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The NifX Protein is Involved in the Final Stages of FeMo-cofactor Transport to the MoFe Protein

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Abstract: The nitrogenase enzyme catalyzes the reduction of dinitrogen to ammonia and is composed of the Fe and MoFe proteins. The iron molybdenum cofactor (FeMo-co) of the MoFe protein is the site of active substrate reduction. The NifX protein has also been suggested to have a role in the FeMo-co synthesis, although its exact role is still open to investigation. We attempted to understand the role of NifX by determining the specific interactions it may have with other Nif proteins involved in FeMo-co synthesis, such as NifD, NifK, NifN, NifDK and NifH. Using the BacterioMatch Two-Hybrid System, a translationally fused construct of NifX with the N-terminal α-RNAP of the pTRG target vector was made and its interaction was tested with the NifDK fusion protein, translationally fused to the λCI of the pBT vector. The strength of the interaction, as determined by measuring the β-galactosidase activity, demonstrated that direct protein-protein interaction exists between NifDK and NifX proteins; the extent of interaction between NifK and NifX proteins was much higher than between NifD and NifX, when individually tested; also, reduced interaction was found between NifH and NifX.

Key words: FeMo-cofactor, NifX, bacteriomatch two-hybrid system, protein-protein interactions

INTRODUCTION

The nitrogenase enzyme converts the atmospheric nitrogen into a bioavailable form, thus serving as a focal point in structural, molecular and biochemical studies[1-3]. The molybdenum containing nitrogenases (Mo-nitrogenases) are the best characterized metalloenzymes and are composed of two oxygen sensitive metalloproteins known as the MoFe protein (NifDK) and the Fe protein (NifH). The MoFe protein contains two types of metal centers, the P-cluster and the iron-molybdenum cofactor (FeMoco)[4]. The FeMoco is the active site of the Mo-nitrogenase and composed of seven Fe, nine S, one Mo, one homocitrate and one atom of unidentified nature[5,6]. It is entirely situated in the NifD subunit which provides one Cys and one His ligand for bonding with the Fe and Mo ends of its metallostructure respectively. Considerable research efforts have been directed towards understanding the biosynthesis and sequential assembly of the FeMoco and it is known that various nitrogen fixation (*nif*) genes play a role in the biosynthesis, assembly, transport and insertion of the FeMo-cofactor[7,8]. The products of the *nifB*, *nifV*, *nifQ*, *nifH*, *nifE*, *nifN*, *nifX* and *nafY* genes have been identified to be the prime players in these processes[9-11].

Two different pathways for the biosynthesis of FeMoco could be pictured: (i) the FeMoco units could be sequentially assembled on the apo-MoFe or (ii) the FeMoco could be separately synthesized and then inserted onto the apo-MoFe protein. However, as established by past experiments, the second pathway proved to be accurate, whereby mutations in either *nifB*, *nifN* or *nifE,* that are involved in the biosynthesis of FeMoco, resulted in very low molybdenum accumulation and a molybdenum-free MoFe protein[12,13]. Thus it was demonstrated that an active FeMoco could be synthesized in the absence of the MoFe protein and that the FeMoco could accumulate on a different protein, such as an intermediate in