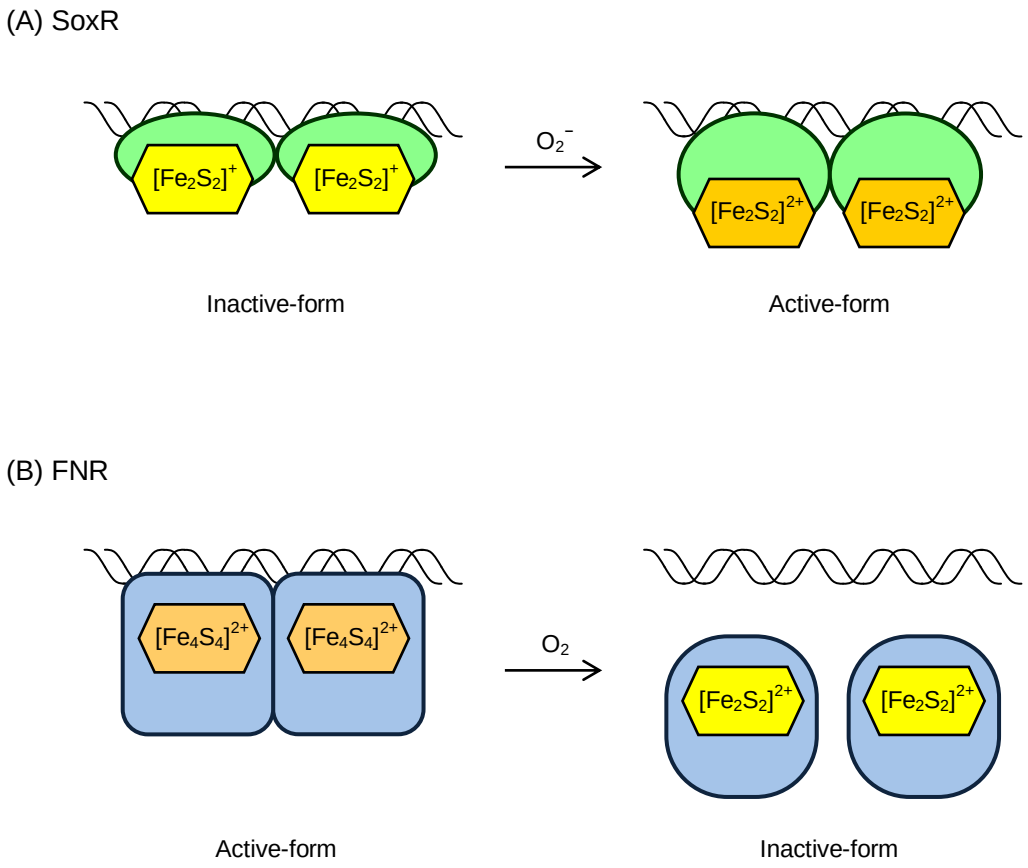


Fig. 8. Fe-S cluster-based transcriptional regulation. (A) SoxR. Under aerobic conditions, the $[Fe_2S_2]$ of SoxR is maintained in the reduced state. The exposure to O_2^- causes oxidation of the cluster to turn SoxR transcriptionally active-form. (B) FNR. Under anaerobic conditions, FNR is a transcriptionally active dimer. In the presence of O_2, the $[Fe_4S_4]^{2+}$ are converted to $[Fe_2S_2]^{2+}$. This drives conformational changes that result in monomerization and inactivation of FNR.

($[Fe_4S_4]^{2+}$) ligated by cysteines in CRM (Cys^{20}-XX-Cys^{23}-XXXXX-Cys^{29} and Cys^{122}), which promotes formation of the transcriptionally active dimer. The reaction of $[Fe_4S_4]^{2+}$ with O_2 results in one-electron oxidation to yield an $[Fe_3S_4]^+$ intermediate with release of one Fe^{2+} ion, followed by spontaneous rearrangement to $[Fe_2S_2]^{2+}$ with release of additional one Fe^{3+} and two S^{2-} ions. The process leads to the dissociation of the protein dimer into transcriptionally inactive monomers. Thus, FNR serves as a sensor of O_2 level via oxidation reaction of the Fe-S cluster.

As surveyed here, the major role of the Fe-S clusters in transcriptional regulators is to sense various environmental factors. The sensing events are reflected in the transcriptional activity of each regulator through various molecular mechanisms.

5. Significance of investigation on VnfA

Since N_2 fixation is a process of fundamental importance to life on Earth, studies on the biological N_2 fixation system have attracted a large number of scientists for many years (77). In contrast to the energy-intensive Haber-Bosch synthesis (78), ammonia production by using N_2-fixation organism occurred at ambient temperature and pressure has been recognized recently as a fascinating eco-friendly alternative. For the application of N_2-fixation organisms, it is essential to understand whole N_2 fixation system. However, even in the N_2 fixation system of the diazotroph *A. vinelandii* which is a model organism for the research on the system, there are still full of problems. One of the problems is in the regulatory mechanism of *nitrogenase-2* gene expression by VnfA.

The Fe-S cluster in VnfA is also fascinating. The most common forms of the clusters in the Fe-S proteins are $[Fe_2S_2]$, $[Fe_3S_4]$ and $[Fe_4S_4]$ (69). Among them, there has been no reports regarding the transcriptional regulator which harbors $[Fe_3S_4]$ in an intact form except for VnfA. Accordingly, the study on $[Fe_3S_4]$ in VnfA would provide a new insight into biological versatility of the Fe-S proteins.

Based on these backgrounds, this thesis discusses the regulatory mechanism of *nitrogenase-2* gene expression by VnfA and the role of the Fe-S cluster. I hope that my study contributes to further understanding of the N_2 fixation system by clarifying the regulatory mechanism of VnfA. To open discussion about a novel aspect of the Fe-S protein is another objective of this thesis.

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Chapter II

Expression, Purification and Characterization of VnfA

Introduction

The diazotroph *Azotobacter vinelandii* contains three distinct nitrogenases. Nitrogenase-1 is a conventional molybdenum nitrogenase that bears a metal-sulfur cluster consisting of molybdenum and iron as the reactive site. The active center of nitrogenase-2 consists of vanadium and iron, and that of nitrogenase-3 contains only iron (1,2). The expression of each set of structural genes is regulated by specific transcriptional activator proteins, namely, NifA, VnfA and AnfA, which regulate *nifHDK* (nitrogenase-1), *vnfDGK* (nitrogenase-2) and *anfHDGK* (nitrogenase-3), respectively (3).

Gene analyses suggest that these activators belong to σ^N-dependent regulatory proteins generally consisting of three major domains (4). The N-terminal domain termed GAF (i.e. cGMP-specific and -stimulated phosphodiesterases, *Anabaena* adenylate cyclases and *Escherichia coli* FhlA) is considered to be a sensory domain (5). The central AAA+ (ATPase associated with a variety of cellular activities) domain is responsible for the interaction of VnfA with σ^N-RNA polymerase and ATP hydrolysis which are necessary to form the transcriptionally active open complex. The C-terminal DNA binding domain containing a helix-turn-helix motif enables the activator to bind to specific DNA region. The primary structure of the GAF domain is highly conserved in VnfA and AnfA, whereas NifA shares little homology with them, suggesting that the sensor structure of NifA is distinct from that of VnfA and AnfA (3). Indeed, the GAF domain of NifA forms a complex with another sensory protein, NifL, which contains a flavin moiety that serves as an O_2 sensor in the cytosol (6-8), whereas VnfA and AnfA work independently and do not have proteins corresponding to NifL (3,9-11). Instead, there are characteristic Cys-rich motifs, Cys-X-Cys-XXXX-Cys and Ser-X-Cys-XXXX-Cys, preceding the GAF domains of VnfA and AnfA, respectively (3). These motifs have been suggested to form active centers in the sensory domains containing metal atoms or clusters as prosthetic groups. In fact, a previous study of AnfA variants

in vivo revealed that AnfA requires Cys residues in the N-terminus and iron ions for transcriptional function (12). Similar inferences have been proposed for nitrogenase regulatory proteins isolated from other diazotrophs, such as *Herbaspirillum seropedicae* (13,14) and *Bradyrhizobium japonicum* (15,16). These regulatory proteins also have Cys-rich motifs in their central domains and have a specific requirement for iron ions to allow activation of the transcription of nitrogenase genes in their host cells, whereas it is still obscure whether the Cys-rich motifs are associated with the requirement for iron.

By contrast to a number of studies conducted *in vivo* (9,12,17-21), there have been essentially no structural and functional analyses of VnfA and AnfA conducted *in vitro* because of the insolubility of the proteins as well as difficulty in overexpressing their genes using recombinant systems. This has hampered their isolation by conventional purification methods such as column chromatography. An exceptional success is the purification of an AnfA variant reported by Austin *et al.* (22). In their study, the N-terminal domain of AnfA was truncated to prevent the intrinsic aggregation of the intact form during purification. The obtained variant retained transcriptional activity and provided fundamental information about the function of AnfA, including the binding sequence in the *anfH* promoter region and prerequisites for transcriptionally active AnfA. However, the sensing mechanism that may reside in the GAF domain remains unknown due to the absence of the N-terminal domain in this variant. Because sensing is a principal function of regulatory proteins, the isolation of VnfA and AnfA with their sensor (GAF) domains is highly desirable.

In Chapter II, I describe the successful production and purification of recombinant full-length VnfA in both *Strep*-tag attached and tag-less forms in *E. coli*. Spectroscopic and biochemical characterization of the recombinant VnfA both *in vitro* and *in vivo* show that VnfA function requires iron-sulfur (Fe-S) clusters as a prosthetic group. I also describe a functional form of VnfA including the number of subunits in the native form and the type, as well as the stoichiometry of the

cluster. Activity assay conducted *in vivo* allows discussion of a role for the Fe-S cluster in the transcriptional function of VnfA.

Results

Expression of *vnfA* in *E. coli*

The production of tag-less VnfA (VnfA) in *E. coli* is sensitive to the cultivation temperature. When induction by isopropyl thio-β-D-galactoside (IPTG) was performed above 25 °C, most of the produced protein was found in the insoluble fraction, whereas, below 25 °C, soluble VnfA can be obtained after cell lysis by sonication and subsequent centrifugation of the cellular debris. Therefore, I cultivated the cells for 16 h at 20 °C to allow efficient induction of soluble VnfA. The amount of O_2 in the culture had little effect on the production; VnfA was produced similarly under both aerobic and micro-aerobic growth conditions. As described below, VnfA produced under these conditions could be purified through a combination of column chromatography and ammonium sulfate fractionation.

Having established the culture conditions that allow the accumulation of VnfA in the cytosol of *E. coli*, EPR spectroscopy with whole *E. coli* cells which overexpressed *vnfA* was attempted to obtain information regarding the metals present in the prosthetic group. The results obtained are shown in Fig. 1. Regardless of the aeration level of the culture, the cells produced distinctive signals at g = 2.03 and 2.01 at 10 K (aerobic cultures are shown Fig. 1A), and this is different from the native signals of *E. coli*, which are mainly the result of high-spin Mn^{2+} species and free organic radicals (23) (Fig. 1B). Figure 1D shows the overall shape of the signals obtained by subtraction of Fig. 1B from Fig. 1A. The signals are consistent with an oxidized 3Fe-4S type cluster ($[Fe_3S_4]^+$) found in metalloproteins, such as inactive cytosolic aconitases, ferredoxin and enzymes bearing

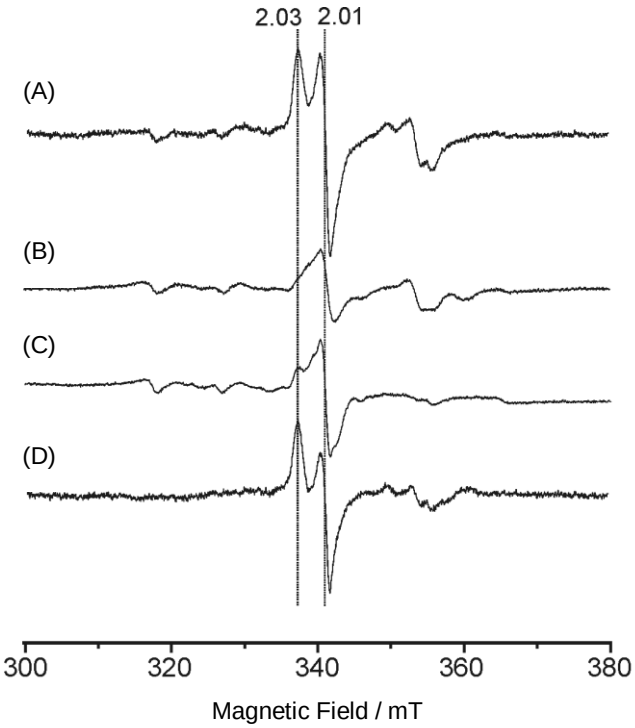

Fig. 1. Whole cell EPR spectra of *E. coli* JM109 strain cultured under aerobic conditions: (A) overexpressing *vnfA* recorded at 10 K, (B) transformed with pKK223-3 carrying no structural gene of VnfA and (C) overexpressing *vnfA* recorded at 50 K. (D) Difference spectrum obtained from (A) – (B). Spectra were recorded at 2.5 mW microwave power and a field modulation of 0.8 mT. The intensities of the spectra were normalized with native signals of Mn^{2+} species from *E. coli*.

$[Fe_3S_4]$ (24). The temperature dependence of the signal intensity also supports the presence of an Fe-S cluster. Weaker signals are observed at higher temperature and almost disappear at 50 K (Fig. 1C). Thus, the EPR results indicate the accumulation of $[Fe_3S_4]^+$ in the expression system of *vnfA* (i.e. the involvement of $[Fe_3S_4]^+$ in VnfA). However, the EPR data cannot exclude possible presence of other types of Fe-S clusters, such as 4Fe-4S ($[Fe_4S_4]$) and 2Fe-2S ($[Fe_2S_2]$), because the Fe-S clusters could be EPR-silent depending on their oxidation state. To address this measurement problem encountered *in vivo*, I purified and characterized VnfA *in vitro*.

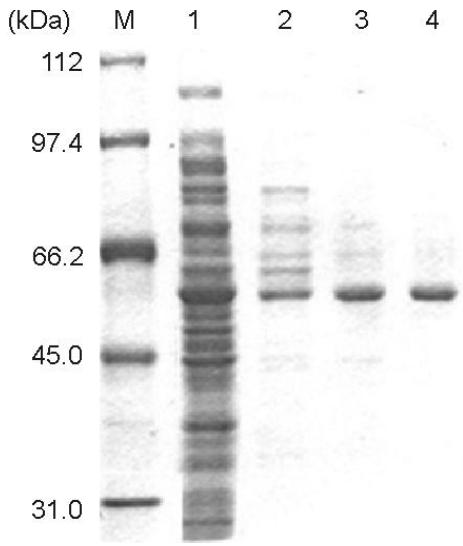

Fig. 2. SDS-PAGE of fractions containing VnfA after each purification step. 1, Q-Sepharose; 2. Ammonium sulfate fractionation; 3. Butyl Sepharose; 4. Heparin Sepharose; M, protein marker bands. The calculated molecular weight of the band in lane 4 is 58 kDa.

Purification of recombinant VnfA

VnfA produced in the cytosol of *E. coli* was purified by column chromatography and ammonium sulfate fractionation under aerobic conditions. The addition of 1 mM dithiothreitol throughout the procedure and 0.2% (v/v) Triton X-100 after the final step (heparin Sepharose column chromatography) was, however, essential for suppressing aggregation of the protein. In the absence of dithiothreitol and Triton X-100, purified VnfA precipitated after several hours, even at 4 °C. Complete elimination of *E. coli* chromosomal DNA during the first pass through an anion exchange column was also crucial for the subsequent purification steps because VnfA cannot be resolubilized once coprecipitated with DNA. An almost homogeneous band was obtained after the final step, comprising heparin column chromatography on SDS-PAGE (Fig. 2). The estimated molecular mass of the band was 58 kDa, in agreement with the calculated value of VnfA (57,608 Da)

based on the nucleotide sequence of *vnfA* (3). Conclusive evidence was obtained from N-terminal amino acid sequence analysis of the first ten residues of the purified protein, providing the sequence MSSLPQYCEC, which is identical to the sequence of VnfA. The yield of purified protein after the final step was approximately 3 mg if started with 20 g of cell pellets. Thus, I have successfully purified a recombinant VnfA that is amenable to further investigation *in vitro*.

Reconstitution of the Fe-S cluster in apo-VnfA

By contrast to the results of the EPR performed *in vivo*, the UV-visible spectrum of aerobically purified VnfA shows no features arising from any Fe-S clusters (Fig. 3A, dotted line) other than an unidentified shoulder band observed at 330 nm. Because some Fe-S clusters in proteins are unstable in atmospheric oxygen, the vanishment of the Fe-S cluster from purified VnfA could be a result of the disassembly of the cluster during aerobic purification. Fe-S clusters in apo-proteins *in vitro* are commonly reconstituted to regenerate their original structures and functions (25-27). Therefore, I attempted the reconstitution of purified VnfA under anaerobic conditions. Enzymatic production of S^{2-} from L-cysteine by cysteine desulfurase (IscS) from *A. vinelandii* (28) was used rather than Na_2S to avoid coprecipitation of VnfA with a large amount of Fe-S colloids formed during the reaction. After reconstitution and subsequent purification using desalting columns, fractions containing VnfA showed an apparent shoulder and broad bands at 310 and 420 nm, respectively (Fig. 3A, solid line). The latter band was bleached upon the addition of the reductant, dithionite salt (Fig. 3A, dashed line). These characteristic properties indicate that apo-VnfA is reconstituted with $[Fe_3S_4]^+$ and/or $[Fe_4S_4]^{2+}$. EPR spectroscopy provides further information on the nature of the Fe-S cluster. The reconstituted holo-VnfA gave a signal with a *g*-value of 2.01, which disappeared upon the addition of the reductant (Fig. 3B). Although the rhombicity of the spectrum found in the whole cell measurement vanishes, the observed properties are common to $[Fe_3S_4]^+$.

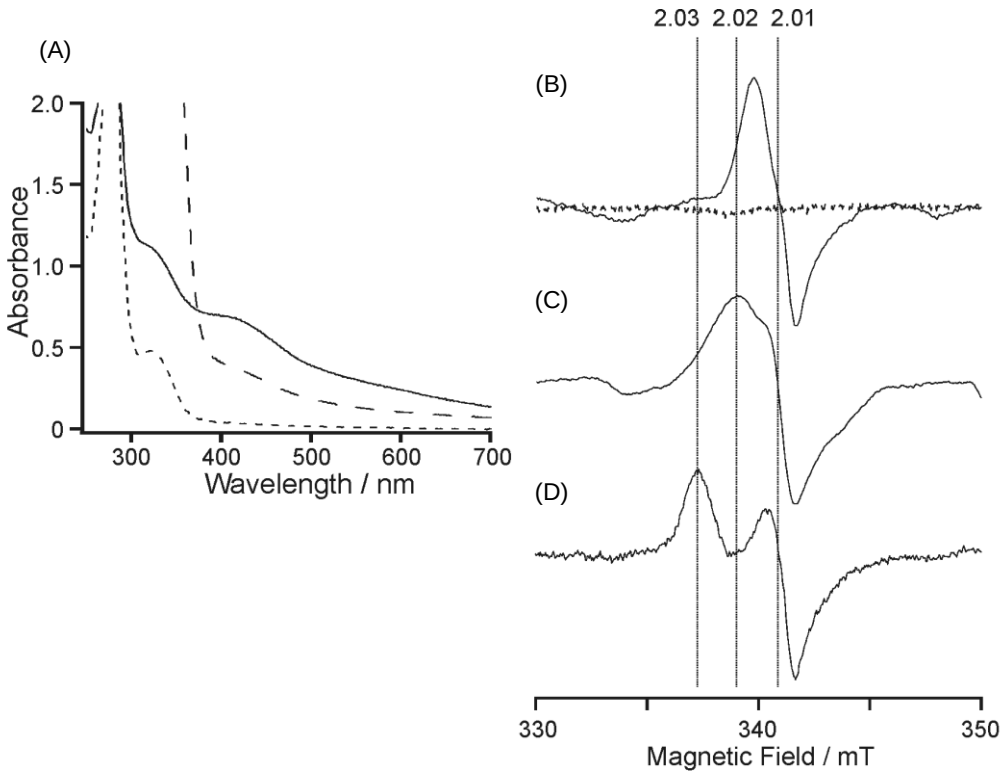

Fig. 3. (A) UV-visible spectra. Dotted line, aerobically purified VnfA (apo-form); solid line, after reconstitution with an Fe-S cluster; dashed line, the reconstituted VnfA (holo-form) after addition of the reductant, dithionite salt. (B) EPR spectrum of the holo-VnfA with a *g*-value of 2.01 (solid line) that disappeared following reduction with dithionite salt (dotted line). (C) EPR spectrum of the holo-VnfA after the addition of 1 mM AMP-PNP. (D) EPR spectrum reproduced from Fig. 1D for facile comparison with the spectra (B) and (C). The concentration of VnfA for both UV-visible and EPR measurements was 50 μM in 20 mM HGDT buffer (determined by the BCA method). EPR spectra were recorded at 10 K using 2.5 mW microwave power and a field modulation of 0.8 mT.

Quantification of the signals using Cu(II)EDTA as a standard indicated that the concentration of $[Fe_3S_4]^+$ was approximately 34 μM, which corresponds to approximately 70% of the VnfA monomer concentration (50 μM) determined by the bicinchoninic acid (BCA) method. The iron and sulfur contents in the reconstituted holo-VnfA were determined by inductively coupled plasma–optical emission spectroscopy (ICP-OES) and acid labile sulfide analysis, respectively. The reconstituted holo-VnfA was found to contain 2.8 ± 0.1 equivalents of iron and 3.5 ± 0.3 equivalents of sulfur per

Table 1. Quantification of iron and sulfur in the reconstituted VnfA.

	[Fe][a] (μM)	[S][b] (μM)	VnfA[c] (monomer) (μM)	[Fe] / VnfA (monomer)	[S] / VnfA (monomer)
Reconstituted holo-VnfA	27.0 (3.1)	32.5 (3.9)	9.5 (1.1)	2.8 (0.1)	3.5 (0.3)
Aerobically purified apo-VnfA	1.5	N.D.[d]	11.5	0.13	N.D.

[a] Determined by ICP-OES using $Fe(NO_3)_2$ as a standard.

[b] Determined as described in ref. (43).

[c] Determined by the BCA method using Bovine serum albumin as a standard.

[d] Not determined.

monomer (Table 1), corresponding to one monomer bearing one Fe-S cluster. These quantitative results indicate that $[Fe_3S_4]^+$ is a major species found in VnfA reconstituted under the present conditions. No EPR signals assignable to $[Fe_4S_4]^{2+}$ were observed, either before or after reduction by dithionite salt.

The lost rhombicity in the EPR spectrum was partially recovered by the addition of 5'-adenylyl-β,γ-imidodiphosphate (AMP-PNP) to the reconstituted holo-VnfA, although the signal at g = 2.03 *in vivo* was still shifted to 2.02 (Fig. 3C). AMP-PNP is a nonhydrolyzable ATP analog that is used to trap an ATP binding state of ATP hydrolases. Some ATPases associated with various cellular activities (AAA+) proteins are known to bind AMP-PNP and reproduce their conformational changes to exert the original functions of the proteins (29,30). Although the ATPase activity has not been reported for VnfA so far, the central domain of VnfA is deduced to be an AAA+ domain based on high homology to the AAA+ domain of NifA (3). Therefore, it is likely that VnfA binds AMP-PNP in the central domain to initiate a conformational change required for the subsequent hydrolysis. Indeed, limited protease digestion assays with either apo- or reconstituted holo-VnfA have provided results that reveal several conformations of VnfA corresponding to a combination of

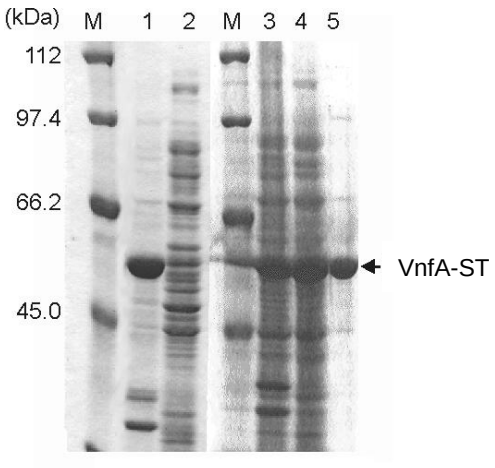

Fig. 4. SDS-PAGE of *E. coli* JM109 overexpressing VnfA-ST. Lanes 1 and 2, insoluble and soluble fractions of the cell lysate, respectively; Lanes 3 and 4, insoluble and soluble fractions, respectively, of lysates from cells co-transformed with *suf* genes; Lane 5, VnfA-ST purified by a streptavidin affinity column; M, protein marker.

the presence and absence of AMP-PNP and the Fe-S cluster (*vide infra*). This could help to solve the problem; why binding AMP-PNP has an influence on the Fe-S cluster detected in the EPR measurement? This point will be discussed subsequently.

The study with the reconstitution of Fe-S clusters in aerobically purified apo-VnfA supports the presence of $[Fe_3S_4]^+$ in VnfA. In order to obtain further evidence demonstrating the involvement of the Fe-S cluster in *in vitro* experiments, I attempted the anaerobic purification of VnfA attached to a *Strep*-tag at the C-terminus of the protein (VnfA-ST).

Anaerobic purification of *Strep*-tag attached VnfA (VnfA-ST)

Attempts to purify VnfA as a fusion protein to glutathione *S*-transferase, thioredoxin or His-tag were unsuccessful because the produced proteins were insoluble, despite manipulation of the aeration and temperature in the culture conditions. VnfA conjugated with *Strep*-tag at the

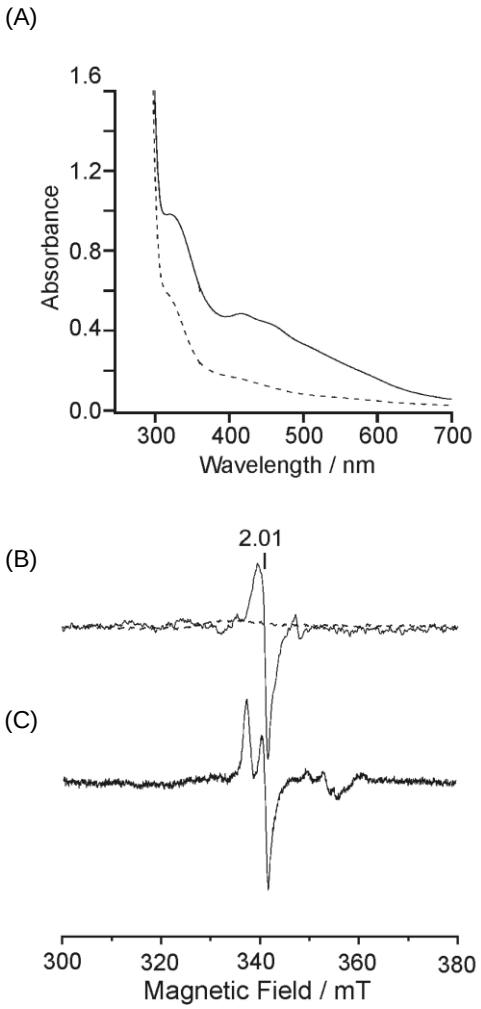

Fig. 5. (A) UV-visible and (B) EPR spectra of anaerobic purified VnfA-ST. The solid lines represent the spectra of purified VnfA-ST. The dotted line represents the spectra following reduction with dithionite. (C) EPR spectrum reproduced from Fig. 1D for facile comparison with the spectra in (B). The EPR spectra were recorded at 10 K using 2.5 mW microwave power and a field modulation of 0.8 mT.

C-terminus (VnfA-ST) yielded a small amount of soluble protein in the cell-free lysate (Fig. 4). However, the solubility of VnfA-ST was markedly improved when the SUF proteins, which are known to be involved in biological Fe-S cluster assembly (31,32), were co-produced with VnfA-ST. After single-step purification under anaerobic conditions using streptavidin attached to an affinity column, VnfA-ST provided an almost homogeneous band on SDS-PAGE.

The UV-visible spectrum of anaerobically purified VnfA-ST showed bands at 330 and 420 nm (Fig. 5A, solid line), which diminished upon the addition of dithionite salt (dotted line). Featureless absorption observed at wavelengths longer than 500 nm might indicate the participation of some $[Fe_2S_2]^{2+}$ species. However, the EPR measurement for VnfA-ST showed a single signal characteristic of $[Fe_3S_4]^{+}$ at g = 2.01 before the reduction (Fig. 5B, solid line) and no signal assignable to $[Fe_2S_2]^{+}$ even after the reduction (dotted line). Although the rhombicity of the EPR signal of VnfA-ST is still unclear, the overall shape is rather similar to that observed in the whole cell measurements. Thus, VnfA-ST purified under anaerobic conditions affords additional support for the involvement of $[Fe_3S_4]^{+}$ in VnfA as a prosthetic group.

Limited protease digestion assay

To obtain experimental evidence for a conformational change of VnfA triggered by AMP-PNP binding, VnfA of either the apo- or reconstituted holo-form was subjected to limited trypsin digestion in the presence and absence of AMP-PNP. Figure 6 shows the time course of proteolysis for VnfA under each set of conditions. AMP-PNP afforded a higher resistance to the proteolysis for both the apo- and holo-forms, as demonstrated by a much slower digestion of the original bands under +AMP-PNP conditions, whereas the digestion patterns of both the apo- and holo-form appeared to be little affected by the presence or absence of AMP-PNP. By contrast, effect of the Fe-S cluster on the proteolysis was not found in the sensitivity to the digestion but was observed with respect to the alteration of the digestion patterns (i.e. digestion sites in apo- and holo-VnfA). One particular change in the digestion pattern was found between 31 and 45 kDa in which two major fragments in the apo-form were not observed in the holo-form, whereas the fragment at 28 kDa in the holo-form was scarce in the apo-form. A fragment at 19 kDa in the holo-form is the other major difference although this was hardly observed in the apo-form. Regarding the effect of

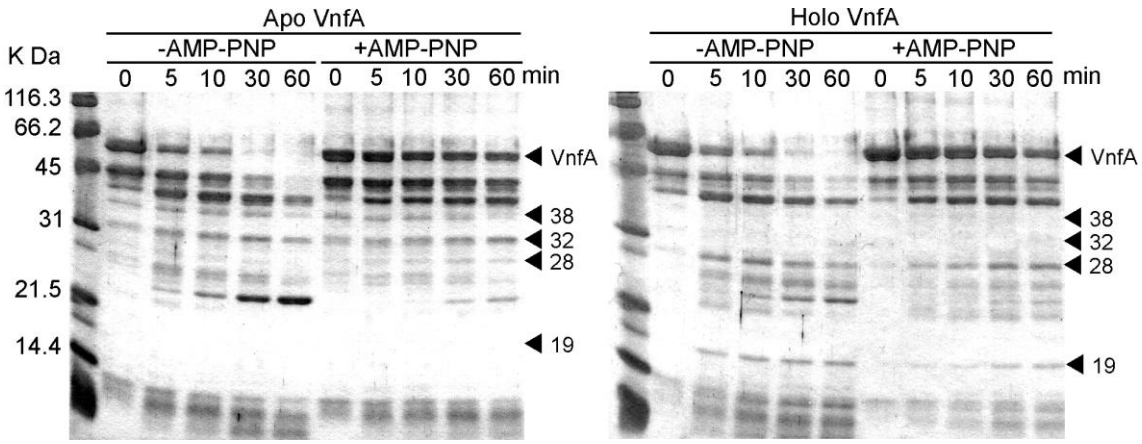

Fig. 6. Limited tryptic digestion assays with VnfA of either apo- or reconstituted holo-form in the presence or absence of AMP-PNP. The reactions were analyzed on 15% polyacrylamide gels. Digestion fragments were obtained by the reaction with trypsin (weight ratio 1 : 180) at 20 °C for 60 min. Details of the reaction conditions are provided in the Experimental Section.

AMP-PNP on the proteolysis of VnfA, a similar effect of the nucleotide binding was reported in a study of the limited trypsin digestion with NifA + MgADP, in which binding MgADP to the central AAA+ domain is ascribed to the trigger of a conformational change of NifA to avoid further proteolysis (33,34). By analogy with the study on NifA, the observed transformation of VnfA to a more resistant form to proteolysis is ascribed to a conformational change induced by binding AMP-PNP, presumably at the central domain of VnfA. Similarly, the changes in the fragmentation depending on the Fe-S cluster can be accounted by a conformational change caused by the cluster formation in VnfA. The variation in the digestion patterns corresponding to a combination of the presence and absence of AMP-PNP and the Fe-S cluster suggests that the conformational changes by the Fe-S cluster and AMP-PNP are interdependent.

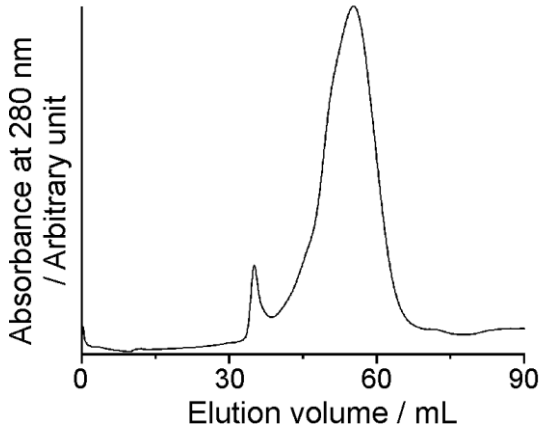

Fig. 7. Representative elution profile of GPC on Superdex-200 with purified apo-VnfA monitored at 280 nm. The profile shows a single elution peak corresponding to a molecular mass of 224 kDa.

Number of subunits in native VnfA

The molecular mass of apo- and holo-VnfA was determined to characterize the quaternary structure of VnfA. Gel permeation chromatography (GPC) of purified apo-VnfA eluted in a single and somewhat broad peak that corresponds to a molecular mass of 224 kDa (Fig. 7). This value is 3.9-fold higher than that of the VnfA monomer (57,608 Da, calculated from the inferred amino acid sequence). Because of technical difficulties in performing GPC under fully anaerobic conditions, the mass of reconstituted holo-VnfA could not be measured by GPC. Instead, holo-VnfA was subjected to anaerobic blue native PAGE (35) using degassed electrophoresis buffers and an argon atmosphere. Holo-VnfA provided a homogeneous band with a molecular mass of 213 kDa, which corresponds to a 3.7-fold higher mass of the subunit (Fig. 8). Thus, the mass analyses of VnfA confirm a tetrameric configuration both in the presence and absence of the Fe-S cluster. As described for the reconstitution of VnfA with the Fe-S cluster, quantitative analyses for iron and acid labile sulfur in the reconstituted VnfA indicated one Fe-S cluster in each monomer, as well as the stoichiometry of four Fe-S clusters in VnfA.

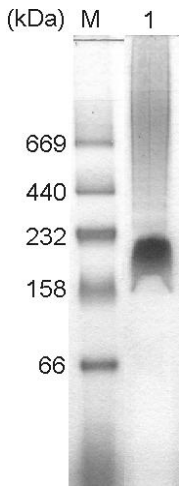

Fig. 8. Blue Native PAGE of the reconstituted holo-VnfA. Electrophoresis was carried out in a glove box under Ar using degassed buffers. Lane 1, the reconstituted holo-VnfA; M, protein marker.

Transcriptional activity of VnfA in the presence or absence of the Fe-S cluster

To clarify the roles of the Fe-S cluster found in VnfA, I performed *in vivo* assays under various growth conditions by using a heterogeneous reporter system carrying the *lacZ* gene preceded by the *vnfH* promoter in the *E. coli* JM109 strain. In the view of immunological detection of produced VnfA, I employed VnfA-ST as a source of VnfA for the reporter system. A similar heterogeneous reporter system has been reported and was shown to be valid for elucidating the biological properties of VnfA and NifAL (6,19).

To determine whether the Fe-S cluster is required for transcriptionally active VnfA, I employed *o*-phenanthroline (*o*-phen) as a metal chelator for the assay, which is expected to permeate cell membranes and restrict iron atoms available for Fe-S cluster assembly in the cell (36,37). Activity was determined by the transcript level of the *lacZ* gene immediately after the addition of *o*-phen to minimize the effect of the growth inhibition by *o*-phen on the transcriptional activity of VnfA under micro-aerobic conditions (Fig. 9). Five minutes after the addition of *o*-phen, the activity began to

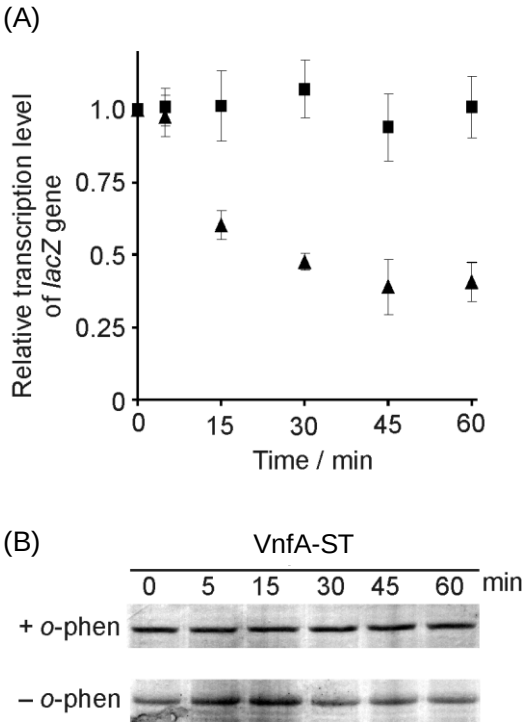

Fig. 9. (A) Time course of the VnfA activity assessed by *lacZ* transcript level at early exponential phase after the addition of 150 μM *o*-phen (▲, +*o*-phen); no addition of *o*-phen (■, −*o*-phen). Each plot presents the mean values from three independent experiments, normalized with the activity at 0 min. (B) Western blot analyses for VnfA-ST recorded at a time corresponding to the performed assays.

decrease and reached 30% of the initial level in 45 min, whereas a control assay under the same conditions without the addition of *o*-phen showed virtually no alteration in the *lacZ* gene transcript. Because a western blot analysis confirmed the constant level of VnfA-ST during the assays both in the presence and absence of *o*-phen, it would be reasonable to ascribe the drop in the *lacZ* transcript to the repression of the transcriptional activity of VnfA-ST. The specific EPR signals of $[Fe_3S_4]^+$ observed for VnfA disappeared after *o*-phen treatment. Instead, a signal of free ferric iron emerged at $g = 4.3$. This indicates that the reaction of *o*-phen brings about disassembly of the Fe-S cluster in transcriptionally active VnfA-ST. Thus, I conclude that the Fe-S cluster is essential for

transcriptionally active VnfA and disassembly and/or that deformation of the Fe-S cluster turns active VnfA inactive.

Discussion

The EPR data of whole *E. coli* cells which produces VnfA suggested the involvement of a 3Fe-4S type cluster in VnfA, which was supported by the spectroscopic analyses for the reconstituted VnfA and anaerobically purified VnfA-ST. The quantitative analyses for the reconstituted VnfA suggest approximately 70% of apo-VnfA being reconstituted with $[Fe_3S_4]^+$, and it indicates that $[Fe_3S_4]^+$ is a major species in VnfA reconstituted under the present experimental conditions. However, the UV-visible spectrum indicated the partial participation of some 2Fe-2S cluster species in the purified VnfA-ST, which offers the possible involvement of other types of Fe-S clusters in VnfA. For future studies, I would like to leave the further identification of the Fe-S cluster in transcriptionally active VnfA including its conformation and oxidation states.

The EPR spectrum of the reconstituted VnfA showed a signal (g = 2.01) of different rhombicity from those observed in the whole *E. coli* cell measurement (g = 2.01 and 2.03). The addition of AMP-PNP to the reconstituted VnfA served to recover the rhombicity. Although the signal at g = 2.03 still shifted to 2.02 and a fully identical spectrum to that observed in the whole cell measurement has not been reproduced under the present reconstitution conditions, the partial recovery of the rhombicity implies that VnfA can bind a nucleotide, and the whole cell EPR spectrum might reflect VnfA of the nucleotide binding form. It has been reported that the binding of ATP or ADP to NifA of *A. vinelandii* leads to rearrangement of interaction between the GAF and AAA+ domains. Such conformational change is considered to be coupled with transmission processes of the sensing events (33). Considering the functional and structural analogies to NifA, it

is presumably rational to expect that VnfA also causes a conformational change in a similar manner to NifA; the binding of an ATP analog induces the rearrangement of the GAF and possible AAA+ (the central domain) domains in VnfA. Indeed, the limited protease assays confirmed that the conformational changes are dependent on a combination of the presence and absence of AMP-PNP and the Fe-S cluster. Consequently, the binding of the ATP analog and the subsequent conformational change affects the electronic condition of the Fe-S cluster through the protein scaffold, resulting in an alteration of the signal rhombicity of the EPR spectrum. The divergence of the g-value from that of the whole cell spectrum remains to be solved. The spectra may be affected by conditions during biosynthetic assembly *in vivo* and artificial reconstitution *in vitro*. For example, the signal intensity ratio of the EPR spectra of $[Fe_3S_4]^+$ changes in response to the buffer composition, such as the concentration of glycerol (38). In ferredoxin II from *Desulfovibrio gigas*, a change of the purification conditions causes variation in the shape of the EPR spectrum of $[Fe_3S_4]^+$ (39). Further modification of the reconstitution procedure is still in progress to obtain an EPR spectrum identical to that observed in the whole cell measurement.

Native molecular mass analyses by native PAGE and GPC show that VnfA remains tetrameric both in the presence and absence of the Fe-S cluster. A similar oligomeric configuration has been reported for truncated AnfA, which is in equilibrium between the dimeric and tetrameric forms, whereas NifA of *A. vinelandii* is known to exist as a dimer (22). A previous investigation of the *vnfH* promoter revealed that the binding site of VnfA consists of two dyad upstream activator sequence (UAS) motifs (5'-GTAC-N6-GAAC-3' and 5'-GTAC-N6-GTAC-3') that lie on top of each other on the same face of the DNA helix (11,17,19). Similar features are commonly required for promoters of σ^N-dependent transcriptional regulators, although there are several variations with respect to the number and distance of the dyad UAS motifs. In most cases, the regulators in a dimeric form bind to each dyad UAS motif cooperatively to associate with the target promoters (40).

However, such a binding mode is unlikely for tetrameric VnfA because it has four DNA binding parts. Simultaneous binding to all four UAS motifs on the *vnfH* promoter is, therefore, the most plausible association mode for single native VnfA.

In summary, this chapter described successful production and purification of recombinant full-length VnfA in *E. coli*. The Fe-S cluster (3Fe-4S type) was found in each subunit of tetrameric VnfA as a prosthetic group which was required for transcriptional active VnfA.

Experimental Section

Chemicals

All chemicals were purchased from Nakalai Tesque (Kyoto, Japan) and Wako Co. (Tokyo, Japan) and were used without further purification.

Overexpression and purification

Tag-less VnfA (VnfA)

Overexpression of the tag-less *vnfA* gene was achieved using a recombinant system. The *vnfA* gene was amplified by PCR using *A. vinelandii* chromosomal DNA as a template. Oligonucleotide primers used for PCR were: 5'-GAATTCTCCAGCCTCCCCCAATACTGCGAATGC-3' and 5'-GA ATCCTCAGCGGTAGTCCTTGTAGTTGAGGTTG-3'.

The PCR product was cloned into pCR4 vector (Invitrogen, Carlsbad, CA, USA) to give pUC-VnfAE and then digested with *Eco*RI to provide an *Eco*RI fragment carrying the *vnfA* gene. The fragment was inserted into an *Eco*RI site in the pKK223-3 expression vector to afford pKKVnfAE and then subsequently used to transform the *E. coli* strain, JM109.

JM109 bearing pKK-VnfAE was cultured in LB medium containing 50 μg/mL ampicillin at

37 °C until OD_{600} of ~ 0.8 was reached. To induce VnfA, the culture temperature was lowered to 20 °C, and then IPTG was added to a final concentration of 0.5 mM and the culture was incubated for 16 h. The cells were harvested by centrifugation (6,000 *g* for 5 min at 4 °C) and the cell pellets were frozen using liquid N_2 and stored at −80 °C.

The first step in the purification of VnfA was column chromatography in TGD buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 1 mM dithiothreitol). After breaking the cells by sonication, the pellets were centrifuged (29,000 *g* at 4 °C for 30 min) to give a crude cell extract that was applied to a Q-Sepharose column (GE Healthcare, Milwaukee, WI, USA) pre-equilibrated with TGD buffer. VnfA was chromatographed using a linear gradient of 150–550 mM KCl and the fractions between 360–500 mM KCl were collected. $(NH_4)_2SO_4$ was added to the obtained fractions to 35% saturation, followed by incubation on ice for 30 min. VnfA was obtained as a precipitate and collected by centrifugation (20,000 *g* for 10 min at 4 °C). The precipitate was dissolved in HGD buffer (20 mM HEPES-KOH, pH 8.0, 10% glycerol, 1 mM dithiothreitol) + 1 M KCl, and the insoluble fraction was removed by centrifugation (20,000 *g* for 10 min at 4 °C). Then, the obtained solution was loaded onto a butyl sepharose column pre-equilibrated with HGD + 1 M KCl + 0.5 M $(NH_4)_2SO_4$ buffer. VnfA was eluted with simultaneous linear gradients of KCl from 1 M to 0 M and $(NH_4)_2SO_4$ from 0.5–0 M. The fractions containing VnfA were combined and desalted using HGD buffer. The final purification was achieved using a heparin-agarose column preequilibrated with HGD buffer. The adsorbed proteins were eluted using a linear gradient of KCl of 150–750 mM; VnfA eluted at approximately 450 mM KCl. Each purification step was monitored by SDS-PAGE. Protein concentrations were determined using the BCA method (Bicinchoninic Acid Protein Assay Kit, Sigma, Saint Louis, MO, USA) with BSA as a quantitative standard. After the final purification step, VnfA was transferred to HGD buffer + 0.2% Trion X-100 (HGDT buffer) for subsequent experiments.

IscS (cysteine desulfurase)

The *iscS* gene was amplified by PCR using *A. vinelandii* chromosomal DNA as the template. The oligonucleotide primers used for PCR were: 5'-GAATTCATGAAATTACCGATTTATCTCG-3' and 5'-GAATTCCTATGTGCCAGCTCGTCGTTCAGC-3'.

The PCR product was cloned into pCR2.1 (Invitrogen) vector arranged for the TA cloning system to afford pCR*viscS*. The obtained plasmid was used to produce the IscS enzyme. IscS produced in the JM109 strain was purified as described previously (41), and the purified protein was stored in small aliquots in HGD buffer at −80 °C.

***Strep*-tag attached VnfA (VnfA-ST)**

To clone the *vnfA* gene into the *Strep*-tag fusion vector, pASK-IBA3plus (IBA Co., Göttingen, Germany), a DNA fragment encoding VnfA was amplified by PCR from pKK-VnfAE using the primers containing a *Bsa*I restriction site (underlined): 5'-CAAAAGGTCTCGAATGTCCAGCCTC CCCCAATA-3' and 5'-CAAAAGGTCTCAGCGCTGCGGTAGTCCTTGTAGTTGA-3'.

After digestion with *Bsa*I, the PCR product was ligated into the *Bsa*I site of pASK-IBA3plus. The resulting plasmid, pASK-IBA3plus-*vnfA*, was used to produce recombinant VnfA fused with *Strep*-tag at the C-terminus (VnfA-ST). The JM109 strain was sequentially transformed with plasmids pASK-IBA3plus-*vnfA* and pRKSUF017, which are an expression system for recombinant SUF proteins (32). The cells were grown at 37 °C in LB medium supplemented with 100 μg/mL ampicillin until OD_{600} of ~ 0.8 was reached. Then, 0.5 mM IPTG was added to initiate expression of the SUF proteins. After 1 h of further growth at 37 °C, 0.1 μg/mL anhydrotetracycline was added to initiate the expression of VnfA-ST from the *tet* promoter. After 20 h at 20 °C, the cells were harvested by centrifugation at 6,000 *g* for 5 min at 4 °C, and the collected cells were stored at −80 °C.

Anaerobic purification of VnfA-ST was manipulated under an argon atmosphere in a glove box, except for centrifugation. Cell pellets (2 g) were resuspended in 20 mL of HGDT buffer flushed with argon. After sonication, the suspension was dispensed to centrifuge tubes, which were sealed with screw caps and a plastic film, and centrifuged at 10,000 *g* for 20 min at 4 °C to remove the cell debris. The supernatant (5 mL) was passed through a 0.5 mL *Strep*-Tactin column (IBA Co.) pre-equilibrated with degassed HGDT buffer. Unbound proteins were removed with three, 1 mL aliquots of HGDT buffer, and affinity-bound VnfA-ST was eluted with 3 mL of HGDT buffer + 2.5 mM desthiobiotin. Purified VnfA-ST was stored under argon at 4 °C.

Spectroscopy

UV-visible spectra were monitored on a MultiSpec-1500 spectrophotometer (Shimadzu Corp., Kyoto, Japan). X-band EPR spectra were recorded on an E500 X-band CW-EPR (Bruker, Ettlingen, Germany). A cryostat (ITC503; Oxford Instruments Co., Abingdon, UK) was used for measurements at low temperature. For whole-cell EPR measurements, 500 mg of cell pellet were resuspended in 1 mL of water and transferred to sample tubes. The tubes were centrifuged at 200 *g* for 10 min to concentrate the cells at the bottom of the tube. The supernatant was removed and the tubes were frozen and stored in liquid nitrogen.

Spin quantification was performed with 10 μM Cu(II) EDTA as a concentration standard under nonsaturating conditions. Values obtained by double integration of the signals were divided by a correcting factor that is a function of the principal *g*-values (42).

Native molecular mass analyses

The native molecular mass of VnfA without the Fe-S cluster was determined by GPC using Superdex-200 and HGDT buffer under aerobic conditions. The molecular mass was calculated by

comparison with protein markers: thyroglobulin (669,000 Da), ferritin (440,000 Da), catalase (232,000 Da), γ-globulin (158,000 Da) and BSA (66,000 Da). Blue native PAGE, performed to determine the molecular mass of the reconstituted VnfA, was conducted under argon in a glove box using a 4–16% Novex Bis-Tris Gel System (Invitrogen). The gel plates were preelectrophoresed for 30 min with degassed electrophoresis buffer + 1 mM dithiothreitol to purge O_2 from the gel. The molecular mass was calculated by comparison with the same protein markers that were used for the chromatography.

Reconstitution of the Fe-S cluster

The entire reconstitution procedure was carried out under argon in a glove box, and all buffers were degassed and equilibrated with argon prior to use. Aerobically purified VnfA (5 μM) in HGDT buffer was incubated with 2 mM dithiothreitol and 0.1 mM L-cysteine for 40 min at room temperature. After the addition of 0.1 mM $FeCl_2$ and 0.1 μM IscS, the mixture was incubated further for 5 h at room temperature. The reaction was monitored using UV-visible spectroscopy to determine the end point of the reconstitution process. The reconstituted protein was desalted successively over a PD-10 column, a G-25 desalting column (5 mL) and a MicroSpin S-200 HR column (GE Healthcare), all equilibrated with degassed HGDT buffer, to remove adventitiously bound iron and sulfide completely from the reconstituted VnfA.

Quantitative analyses of protein, iron and acid labile sulfur in reconstituted VnfA

The BCA assay was used to quantify the amount of VnfA and VnfA-ST proteins. A calibration curve was constructed using BSA as a standard. Quantification of acid labile sulfur was carried out as described previously (43), and inductively coupled plasma–optical emission spectroscopy was employed to quantify iron content. Reconstituted VnfA (typically 1.5 mL in

HGDT buffer) was mixed with an equal volume of 60% HNO_3 in a glass flask that had been pre-treated with 60% HNO_3 and rinsed several times with distilled water. The solution was boiled for 30 min and cooled to room temperature. Distilled water was added to adjust the solution to 4 mL. Any precipitate formed was removed by centrifugation (20,000 *g* for 10 min). A 3 mL aliquot was placed in a spectrometer (Vista-Pro; Varian Inc., Palo Alto, CA, USA) zeroed against HGDT buffer. A calibration curve for Fe was constructed using an $Fe(NO_3)_2$ standard (100 mM) purchased from Wako Co.

Limited protease digestion assay

Limited protease digestion assays were carried out in a mixture containing 50 mM Tris-acetate (pH 8.0), 100 μM potassium acetate, 8 mM magnesium acetate and 1 mM dithiothreitol at 20 °C in the presence or absence of 3 mM AMP-PNP according as described previously (33). After preincubation of apo- or reconstituted VnfA (18 μg) with or without AMP-PNP for 2 min, the reactions were started by the addition of Trypsin (0.1 μg). Aliquots (15 μL each) were withdrawn from the reaction mixture at 0 and 60 min to tubes containing 5 μL of gel loading buffer [250 mM Tris-HCl (pH 8.0), 25% glycerol, 7.5% SDS, 60% β-mercaptoethanol, 0.0003% bromophenol blue]. Electrophoresis on 15% SDS-PAGE gels was carried out to resolve low molecular mass digestion products.

In vivo transcription assay

A reporter strain was constructed by transcriptional fusion of the *vnfH* promoter region to the *lacZ* gene. A 258 bp DNA fragment carrying the *vnfH* promoter region corresponding to the *vnfH* −231 to +27 transcript (17) was amplified by PCR with *A. vinelandii* chromosomal DNA using the primers: 5'-TCCGGCGCCGTCGAGCACCCCAGTACCATG-3' and 5'-GATTCGTTGGCGTTTTG

ATTTGTGCCGACG-3'.

The PCR product was cloned into the pCR2.1 vector. A 276 bp insert was excised from the plasmid with *Eco*RI and cloned into the *Eco*RI site of pRS551. This plasmid-borne fusion was transferred to λRS74 phage vector by homologous recombination in *E. coli* P90C as described previously (44), followed by preparation of the phage lysate containing the recombinant phage. Lysogens were obtained by infecting JM109 cells with this phage lysate and selecting transformants on LB agar plates containing kanamycin. A clone bearing a single copy of the recombinant λ-prophage was selected and used in the subsequent procedures. This strain was named *E. coli* λPvnfH. The prophage copy number was determined by PCR as described previously (45).

To perform the assays, λPvnfH was transformed with a VnfA-ST expression plasmid, pASK-IBA3plus-*vnfA*. The cells were grown in LB medium supplemented with 100 μg/mL ampicillin and 30 μg/mL kanamycin at 37 °C for 7 h, then harvested by centrifugation (6,000 *g* at 4 °C for 10 min). The cell pellets were washed twice with NFDM medium (70 mM K_2HPO_4, 25mM KH_2PO_4, 9mM NaCl, 1 mM $MgSO_4$, 100 mM glucose, 2 mM glutamine, pH 7.0) (46), and resuspended in NFDM medium supplemented with 100 μg/mL ampicillin, 30 μg/mL kanamycin and *o*-phen for the assay in a 500 mL conical flask. The prepared NFDM suspension was used for the *lacZ* transcript assays described below.

The amount of *lacZ* transcript was monitored by modification of the RT-PCR method described previously (47). After appropriate period of the culture in the NFDM medium, the harvested cells were used for isolation of total RNA by RNAiso Plus (Takara Bio Inc., Otsu, Japan) in accordance with the manufacturer's instructions. For RT-PCR, SuperScript III One-Step RT-PCR System with Platinum *Taq* High Fidelity (Invitrogen) was used with the primers: 5'-CCCAACTTAATCGCCTTG CAGCACA-3' and 5'-CGGTTTATGCAGCAACGAGACGTCA-3'.

RT-PCR was carried out in 25 μL volumes containing 0.5 μL of the enzyme mix, 12.5 μL of the

2× reaction mix, 0.2 μM of each primer and 0.5 μg of total RNA. Following cDNA synthesis at 55 °C for 30 min and pre-denaturation at 94 °C for 2 min, the reaction was subjected to 30 cycles of amplification at 94 °C for 15 s, 55 °C for 30 s, 68 °C for 1 min, and a final extension for 5 min. Ten microliters of each reaction was size-fractionated by 1% (w/v) agarose gel electrophoresis, in which the gel was stained with ethidium bromide and photographed under UV light. As a positive control in the RT-PCR experiments, constitutively expressed 16S rRNA was RT-PCR amplified in tandem with experimental samples from all RNA samples assayed using the primers: 5'-CAGCGGGGAGG AAGGGAGTAAAGT-3' and 5'-CCACATGCTCCACCGCTTGT-3'.

The RT-PCR conditions employed for detection of 16S rRNA were the same as those for RT-PCR of *lacZ* mRNA, except that there were six cycles of amplification.

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Chapter III

Functions of the Domains in VnfA

Introduction

In Chapter II, I described the successful isolation of VnfA as a recombinant protein. The study with the isolated protein revealed that VnfA bears an Fe-S cluster (3Fe-4S type) which takes a key role in regulating the transcriptional function of VnfA. In the presence of the cluster, VnfA is transcriptionally active, while disruption of the cluster turns VnfA to the inactive form. A limited protease digestion assay revealed the conformational change between apo- and holo-forms of VnfA. These findings allowed me to assume the signal transduction mechanism of VnfA; the Fe-S cluster serves as a sensory moiety which is required of transcriptionally active VnfA. Upon the reaction with the environmental factor, the active VnfA loses the Fe-S cluster, which triggers a conformational change of VnfA to turn off its transcriptional activity. Thus, my experimental data proved the significance of the Fe-S cluster in the regulatory mechanism of VnfA as is inferred from the amino acid sequence alignments for three transcriptional regulators (NifA, VnfA and AnfA) in *A. vinelandii*. Meanwhile, participation of the cysteine residues of a cysteine-rich motif (CRM: Cys^{8}-X-Cys^{10}-XXXX-Cys^{15}) in binding to the cluster has not been confirmed. In addition, roles of the GAF domain and the central (possible AAA+) domain have not been assigned clearly in the signal transduction mechanism although the sequence analysis based on other σ^{N}-dependent transcriptional regulators has predicted that the central domain exerts the nuclease activity and provides an interaction site with the RNA polymerase.

In Chapter III, I discuss the functions of the domains based on results obtained from three truncated variants of VnfA. These variants are designated VnfAΔCRM, VnfAΔGAF, and GAF_{VnfA}. The former two variants lack the N-terminal CRM and the whole GAF domain, respectively, but retain whole parts of the central and C-terminal DNA binding domains. The last consists of only the GAF domain (the initial to 208^{th} amino acid residues). The *in vitro* reconstitution of the Fe-S cluster in the variants confirms the participation of the GAF domain in binding to the Fe-S cluster,

which is consistent with the results of *in vivo* transcription assay with cysteine variants. The *in vivo* transcription assay and ATPase analysis with the truncated variants propose that the bound Fe-S cluster releases the inhibitory effect of the GAF domain on the ATPase activity of the central domain to turn VnfA to the transcriptionally active form.

The variants employed here are conjugated with *Strep*-tag for facile purification and detection of the proteins.

Results

Sequence analysis of the N-terminal GAF domains of nitrogenase transcriptional regulators

Figure 1 summarizes classification of 28 presumed orthologues of the nitrogenase transcriptional regulators based on their sequences of the N-terminal GAF domains. VnfA forms a clade (identified as the VnfA clade) with five other transcriptional regulators including AnfA from *A. vinelandii*. These regulators are responsible for the regulation of nitrogenase-2 or -3 which bears a V/Fe or Fe only active center, respectively (1). NifA from *A. vinelandii* belongs to a different clade. A distinguished property of this clade is the two-component system in which NifAs cooperatively work with their sensory proteins (2-5). CRM in a central domain is a common property of the regulators in the rest clade although there is no specific conservation in the sequences between the cysteine residues.

VnfA from *A. vinelandii* has six cysteine residues (Cys at position 8, 10, 15, 107, 134 and 267). The first five residues are located in the N-terminal GAF domain, and the last one is in the central domain (1). Comparison of the amino acid sequences of the GAF domain among the six regulators in the VnfA clade reveals that Cys^{10} and Cys^{15} in the CRM of VnfA are highly conserved in all the

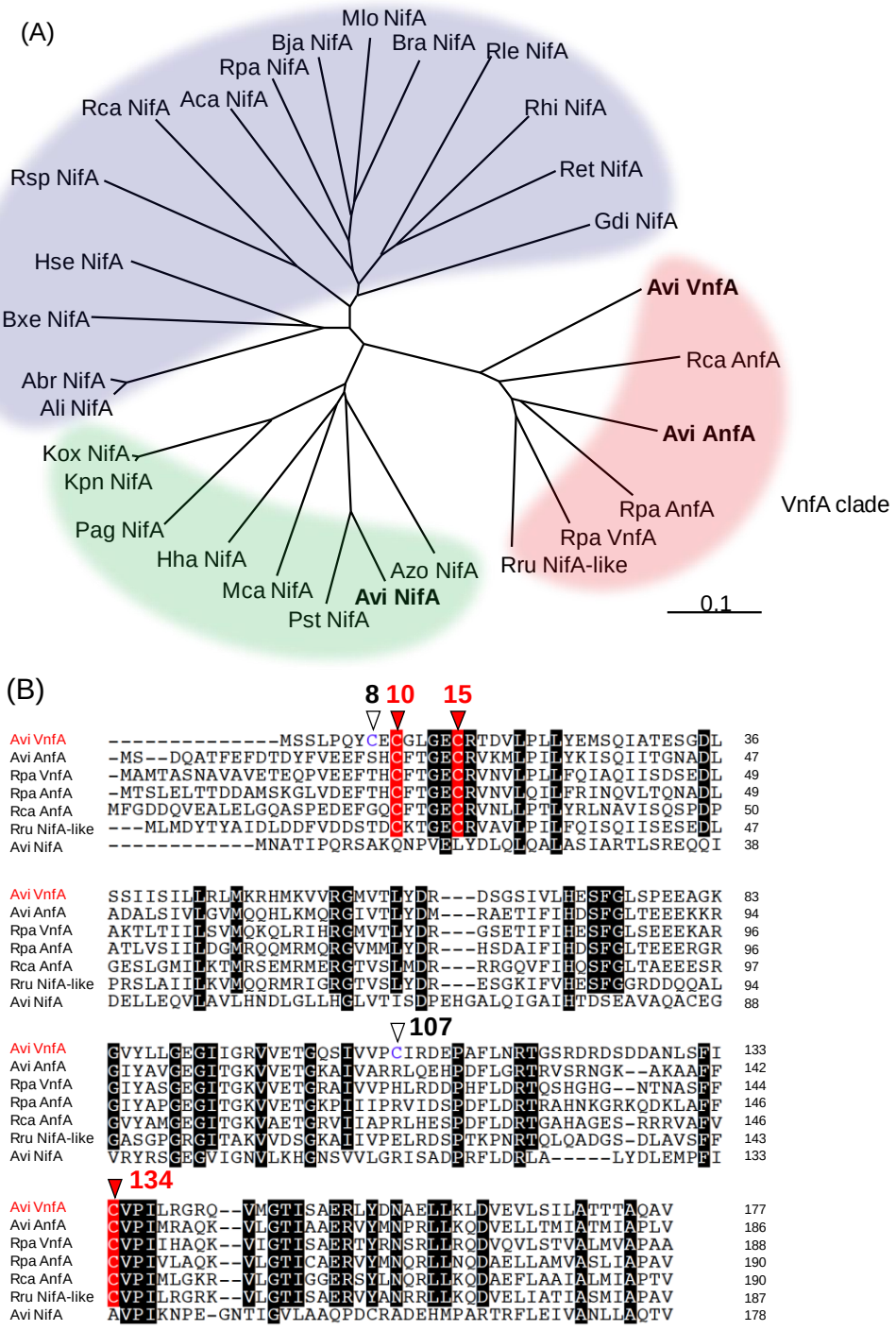

Fig. 1. (A) A summary diagram of 28 nitrogenase transcriptional regulators classified on the basis of amino acid sequences of N-terminal GAF domains. VnfA from *A. vinelandii* forms a clade (identified as VnfA clade) with other five orthologous regulators. (B) Amino acid sequence alignments for the GAF domains of the regulators in the VnfA clade. An alignment for NifA from *A. vinelandii* is also shown for reference.

regulators in a common sequence, C-XX-GECR (Fig. 1). In contrast, Cys^{8} is unique to VnfA and mainly either a serine or threonine residue occupies the corresponding loci in the other regulators. It has been reported that AnfA from *A. vinelandii* requires no specific properties of the corresponding residue to exert the transcriptional function (6). In the downstream region of the CRM, the fourth cysteine residue in VnfA (Cys^{107}) is replaced with residues of different properties in other regulators in the VnfA clade. This suggests less significance of the cysteine residue at this position. The cysteine residue corresponding to Cys^{134} of VnfA is conserved in the all regulators in the VnfA clade. All Cys residues conserved in the VnfA clade are replaced by other residues of different properties in the conventional nitrogenase regulator, NifA from *A. vinelandii*. This counterpoint gives inference that the regulators in the VnfA clade work under a common regulatory mechanism involving the corresponding cysteine residues, which is distinguished from the well-known mechanism for NifA from *A. vinelandii*.

Transcriptional activity of cysteine variants

The findings obtained in Chapter II indicated that VnfA bears a 3Fe-4S type cluster as the prosthetic group. The involvement of some metal ions as a prosthetic group was originally deduced from the characteristic CRM in the N-terminal region of VnfA and a mutagenesis study on AnfA (1,6). Therefore, it is likely that these cysteine residues participate in binding to the cluster. However, VnfA has additional three cysteine residues at position 107 and 134 in the GAF domain and at position 267 in the central domain. Accordingly, to determine which Cys are associated with the binding of the Fe-S cluster, I have prepared six Cys variants of VnfA (C8A, C10A, C15A, C107A, C134A and C267A, in which each cysteine residue was replaced with alanine) and performed the *in vivo* transcription assay for each variant (Fig. 2). The result apparently classifies the variants in two parts. Three variants of the N-terminal Cys residues (C8A, C10A and C15A)

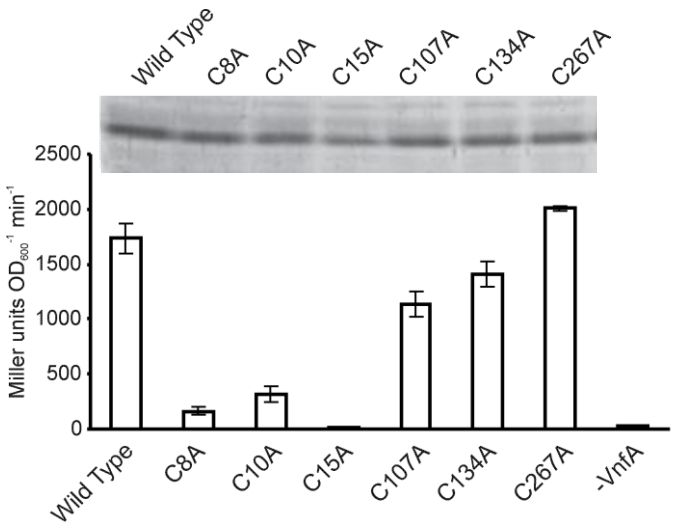

Fig. 2. Conventional *in vivo* β-gal activity assays for wild-type VnfA and Cys variants under aerobic conditions. The upper panel shows the stability of the wild-type and variants as monitored by western blot analysis. –VnfA corresponds to the λPvnfH strain transformed with the plasmid, pASK-IBA3plus, carrying no structural gene of VnfA.

show significantly low transcriptional activities corresponding to 12%, 23% and 1% of that of the wild-type, respectively. On the other hand, the remaining variants (C107A, C134A and C267A) retain almost original or rather higher activities (65%, 81% and 117% of that of the wild-type, respectively). Western blot analysis shows approximately the same stability of the variants compared to that of the wild-type, confirming that the difference in activity of the variants reflects the intrinsic ability of the variants compared to the transcriptional regulator. The result indicates that the CRM serves to harbor the Fe-S cluster in VnfA.

Reconstitution of the Fe-S cluster in truncated variants

The transcription assay for Cys variants of VnfA reveals that the removal of cysteine residues in the CRM (Cys at position 8, 10 and 15) results in the significant drop in the transcriptional activity

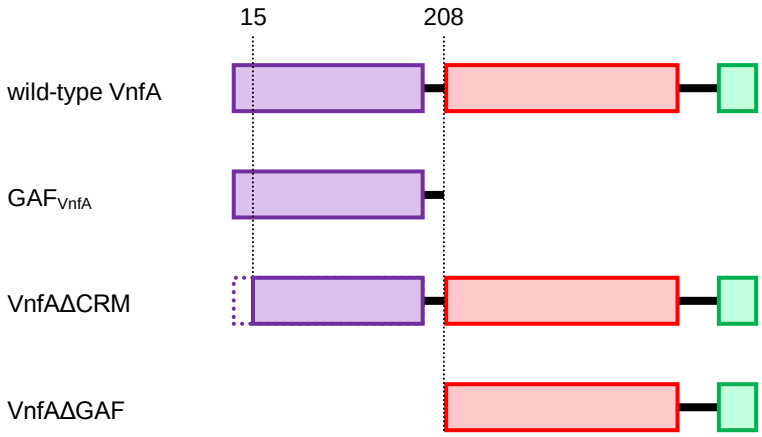

Fig. 3. Schematic diagram of the domain structure of wild-type and truncated variants of VnfA. The N-terminal (GAF), central (possible AAA+) and C-terminal DNA-binding domains are shown as purple, red and green rectangles, respectively. Numbers indicate the amino-acid position in wild-type VnfA sequence. GAF_{VnfA} consists of only the GAF domain. VnfAΔCRM and VnfAΔGAF lack the first 15 (the CRM) and 208 (the GAF domain) amino acid residues, respectively.

while no depressive effects on VnfA are observed by the mutagenesis of other cysteine residues (Cys at position 107, 134 and 267). The result indicates that the CRM is involved in regulation of the VnfA activity presumably through holding and losing the Fe-S cluster at the motif. In order to obtain details about the regulatory mechanism of VnfA, I have prepared N-terminally (VnfAΔCRM and VnfAΔGAF) and C-terminally (GAF_{VnfA}) truncated variants (Fig. 3). The VnfAΔCRM and VnfAΔGAF variants lack the first 15 (the CRM) and 208 (the GAF domain) amino acid residues, respectively, but bear the complete central and DNA binding domains. The GAF_{VnfA} variant consists of only the GAF domain (1^{st}-208^{th} amino acid residues).

As mentioned in Chapter II, aerobic purification of the recombinant VnfA from *E. coli* afforded the apo-form with no vestige of [Fe_3S_4], which, however, could be restored by the enzymatic reconstitution using cysteine desulfurase from *A. vinelandii*. Similar to the wild-type, all variants are obtained with no Fe-S clusters by the aerobic purification. Purified VnfAΔGAF is stable and

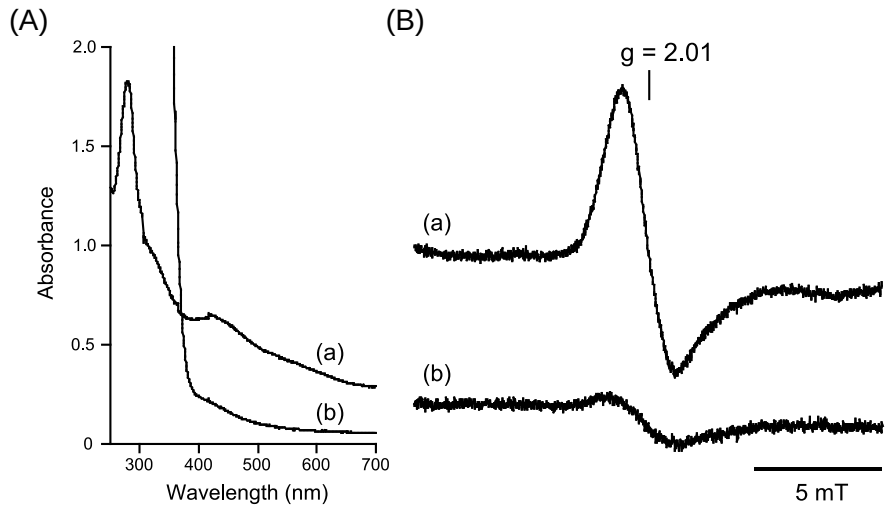

Fig. 4. (A) UV-visible and (B) EPR spectra of reconstituted GAF_{VnfA} as purified (a) and reduced with dithionite salt (b). EPR spectra were recorded at 15 K using 2.5 mW microwave power and a field modulation of 0.8 mT.

takes a tetrameric form in quaternary structure as found for the wild-type, whereas the reconstitution process applied to the wild-type results in no restoration of Fe-S clusters in the variant. On the other hand, purified GAF_{VnfA}, whose oligomerization state is in equilibrium of tetramer and octamer, is able to hold the Fe-S cluster showing characteristic features of $[Fe_3S_4]^+$ in the UV-Vis and EPR spectra (Fig. 4). Thus, the reconstitution study with the truncated variants confirms that the GAF domain is responsible for holding the Fe-S cluster, and that the central domain which has the 6^{th} cysteine residue (Cys^{267}) is unlikely to participate in holding the Fe-S cluster. Unexpectedly, purified VnfAΔCRM shows a trimeric conformation different from the wild-type. This suggests that VnfA ΔCRM doesn't exert VnfA function appropriately since the VnfA binding sequences on DNA require four DNA binding motifs to form the tight VnfA-DNA complex.

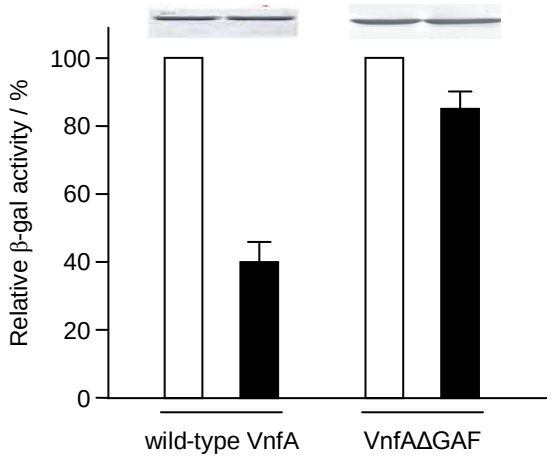

Fig. 5. Relative β-gal activities for wild-type VnfA and VnfAΔGAF in the absence (open bars) or presence (filled bars) of 500 μM EDTA. Each activity is normalized with that in the absence of EDTA. Error bars show standard deviation between repeat experiments.

Transcriptional activity of a truncated variant

Figure 5 shows the β-gal activities of the reporter strains producing wild-type VnfA and VnfAΔGAF. As demonstrated in Chapter II, wild-type VnfA bearing [Fe_3S_4] is transcriptionally active and turns into an inactive form upon the loss of the cluster under Fe depletion conditions. Consistent with the result, the β-gal activity of wild-type VnfA is repressed by a metal chelator, EDTA. Since the expression level of wild-type VnfA remains constant, the observed decrease in the β-gal activity is ascribed to inactivation of VnfA. In contrast to wild-type VnfA, EDTA shows no significant effect on the β-gal activity of VnfAΔGAF. The western blot assay shows that the expression level of VnfAΔGAF is largely unaffected by EDTA. Therefore, the transcriptional activity of VnfAΔGAF is dominantly positive irrespective of the Fe availability in the host cells. The observation of the dominantly positive activity for VnfAΔGAF supports that the GAF domain acts as an inhibitor in the transcriptional function of VnfA. The inhibitory effect of the GAF

domain is commonly found among σ^N-dependent transcriptional regulators (3,7-9). In these regulators, the depressed transcriptional activity can be retrieved by reactions of the GAF domain with environmental factors, followed by a conformational change in the domain and/or a linker region between the GAF and AAA+ domains. In the case of VnfA, binding of the Fe-S cluster to the N-terminal CRM could correspond to the process. Accordingly, the results imply that the Fe-S cluster bound to the GAF domain through CRM relieves the transcriptional activity of VnfA from the inhibition by the GAF domain.

ATPase activity of wild-type and truncated variants

Modulation of the nucleoside triphosphatase activity of the AAA+ domain has been known as a common mechanism to regulate the functional activity of the σ^N-dependent transcriptional regulators (10). NifA also has an analogous mechanism to regulate its activity, in which the inhibitory effect of the GAF domain on the nuclease activity is induced by an intermolecular interaction between NifA and NifL (3,8). Taken high homology of the possible AAA+ domain (the central domain) in VnfA to the AAA+ domain of NifA (1), it would be rational to deduce that the possible AAA+ domain in VnfA has the nuclease activity which is involved in the regulatory mechanism by the GAF domain and the Fe-S cluster. I, therefore, investigated the nuclease activity for wild-type and N- and C-terminally truncated variants of VnfA using ATP as a substrate.

Figure 6 shows the ATPase activity of wild-type VnfA including both the holo- and apo-forms at various ratios. Reduction of the catalytic activity is parallel to decrease in the ratio of the holo-form. The catalytic activity of the fully reconstituted VnfA is determined to be 1.4 min^{-1} which is virtually same to that of NorR (1.3 min^{-1}), a σ^N-dependent transcriptional regulator bearing a non-heme Fe as a nitric oxide sensor (9). Due to aggregation of VnfA at a higher ratio of the apo-form than 50 % under the present reaction conditions, the catalytic activity of apo-VnfA has

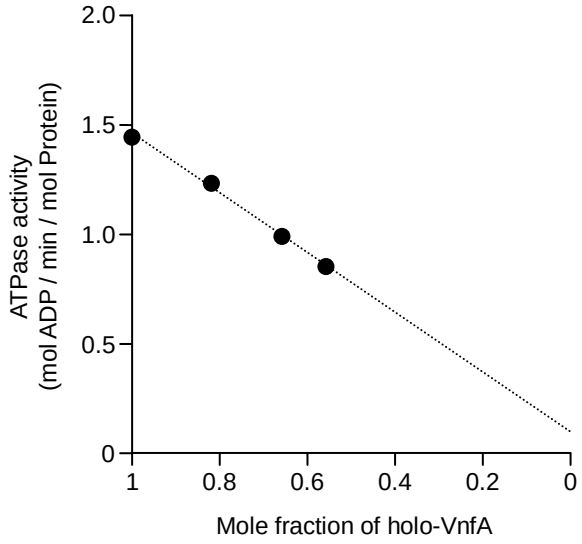

Fig. 6. ATPase activity of VnfA at various ratio of holo-form. A metal chelator, EDTA was added to reduce the ratio since EDTA induces disruption of the Fe-S cluster. ATPase activities are expressed as specific activity relative to protein concentration as monomer. The dotted line was obtained by the least squares method.

Table 1. ATPase activities of wild-type and variants of VnfA

	VnfAΔGAF	wild-type VnfA		GAF_{VnfA}
		holo-form	apo-form	
ATPase activity (mol ADP / min / mol Protein)	4.43 ± 0.12	1.42 ± 0.04	0.1	0.02 ± 0.02

ATPase activities are expressed as specific activity relative to protein concentration as monomer.

The ATPase activity of apo-VnfA was calculated by extrapolation as described in Fig. 6.

not been determined experimentally, while assuming linear correlation between the catalytic activity and the holo/apo ratio, extrapolation in Figure 6 gives 0.1 min^{-1} to the ATPase activity of apo-VnfA. Thus, the holo-form of wild-type VnfA has the ATPase activity which is depressed by the removal of the Fe-S cluster. This result agrees with the data of *in vivo* transcription assay described in Chapter II. The ATPase activity of VnfAΔGAF is also observed (4.4 min^{-1}), which is relatively higher than that of the wild-type, while the catalytic activity of GAF_{VnfA} consisting of only the GAF domain is negligible (Table 1). Taken these observations, it is probable that the possible AAA+ domain is ascribed to the nuclease activity of VnfA, which is negatively regulated by the GAF domain. The role of the Fe-S cluster is to release the catalytic activity from the inhibitory effect of the GAF domain. The results of the ATPase activity are coincident with those of the *in vivo* transcription assays (*vide supra*). This indicates that the regulation of the nuclease activity of the possible AAA+ domain closely related to the regulatory mechanism of the transcriptional activity of VnfA.

DNA binding ability

A fluorescence polarization-based assay was carried out to study the interaction between VnfA and a 55 bp DNA containing the *vnfH* promoter region. Figure 7 illustrates the binding isotherm of VnfA reconstituted with the Fe-S cluster in the presence of the DNA (10 nM). The titration experiment indicates that the reconstituted VnfA binds to the DNA with a dissociation constant (K_d) of 84.4 ± 13.5 nM. Due to a propensity for severe aggregation of apo-VnfA at a higher concentration, an attempt to determine the K_d value by the assay with apo-VnfA is unsuccessful. Hence, I decide to produce the DNA bound apo-form from the DNA bound holo-form by disassembling the Fe-S cluster with EDTA. This procedure seems rational in light of reconstructing the physiological conditions since the holo-form lying on the promoter senses an environmental factor and turns to the transcriptionally inactive apo-form. Even after 50% removal of the Fe-S

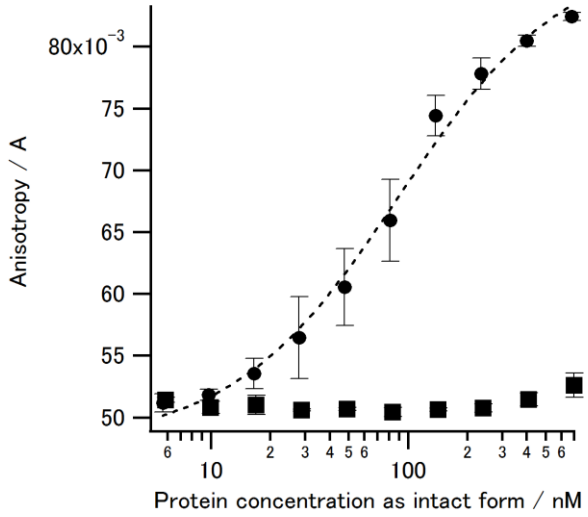

Fig. 7. The binding isotherm of holo-VnfA to the fluorescein isothiocyanate-labeled DNA carrying the *vnfH* promoter sequences (●). Non linear curve fitting of the binding data (dotted line) gives dissociation constant (K_d) of 84.4 ± 13.5 nM. The plots of closed rectangular (■) represent the control experiment with BSA.

cluster from holo-VnfA, virtually no alteration is observed in the DNA binding ability (K_d = 106 ± 6 nM). This result demonstrates that the binding ability of VnfA to the promoter sequences is uncoupled with the state of the Fe-S cluster and therefore, is not under the inhibitory effect of the GAF domain. No binding ability is observed for GAF_{VnfA}, which is rational since the variant lacks the DNA binding domain. The K_d value of VnfAΔGAF cannot be determined under the present conditions because of severe aggregation at a higher concentration similarly to apo-VnfA. The binding mode of VnfA to the target promoter sequences is contrast to that of NifA (8). The transcriptionally active NifA exhibits a specific binding ability to DNA which is, however, reduced when NifA forms the inactive complex with the sensory protein, NifL.

Discussion

Amino acid residues binding to the Fe-S cluster

In Chapter II, I described the involvement of an Fe-S cluster (3Fe-4S type) as a prosthetic group in each subunit of the VnfA tetramer, while the locus of the Fe-S cluster remained uncertain. The sequence analysis of N-terminal GAF domains involved in the VnfA clade proposed a characteristic CRM in the GAF domain as a candidate for the cluster binding. In fact, a systematic mutagenesis study with Cys variants of AnfA revealed that Cys^{21} and Cys^{26}, corresponding to Cys^{10} and Cys^{15} in VnfA, respectively, were essential for the transcriptional activity (6). In agreement with this, the *in vivo* transcription assay for the cysteine variants of VnfA indicates that the N-terminal cysteine residues are plausible candidates for the ligands of the Fe-S cluster. The fact that GAF_{VnfA} can harbor the same type of the Fe-S cluster as the wild-type also supports that the CRM in the GAF domain participates in holding the Fe-S cluster. Because a single residue gap between Cys^8 and Cys^{10} is unusual to be ligands for a single Fe-S cluster, it is unlikely that all the N-terminal cysteine residues in the subunit of VnfA bind to the single Fe-S cluster. A possible scenario is that two of three cysteine residues (Cys^{15} and Cys^8 or Cys^{10}) bind to the Fe-S cluster and the remaining residue binds to the Fe-S cluster in neighboring subunit of VnfA. Alternatively, a non-cysteinyl residue such as histidine, aspartate or glutamate could comprise the third ligand. Then, the reduction of the transcriptional activity for C8A or C10A is associated with an indirect influence of mutagenesis at the neighboring residue.

The regulatory mechanism of VnfA

VnfA is one of the σ^N-dependent transcriptional regulators composed of the GAF, possible AAA+ and DNA binding domains. The original AAA+ domain in the σ^N-dependent transcriptional regulators is responsible for not only interaction of the regulators with the σ^N-RNA polymerase

complex but also the ATP hydrolysis (7,11). The hydrolysis is coupled with conversion of double-stranded DNA (dsDNA) to single-strand (ssDNA) to form an open complex with the σ^N-RNA polymerase. I show here that the possible AAA+ domain of VnfA also has the ATPase activity and that the activity is negatively regulated by the GAF domain in response to the state of the Fe-S cluster. As described in the limited protease assay (Chapter II), VnfA has different conformation between the holo- and apo-forms, which implies that the regulation of the ATPase activity by the GAF domain requires a conformational change although the quaternary structure (tetrameric configuration) is not modulated by the Fe-S cluster. A similar regulatory mode of the ATPase activity by the sensory domain (GAF domain) is commonly found in the σ^N-dependent transcriptional regulators (3,8,9). The inhibition of the ATPase activity by the sensory domain results in no conversion of the dsDNA to the ssDNA and the consequent repression of the transcription. Unlike the NifAL system of *A. vinelandii* (8), DNA binding ability of VnfA is little affected by the inhibitory function of the GAF domain. This allows inference that VnfA is always attached on the promoter region of the *nitrogenase-2* genes irrespective of the state of the Fe-S cluster. Consequently, the transcriptional regulation by VnfA should largely depend on the regulation of the catalytic activity by the GAF domain in response to the state of the Fe-S cluster. This is rather different from another nitrogenase regulatory system, NifAL (8), but is similar to an oxidative stress sensor, SoxR (12-15). This regulator exhibits the constant binding ability to its target sequence irrespective of the state of the sensory moiety, a 2Fe-2S type cluster.

In summary, this chapter described that CRM in the GAF domain (Cys^8-X-Cys^{10}-XXXX-Cys^{15}) participates in holding the Fe-S cluster. The bound Fe-S cluster released the inhibitory effect of the GAF domain on the ATPase activity of the possible AAA+ domain to render VnfA to the transcriptionally active, while VnfA retained the DNA binding ability irrespective of the state of the

Fe-S cluster.

Experimental Section

Chemicals

All chemicals were purchased from Nakalai Tesque (Kyoto, Japan) and Wako Co. (Tokyo, Japan) and were used without further purification.

Anoxic Sample Handling

To minimize any exposure to O_2, VnfA variants harboring the Fe-S cluster were manipulated under argon atmosphere. After degassed and sparged with argon gas three times, buffers were allowed to stand in an anaerobic chamber for at least 16 h. All sample-handling devices were rinsed with anoxic buffers. Buffer containing the reductant, sodium dithionite, was used in the case of O_2 being not removed thoroughly.

Construction of strains and plasmids

The strain and plasmids used in this work are listed in Table 2. Construction of pASK-IBA3plus-VnfA plasmid which encodes *Strep*-tag fused VnfA at C-terminus has been described previously in Chapter II. Site-directed mutagenesis of cysteine to alanine in VnfA was carried out with the QuickChange kit (Stratagene, La Jolla, CA, U.S.A.) with the plasmid, pASK-IBA3plus-VnfA, as a template. The following primers were used for site-directed mutagenesis of TGC (Cys) to GCC (Ala) underlined: 5'-CTCCCCCAATACGCCGAATGCGGCCT CG-3' and 5'-CGAGGCCGCATTCGGCGTATTGGGGGAG-3' for C8A, 5'-CAATACTGCGAA GCCGGCCTCGGCGAG-3' and 5'-CTCGCCGAGGCCGGCTTCGCAGTATTG-3' for C10A,

Table 2. Bacterial strain and plasmids employed in this study

Strain, plasmid	Relevant characteristic	Reference
Strain		
E. coli		
λPvnfH	JM109[a] lysogenized with λ(*vnfH* promoter-*lacZ*)	Chapter II
Plasmids		
pASK-IBA3plus-VnfA	pASK-IBA3plus[b] with *Bsa*I fragment containing *vnfA* gene	Chapter II
pASK-IBA3plus- GAF_{VnfA}	pASK-IBA3plus with *Bsa*I fragment encoding the VnfA GAF domain only	This work
pASK-IBA3plus-VnfAΔCRM	pASK-IBA3plus with *Bsa*I fragment encoding the VnfAΔCRM.	This work
pASK-IBA3plus-VnfAΔGAF	pASK-IBA3plus with *Bsa*I fragment encoding the VnfAΔGAF.	This work
pRKSUF017	*Lac* promoter-containing plasmid with *suf* gene	(16)

Purchased from [a)]TOYOBO Co., [b)]IBA Co.

5’-GCCTCGGCGAGGCCCGTACGGACGTC-3’ and 5’-CACGTCCGTACGGGCCTCGCCGAG GC-3’ for C15A, 5’-CATCGTGGTGCCCGCCATCCGCGAC-3’ and 5’-GTAGCGGATGGCGGGC ACCACGATG-3’ for C107A, 5’-CTGTCGTTCATCGCCGTGCCGATCCTGCG-3’ and 5’-CGCAG GATCGGCACGGCGATGAACGACAG-3’ for C134A, 5’-GTCAAGTTCAACGCCGCCTCGCTG CCC-3’ and 5’-GGGCAGCGAGGCGGCGTTGAACTTGAC-3’ for C267A. After verifying the DNA sequences, transformation of *E. coli* λPvnfH with the obtained plasmid was performed.

Three derivatives of pASK-IBA3plus-VnfA containing genes that produce GAF_{VnfA}, VnfAΔCRM and VnfAΔGAF (pASK-IBA3plus-GAF_{VnfA}, pASK-IBA3plus-VnfAΔCRM and pASK-IBA3plus-VnfAΔGAF, respectively) were constructed for overproduction of the truncated variants. To clone the gene which encodes each VnfA variants into the *Strep*-tag fusion vector, pASK-IBA3plus (IBA Co., Göttingen, Germany), the target DNA fragment was amplified by PCR

from pASK-IBA3plus-VnfA using the primers containing a *Bsa*I restriction site (underlined): 5'-CA AAAGGTCTCGAATGTCCAGCCTCCCCCAATA-3' and 5'-CAAAAGGTCTCAGCGCTCTCCA GGGCGACGTTCTCGAC-3' for GAF_{VnfA}, 5'-CAAAAGGTCTCGAATGCGTACGGACGTGTTG CCGCT-3' and 5'-CAAAAGGTCTCAGCGCTGCGGTAGTCCTTGTAGTTGA-3' for VnfAΔCRM, 5'-CAAAAGGTCTCGAATGAACATCATCGGCAATTCCAA-3' and 5'-CAAAA GGTCTCAGCG CTGCGGTAGTCCTTGTAGTTGA-3' for VnfAΔGAF. After the cloning, the *E. coli* JM109 strain was sequentially transformed with the obtained plasmid and pRKSUF017 (16), which is an expression system for recombinant SUF proteins, using similar techniques described in Chapter II.

In vivo transcription assay

The β-galactosidase activity was measured by a modified method of Miller (17). Cells grown in LB medium containing 100 μg/mL ampicillin and 30 μg/mL kanamycin at 37 °C for 7 h were washed twice with NFDM medium (70 mM K_2HPO_4, 25 mM KH_2PO_4, 9 mM NaCl, 1 mM $MgSO_4$, 100 mM glucose, 2 mM glutamine, pH 7.0) (18), and resuspended in NFDM medium supplemented with 100 μg/mL ampicillin and 30 μg/mL kanamycin. After 18 h of further growth at 30 °C, the cells were harvested by centrifugation, resuspended in Z buffer and sonicated. The β-galactosidase activity of the cell extract was determined using *o*-nitrophenyl-β-D-galactopyranoside.

Purification of wild-type and truncated variants of VnfA

Cells carrying pASK-IBA3plus-VnfA were grown at 37 °C in LB medium plus 100 μg/mL ampicillin. When the cells reached an OD_{600} of 0.8, freshly prepared isopropyl-thio-β-D-galactoside was added to a final concentration of 0.5 mM for inducing the SUF protein biosynthesis. After 1 h of further growth at 37 °C, the temperature was lowered to 20 °C and then anhydrotetracycline was added to a final concentration of 100 ng/mL for overproducing

VnfA. The cells were harvested by centrifugation at 12,000 *g* for 15 min at 4 °C after 20 h of further growth at 20 °C. The collected cells were stored at −80 °C.

Cell pellets (2 g) were resuspended in 20 mL of HGDT buffer (20 mM Hepes-KOH, pH 8.0, 10% glycerol, 1 mM dithiothreitol, 0.2% Triton X-100 reduced) and sonicated. The extracts were centrifuged at 13,000 *g* for 20 min at 4 °C to remove cell debris and the resulting supernatant was passed through a 2.0 mL *Strep*-Tactin column (IBA Co., Göttingen, Germany) pre-equilibrated with HGDT buffer. After washing the column with 4 mL of HGDT buffer and then repeated the same for three times, affinity-bound VnfA was eluted with 8 mL of HGDT buffer containing 2.5 mM desthiobiotin. In the case of DNA being contaminated in the fraction, the protein was further purified with Q-Sepharose column (GE Healthcare, Milwaukee, WI, USA). Purified VnfA was dialyzed with HGDT buffer and stored at 4 °C.

Overproduction and purification procedures of GAF_{VnfA}, VnfAΔCRM and VnfAΔGAF are the same as that of VnfA except that 4 g of cell pellets overproducing VnfAΔCRM or VnfAΔGAF was used at sonication step because expression level was less than VnfA.

Reconstitution of the Fe-S cluster

VnfA and GAF_{VnfA} were reconstituted by a modified procedure previously described (19). Aerobically purified 10 μM VnfA or GAF_{VnfA} in HGT buffer (20 mM Hepes-KOH, pH 8.0, 10% glycerol, 0.2% Triton X-100 reduced) was incubated with 270 μM L-cysteine and 540 μM DTT for 20 min under anaerobic conditions at room temperature. After addition of purified IscS protein (which was described in Chapter II) and $FeCl_2$ at a final concentration of 0.135 μM and 270 μM, respectively, further incubation was carried out. The reconstitution reaction was monitored spectrophotometrically and once complete, the reconstituted protein was separated from low molecular weight reactants and other products by size exclusion chromatography on a MicroSpin

S-200 HR column (GE Healthcare) equilibrated with HGT buffer.

Native molecular mass analyses

Analytical size-exclusion chromatography was performed on a HiPrep 16/60 Sephacryl S-300 HR (GE Healthcare) in HGDT buffer at 4 °C. The flow rate was 0.15 mL/min. Size standards eluted at 12 mL (Ferritin, 440 kDa), 19.1 mL (Catalase, 232 kDa), 30.6 mL (Hemoglobin, 64.5 kDa), 46.2 mL (Trypsinogen, 24.0 kDa) and 55.2 mL (Cytochrome *c*, 12.3 kDa). Apo-form of GAF_{VnfA} eluted in two parts at 22.5 mL and 29.4 mL, VnfAΔCRM at 22.0 mL and VnfAΔGAF at 24.7 mL, corresponding to an apparent molecular weight of 166 kDa, 94.1 kDa, 173.2 kDa and 138.6 kDa, respectively.

ATPase assay

The purified protein was dissolved to a concentration of 2 μM in basal reaction buffer, which contained 50 mM Tris-HCl (pH 8.0), 100 mM potassium chloride and 2 mM magnesium chloride. The ATPase reaction was initiated by adding ATP at a final concentration of 3 mM. After incubation at 25 °C, the solution was quickly mixed with an equal volume of 0.1 M hydrochloric acid to terminate the reaction. Then the solution was diluted 5 times with MilliQ water and centrifuged at 20,000 *g* for 5 min. A 20-μL aliquot of clear supernatant was applied to a reverse-phase HPLC column (VP-ODS 250 × 4.6, Shimadzu, Kyoto, Japan) equilibrated with the carrier buffer of 50 mM triethylammonium phosphate (pH 6.8) and 2.5% (v/v) acetonitrile. The absorbance of ADP was monitored at 260 nm using a UV detector.

In order to reduce the holo-/apo-VnfA ratio, the reconstituted VnfA was incubated at 25 °C with 200 equivalent of a metal chelator, EDTA. The concentration of the holo-VnfA was determined from absorbance at 420 nm assignable to the Fe-S cluster in VnfA.

DNA binding assay *in vitro*

DNA binding by VnfA variants was quantified using fluorescence polarization (20,21). Binding assays were performed in $HGT_{0.5}$ buffer (20 mM Hepes-KOH, pH 8.0, 10% glycerol, 0.5% Triton X-100 reduced) with a 55 bp DNA probe involving the *vnfH* promoter region, 5'-CCCCAGTACCATGCGGAACGGATCGCTTCCCGGCTGTACCTGCGGGTACGTCGAC-3', labeled at the 5' end with fluorescein isothiocyanate. The DNA probe (10 nM) and varying concentrations of VnfA variant were mixed in test tubes. The tubes were fitted with rubber septa if needed to protect VnfA variants harboring the Fe-S cluster from inactivation by O_2. The samples were equilibrated at 25 °C for 5 min before measurements. Fluorescence polarization was recorded at 25 °C on a Beacon 2000 fluorescence polarization detector (PanVera Co., Madison, WI, USA). The excitation wavelength was 490 nm, and the fluorescence polarization signal was measured at 520 nm. Dissociation constants were calculated by nonlinear curve fitting of the binding data using the equation of Lundblad *et al.* (20). Control experiments were carried out under the same conditions using BSA instead of VnfA variants.

In order to reduce the holo-/apo-VnfA ratio, the reconstituted VnfA was incubated at 25 °C with 10 nM the DNA probe and 4 mM EDTA. The concentration of the holo-VnfA was determined from absorbance at 420 nm assignable to the Fe-S cluster in VnfA.

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Chapter IV

Environmental Factors of VnfA

Introduction

As described in the previous chapters, VnfA is the transcriptional regulator of the *nitrogenase-2* genes in diazotroph *Azotobacter vinelandii*, and a member of σ^N-dependent transcriptional regulators. The σ^N-dependent regulators commonly have the GAF domain (i.e. cGMP-specific and -stimulated phosphodiesterases, *Anabaena* adenylate cyclases and *Escherichia coli* FhlA), which is believed to sense environmental factors immediately or through interaction with sensory proteins (1,2). In Chapter II, I described that VnfA involves a 3Fe-4S type cluster ($[Fe_3S_4]$) as the prosthetic group which is essential for transcriptionally active VnfA. Therefore, the Fe-S cluster is expected to serve as a sensor for switching the transcriptional activity of VnfA, while little is elucidated regarding environmental factor(s) reactive with the cluster.

The environmental factors of σ^N-dependent transcriptional regulators are diverse, but should correspond to physiological events. For example, XylR and NorR, members of σ^N-dependent transcriptional regulators sense *m*-xylene and nitric oxide (NO) to modulate xylene metabolism and NO detoxification, respectively (3-8). NtrC senses nitrogen starvation to regulate nitrogen assimilation via a phosphorylation cascade involving the sensor kinase, NtrB (9,10). Likewise, an environmental factor of VnfA should be related to the nitrogen fixation. It has been reported that the nitrogen fixation is affected by several environmental factors. Dioxygen is known to inactivate nitrogenase (11,12). The NifAL system in *A. vinelandii* represses the transcription of the *nitrogenase-1* gene in response to O_2 to protect nitrogenase from its inactivation (13,14). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as superoxide (O_2^-) and NO, respectively, also cause the inactivation of nitrogenases through attack on the active centers (15-19). Therefore, the O_2, ROS and RNS molecules are also candidates for the environmental factors of VnfA.

In Chapter IV, I describe identification of environmental factor(s) of VnfA. The experimental

results demonstrate that plausible environmental factors are O_2^- and NO which depress the activity of VnfA by disassembling the Fe-S cluster. In the experiments described in this chapter, I employed a VnfA protein conjugated with *Strep*-tag in place of the intact form because of facile purification and detection of the protein. The transcriptional function of the tag-attached VnfA has been affirmed to be a comparable level to the intact form by other *in vivo* experiments.

Results

Screening of environmental factors of VnfA

Dioxygen

Dioxygen level in a cell is a well-known environmental factor for the nitrogenase transcriptional regulation. In order to estimate an effect of O_2 on the transcriptional activity of VnfA, the VnfA activity is assessed under aerobic and micro-aerobic conditions (Fig. 1). No significant change is observed in the expression level of the *lacZ* transcript after transferring the reporter strain from micro-aerobic to aerobic culture. A consistent result is also obtained for the β-galactosidase (β-gal) activity assay (Table 1). An accumulation of β-gal in the reporter strain is at the same level after the aerobic and micro-aerobic cultures. These findings are contrast to the previous studies on the transcriptional regulation by NifAL. NifL is sensitive to cytosolic O_2 of the aerobically grown cell. Consequently, the transcriptional activity of the NifAL system is affected by aeration conditions of the growth media (13). Thus, the present results indicate that the Fe-S cluster in VnfA is insensitive to O_2 permeating to the cell from the air, which excludes O_2 from the environmental factor of VnfA.

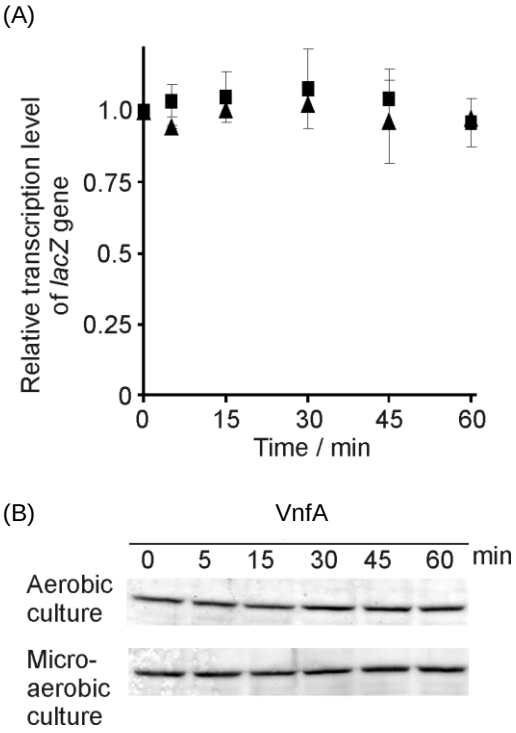

Fig. 1. (A) Time course of the VnfA activity assessed by the *lacZ* transcript level at early exponential phase after culture under micro-aerobic conditions, the cells were divided into aerobic (▲) and micro-aerobic (■) cultures at 0 min for the subsequent assay. Each plot presents the mean values from three independent experiments, normalized with the activity at 0 min. (B) Western blot analyses for VnfA recorded at a time corresponding to the performed assays.

Table 1. Effect of aeration level of culture on the β-gal activity assay.

	Aerobic culture	Micro-aerobic culture
β-gal activity (Miller units / mg of protein)	281(11)	232(15)

Values in parentheses are standard deviations calculated from four independent measurements and rounded off to the whole number.

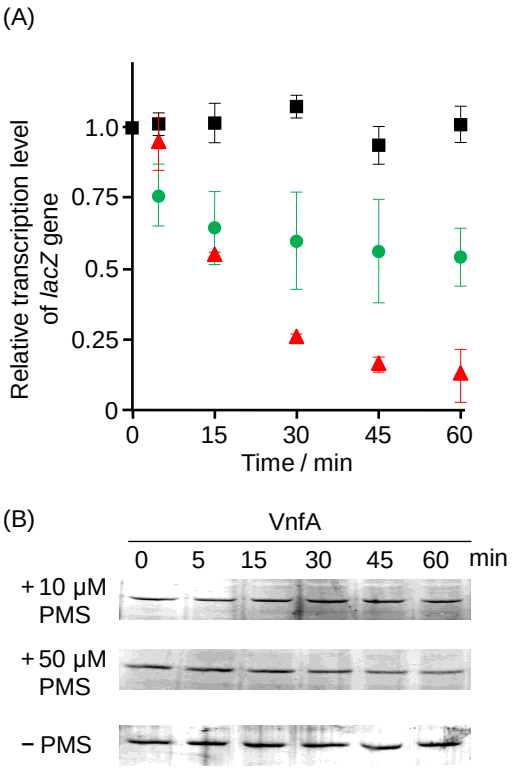

Fig. 2. (A) Time course of the VnfA activity assessed by the *lacZ* transcript level at early exponential phase after the addition of 10 μM PMS (●, +10 μM PMS); 50 μM PMS (▲, +50 μM PMS); no addition of PMS (■, −PMS). Each plot presents the mean values from three independent experiments, normalized with the activity at 0 min. (B) Western blot analyses for VnfA recorded at a time corresponding to the performed assays.

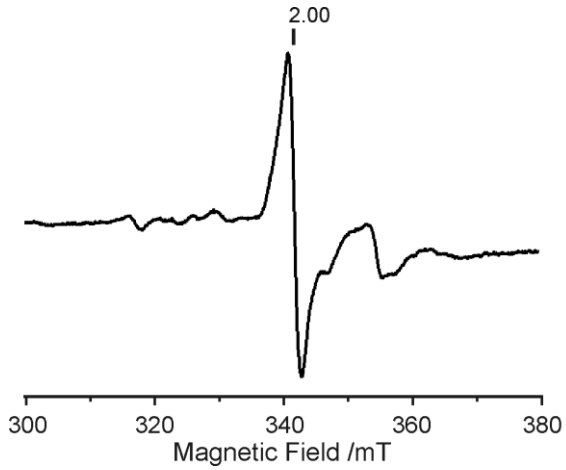

Fig. 3. Effect of PMS on the whole cell EPR spectrum of aerobically grown *E. coli* JM109 overexpressing *vnfA*. Addition of PMS to the NFDM medium (final concentration of 50 μM) was followed by 60 min of further culture and then harvesting. The spectrum was recorded at 10 K using 2.5 mW microwave power and a field modulation of 0.8 mT.

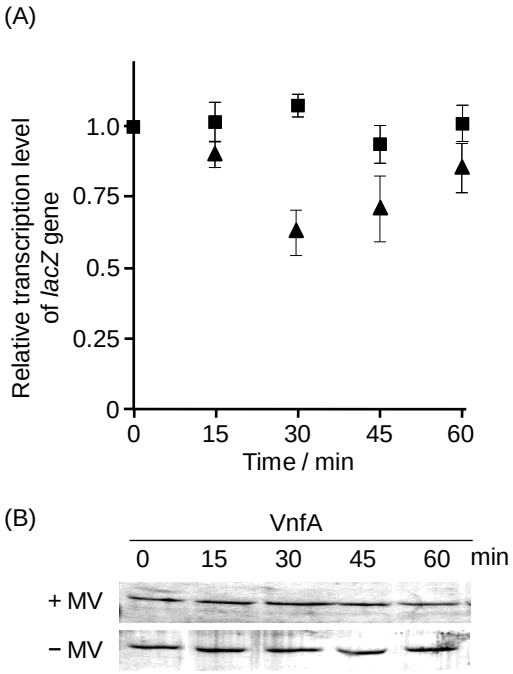

Fig. 4. (A) Time course of the VnfA activity assessed by the *lacZ* transcript level at early exponential phase after the addition of 100 μM MV (▲, +MV); no addition of MV (■, −MV). Each plot presents the mean values from three independent experiments, normalized with the activity at 0 min. (B) Western blot analyses for VnfA recorded at a time corresponding to the performed assays.

Reactive oxygen species (ROS)

Since O_2 is unlikely the environmental factor of VnfA, I subsequently inspected the effect of reactive oxygen species (ROS) on the transcriptional function of VnfA. As shown in Fig. 2, the expression level of the *lacZ* transcript decreases upon the addition of phenazine methosulfate (PMS) which is an efficient O_2^- generator in aerobically grown cell (20). The addition of PMS at a final concentration of 10 and 50 μM gives rise to a drop in the transcript level by 40 and 90% at 60 min, respectively. Because the expression level of VnfA is scarcely affected by PMS during the assay, the observed decrease in the transcript is not associated with the growth inhibition of the strain but is ascribed to immediate inactivation of VnfA by PMS. The EPR spectrum from *E. coli* producing

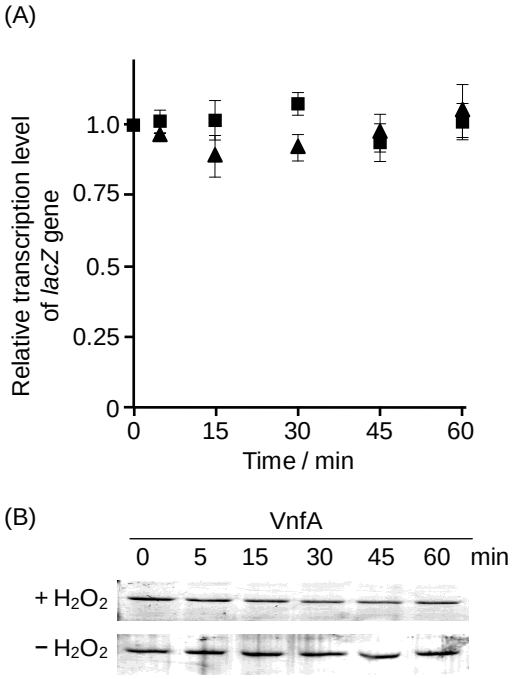

Fig. 5. (A) Time course of the VnfA activity assessed by the *lacZ* transcript level at early exponential phase after the addition of 500 μM H_2O_2 (▲, $+H_2O_2$); no addition of H_2O_2 (■, $-H_2O_2$). Each plot presents the mean values from three independent experiments, normalized with the activity at 0 min. (B) Western blot analyses for VnfA recorded at a time corresponding to the performed assays.

VnfA after the addition of 50 μM PMS exhibits replacement of the signals from $[Fe_3S_4]^+$ with a strong signal at g = 2.00, which is assignable to organic radicals generated by the reaction of amino acid residues with O_2^- (21) (Fig. 3). A similar dropping of the transcriptional activity is observed when adding 100 μM methyl viologen (MV), a moderate O_2^- generator (20), while the transcriptional activity recovers after the dropping (Fig. 4). Superoxide undergoes disproportionation into H_2O_2 and O_2 in aqueous media, which is accelerated by superoxide dismutase (SOD) *in vivo* (22). Therefore, H_2O_2 could be another candidate of the environmental factor of VnfA. In order to confirm this, the transcription assay was attempted in the culture containing H_2O_2. As shown in Fig. 5, even 500 μM H_2O_2 does not affect the expression level of the

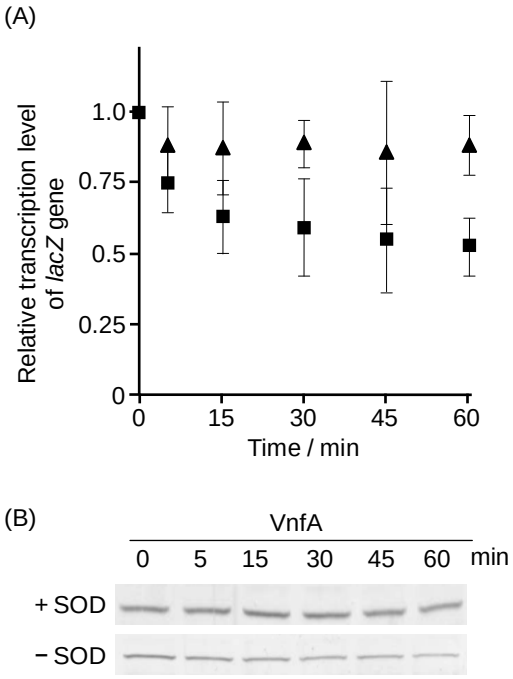

Fig. 6. (A) Time course of the VnfA activity assessed by the *lacZ* transcript level at early exponential phase of *E. coli* overexpressing *sodA* gene (▲, +SOD) and control (■, −SOD) after the addition of 10 μM PMS. Each plot presents the mean values from three independent experiments, normalized with the activity at 0 min. (B) Western blot analyses for VnfA recorded at a time corresponding to the performed assays.

lacZ transcript. In addition, the expression level of VnfA remains constant under the same conditions, indicating that H_2O_2 has effect on neither the function nor the stability of VnfA. Supportive data is also obtained from an additional transcription assay with the reporter strain co-producing SOD which is expected to convert the generated O_2^- by PMS to H_2O_2 in the cell (Fig. 6). The effect of 10 μM PMS in the culture is apparently cancelled in the presence of SOD. These findings demonstrate that O_2^- is the exclusive candidate for the environmental factor among ROS.

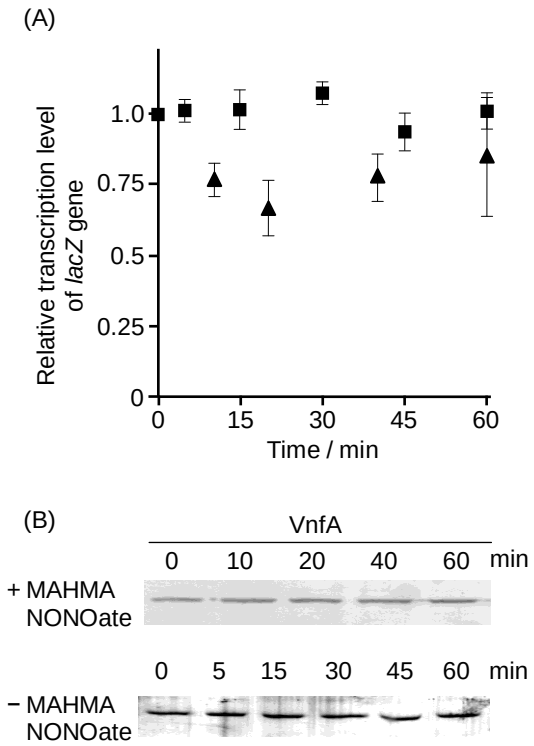

Fig. 7. (A) Time course of the VnfA activity assessed by the *lacZ* transcript level at early exponential phase after the addition of 25 μM MAHMA NONOate (▲, +MAHMA NONOate); no addition of MAHMA NONOate (■, −MAHMA NONOate). Each plot presents the mean values from three independent experiments, normalized with the activity at 0 min. (B) Western blot analyses for VnfA recorded at a time corresponding to the performed assays.

Nitric oxide

Nitric oxide is known to be reactive with Fe-S clusters in proteins such as nitrogenases to depress their catalytic activity (18,23-26). In accordance with this, the *in vivo* transcription assay shows a decrease in the expression level of the *lacZ* transcript when adding a NO releaser, MAHMA NONOate (Sigma Co.) at a final concentration of 25 μM (Fig. 7). Following the maximum dropping by 30% at 20 min, the activity returns to the original level at 60 min. Since the expression level of VnfA during the assay remains virtually constant, the observed decrease in the

Table 2. Effect of Mo and V on the β-gal activity assay.

	Concentration of metal ions in media (μM)						
Mo[a]	0	1	0	1	100	0	100
V [b]	0	0	1	1	0	100	100
β-gal activity (Miller units / mg of protein)	232(15)	237(11)	258(17)	276(10)	241(9)	275(12)	262(13)

Culture was performed under micro-aerobic conditions. Values in parentheses are standard deviations calculated from four independent measurements and rounded off to the whole number.
[a)] Source of Mo is Na_2MoO_4. [b)] Source of V is V_2O_5.

transcript is not associated with growth inhibition of the strain but is ascribed to the inactivation of VnfA by NO supplied from the releaser.

Discussion

Previous studies have reported that neither molybdenum (Mo) nor vanadium (V) show a direct effect on the transcriptional function of VnfA (27-30). I also obtained results consistent with these findings in the β-gal activity assays regarding Mo and V (Table 2). Previous studies on the expression from promoters of *vnfA*, *vnfH* and *vnfDGK* demonstrated that V is not required for the transcription of each promoter (28,29), but is for the translation of the *vnfDGK* transcript (27), and that the repressive effect of Mo on the *vnfH* and *vnfDGK* promoters is mediated through the repression of *vnfA* transcription (30). On the basis of these considerations, I conclude that both Mo and V are excluded from the candidates for the VnfA environmental factor. Dioxygen serves as an environmental factor for nitrogenase transcriptional regulators. This is also true for the NifAL system in *A. vinelandii* (13,14). Recent kinetic studies on FNR, a well-studied O_2 responsive transcriptional regulator bearing a 4Fe-4S type cluster, have proposed transient formation of $[Fe_3S_4]^+$ in FNR upon the reaction with O_2, followed by self-disassembly to $[Fe_2S_2]^{2+}$ and subsequent loss of

the whole Fe-S cluster structure (31,32). Hence, it is possible that $[Fe_3S_4]^+$ observed for VnfA in the EPR measurement (Chapter II) is a stable intermediate generated from EPR silent $[Fe_4S_4]^+$ in the process of O_2 level sensing. However, the *in vivo* assays performed under aerobic and micro-aerobic conditions provide no supportive data of the O_2 level sensing. Instead, the transcription assay reveals that VnfA is reactive with ROS to reduce its transcriptional activity. The co-production of SOD in the reporter strain releases the VnfA function from the repression by ROS. Since SOD produces H_2O_2 from O_2^-, the co-produced SOD may temporarily increase the concentration of H_2O_2 in the reporter strain at the expense of O_2^-. Therefore, this result suggests that VnfA is insensitive to H_2O_2. Another result that externally added H_2O_2 does not affect the transcriptional activity of VnfA is consistent with the effect of the co-produced SOD. Thus, the *in vivo* activity assay indicates that O_2^- is the exclusive environmental factor of VnfA among ROS.

The production of O_2^- by nitrogenases has been proposed as an initial reaction of a possible protection mechanism of the nitrogenases against O_2-induced damage when the respiratory protection mechanism is overloaded (33-36). This proposal appears to support the physiological significance of O_2^- sensing by VnfA. It is, however, still premature to conclude a physiological role for O_2^- sensing by VnfA if a high reactivity of O_2^- to Fe-S clusters is taken into account (37-39). Further study regarding effects of O_2^- on the nitrogenases and whole nitrogen fixation system will be needed.

The problem, whether VnfA has a partner protein for the O_2 level sensing, should be considered. NifL would be a candidate for the partner protein. Similar to the NifAL system, VnfA might form a protein–protein complex with NifL to allow repressive regulation in response to the O_2 level as well as nitrogen status in the cytosol (NifL is the bifunctional sensor for both the O_2 and fixed nitrogen levels.).

Several lines of evidence indicate that redox-sensitive metal clusters bound to cysteine-rich

motif serve as sensors for not only O_2 and Fe (40), but also NO (41). The Fe-S containing proteins like dehydratases have been known as targets of NO as well as O_2^- and H_2O_2 (26,42). Nitric oxide is reactive to Fe-S clusters to form complexes in both model compounds and proteins (18,43,44). Aconitase having $[Fe_4S_4]$ is sensitive to NO, but recently it has been proposed that the peroxynitrite anion ($ONOO^-$), formed in the reaction of NO with O_2^-, is the real reactive species with the Fe-S cluster (45,46). Regulatory proteins containing an Fe-S cluster such as SoxR and mammalian iron-responsive element-binding protein 1 (IRE-BP1) are also reactive with NO (24,40,47,48). This is also true of FNR, a global oxygen sensor in *E. coli*. The present results show that VnfA is inactivated by NO. A possible physiological role of the NO sensing by VnfA is protection of the nitrogenases from the inactivation by NO (17). Although little is reported regarding nitrosative stress on *A. vinelandii*, the structural genes designated *norR* and *hmp* whose products participate in the NO detoxification system in a wide variety of bacteria (7,49-52) are coded in a genome of *A. vinelandii* (53). This implies that *A. vinelandii* suffers toxic NO by chance in the growth environment. Diffusion out of NO from denitrifying microbes likely gives rise to increase of the NO concentration in soil (54-60). The emitted NO might cause the nitrosative stress on *A. vinelandii*.

In the *in vivo* transcription assay, the initial reduction of VnfA activity with a moderate amount of O_2^- or NO is followed by the return to the original level. This suggests that VnfA has a repair system after the reaction with O_2^- or NO. The SUF and ISC proteins which are the biosynthetic machineries of Fe-S cluster assembling are considered to be involved in the general repair system of Fe-S clusters in proteins (61-64). Inactivated VnfA may also employ the SUF and/or ISC machineries to recover the activity. In accordance with this assumption, the *in vivo* transcription assay reveals that increase in the expression level of the SUF proteins is coincident with the recovery of the VnfA activity (Fig. 8).

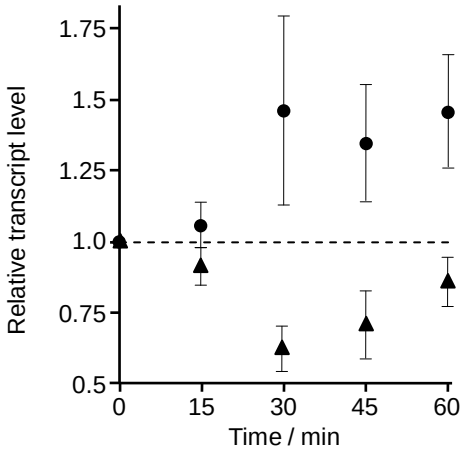

Fig. 8. Time course of the expression level of the SUF proteins assessed by the *suf* transcript level (●). The *lacZ* transcript level (▲) at each time in Fig. 4 is also shown for facile comparison between the expression level of the SUF proteins and the VnfA activity. Each plot presents the mean values from three independent experiments, normalized with the transcript level at 0 min.

In summary, I demonstrated that VnfA regulates the transcription in response to O_2^- and NO. The results obtained here is distinctive of VnfA and significantly different from the NifAL system, which will open up arguments about the physiological role(s) and functional mechanism of VnfA in the regulation of nitrogenase-2.

Experimental Section

Chemicals

All chemicals were purchased from Nakalai Tesque (Kyoto, Japan) and Wako Co. (Tokyo, Japan) and were used without further purification.

Construction of plasmid and strain

For *in vivo* transcription assay, the reporter strain was constructed as described in Chapter II. In order to construct the reporter strain co-producing SOD, a *sodA* expression plasmid, pSTV28-SOD was prepared. The *sodA* gene was amplified by PCR from *E. coli* chromosomal DNA using the primers: 5'-GGAGAATTCGATGAGCTATACCCTGCCATCCCTG-3' (*Eco*RI site underlined) and 5'-GGCAAGCTTTTATTTTTTCGCCGCAAAACGTGCC-3' (*Hin*dIII site underlined). After digestion with *Eco*RI and *Hin*dIII, the PCR product was ligated into the *Eco*RI-*Hin*dIII restriction sites of pSTV28. With the resulting plasmid pSTV28-SOD, the reporter strain expressing *vnfA* was transformed.

In vivo transcription assay

The activity was determined by the expression level of the *lacZ* transcript in the reporter system as described in Chapter II, where H_2O_2, phenazine methosulfate (PMS), methyl viologen (MV) and MAHMA NONOate were used instead of *o*-phen. The PMS and MV are known as O_2^- generators (65) and MAHMA NONOate is one of NO releasers. When micro-aerobic conditions were required, the medium was equipped with several pieces of AnaeroPouch-MicroAero (Mitsubishi Gas Chemical Co., Tokyo, Japan). The AnaeroPouch-MicroAero absorbs O_2 and releases CO_2 to maintain around 6% O_2 in the atmosphere of the flask.

Spectroscopy

UV-visible spectra were monitored on a MultiSpec-1500 spectrophotometer (Shimadzu Co., Kyoto, Japan). X-band EPR spectra were recorded on an E500 X-band CW-EPR (Bruker, Ettlingen, Germany). A cryostat (ITC503; Oxford Instruments Co., Abingdon, UK) was used for measurement at low temperature. For whole-cell EPR measurement, 500 mg of cell pellet was

resuspended in 1 mL of water and transferred to sample tube. The tube was centrifuged at 200 *g* for 10 min to concentrate the cells at the bottom of the tube. The supernatant was removed and the tubes were frozen and stored in liquid nitrogen until use.

Spin quantification was performed with 10 μM Cu(II) EDTA as a standard under nonsaturating conditions. Values obtained by double integration of the signals were divided by a correcting factor, i.e., a function of the principal *g*-values (66).

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Chapter V

Summary and Conclusions

The *vnfA* gene encoding transcriptional regulator of nitrogenase-2 in diazotroph *Azotobacter vinelandii* was found in 1989. The gene analysis suggested that the product of *vnfA* was a member of σ^N-dependent transcriptional regulators and had a unique cysteine-rich motif at the N-terminal (Cys^8-X-Cys^{10}-XXXX-Cys^{15}), which might serve to hold metal ions and/or metal clusters. There had been, however, essentially no structural and functional analysis of VnfA conducted *in vitro* when I started the study on VnfA because of difficulty in overexpressing *vnfA* gene using a recombinant system. Hence my first approach to the VnfA study was the establishment of the recombinant system for the *vnfA* expression.

In Chapter II, I describe successful isolation of VnfA as a recombinant protein using *E. coli*. Spectroscopic and biochemical characterization both *in vivo* and *in vitro* reveal that VnfA takes a tetrameric form and each subunit harbors a 3Fe-4S type cluster as the prosthetic group. The tetrameric configuration is coincident with two dyad upstream activator sequences (5'-GTAC-N6-GAAC-3' and 5'-GTAC-N6-GTAC-3'). The *in vivo* activity assay with *o*-phenanthroline suggests that the Fe-S cluster is required of active VnfA and senses environmental factors to depress the transcription. The characterization of the environmental factors is discussed in Chapter IV.

Chapter III describes functions of domains comprising a VnfA subunit. The early genetic work predicted that the VnfA subunit consisted of the GAF, possible AAA+ and DNA binding domains. In general, GAF is considered to be a sensory domain to modulate activity of transcriptional regulators in response to environmental factors. The AAA+ domain is responsible for interaction of the regulator with the σ^N-RNA polymerase complex. The ATP hydrolysis is another function of the AAA+ domain. The DNA binding domain plays an important role in fixing the regulator at proper position on target DNA sequences.

In the transcriptional activation mechanism by the σ^N-dependent transcriptional regulators, the ATP hydrolysis at the AAA+ domain is coupled with the conversion of double-stranded DNA

(dsDNA) to single-strand (ssDNA), which enables the RNA polymerase to start transcription after the formation of the open complex. In agreement with this mechanism, VnfA shows the ATPase activity at the central domain. The role of the GAF domain in VnfA is to inhibit the ATPase activity and then, repress the transcriptional activity. The inhibitory function of the GAF domain is canceled by the Fe-S cluster bound through the N-terminal cysteine-rich motif.

Chapter IV discusses the environmental factors sensed by the Fe-S cluster in VnfA. The *in vivo* transcription assay suggests that superoxide (O_2^-) and nitric oxide (NO) are presumable environmental factors. Since O_2^- and NO are known to induce inactivation of nitrogenases, VnfA would protect the nitrogenases from the inactivation by O_2^- and NO.

Taken the results obtained in my study and the general transcriptional activation mechanism by the σ^N-dependent transcriptional regulators, I propose the following regulatory mechanism for VnfA (Fig. 1);

1. The DNA binding domain recognizes its specific binding site (5'-GTAC-N6-GAAC-3' and 5'-GTAC-N6-GTAC-3') and binds to it. Independently, σ^N-RNA polymerase also recognizes and binds to the appropriate promoter region whose consensus sequence is TGGCAC-N5-TTGC.
2. VnfA interacts with σ^N-RNA polymerase on the promoter through the central domain. The central domain catalyzes the hydrolysis of ATP, which is coupled with rewinding dsDNA to ssDNA to form the open complex. Then, the RNA polymerase starts the transcription.
3. In order to protect nitrogenase-2 from the inactivation by O_2^- and NO, the Fe-S cluster in the GAF domain of VnfA senses O_2^- and NO as the environmental factors. Upon the sensing, the Fe-S cluster is disrupted to induce the repressive effect of the GAF domain on the ATPase activity of the central domain. The effect results in the suppression of the transcription due to inability of the open complex formation coupled with the ATP hydrolysis.

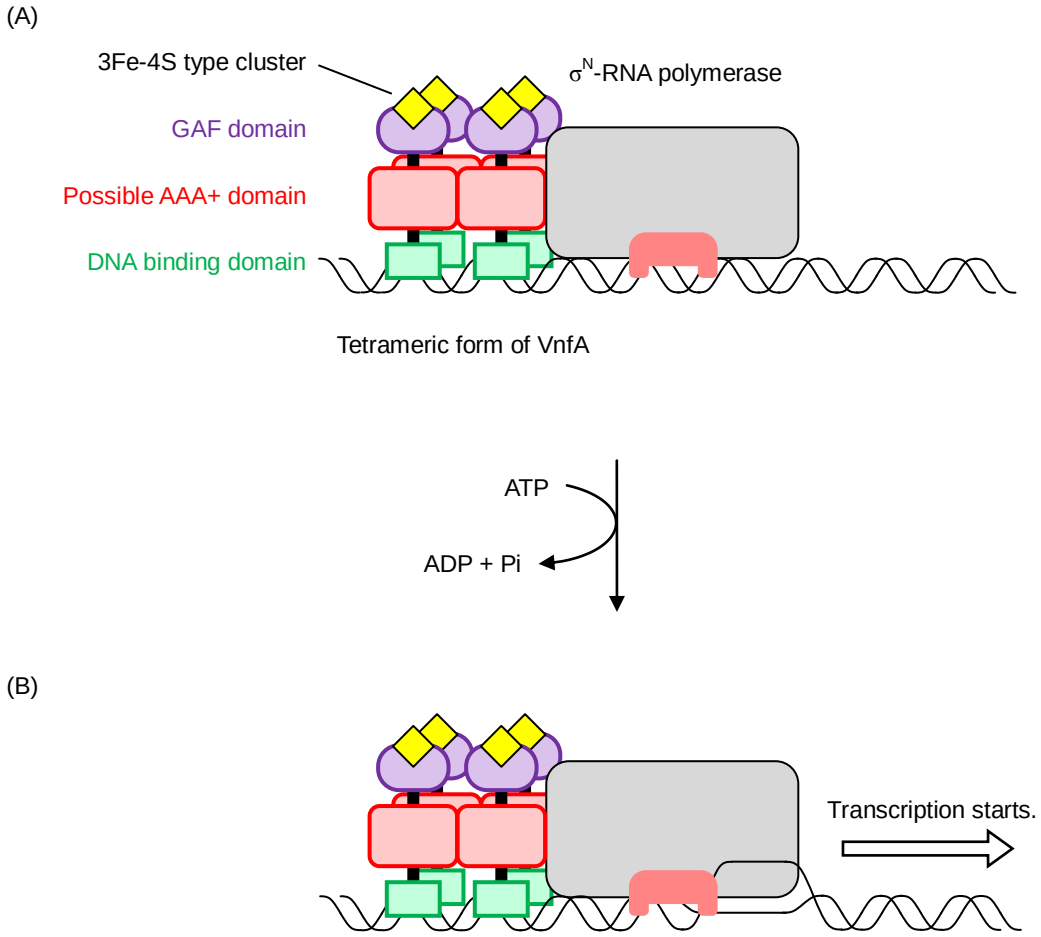

Fig. 1. The proposed transcriptional activation mechanism by VnfA. (A) The σ^N subunit binds to the specific promoter region together with the core RNA polymerase. VnfA also binds to its specific sequences, and then VnfA contacts with the σ^N-RNA polymerase complex through the possible AAA+ domain. (B) The ATP hydrolysis of VnfA is coupled with the open complex formation to initiate the transcription.

The findings in my study must promote studies to understand whole N_2 fixation system further in detail, which contributes to the application of the diazotroph bacteria, particularly *A. vinelandii*, to the artificial ammonium production using the biological system.

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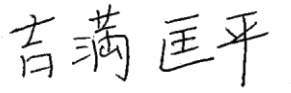

Kyohei Yoshimitsu

List of Publications

1. The role of the Fe-S cluster in the sensory domain of nitrogenase transcriptional activator VnfA from *Azotobacter vinelandii*

   H. Nakajima, N. Takatani, K. Yoshimitsu, M. Itoh, S. Aono, Y. Takahashi and Y. Watanabe (2010) *FEBS J.* **277**, 817-832.

2. The role of the GAF and central domains in VnfA, a transcriptional activator of nitrogenase-2 in *Azotobacter vinelandii*

   K. Yoshimitsu, N. Takatani, Y. Kanematsu, Y. Miura, Y. Watanabe and H. Nakajima

   To be submitted.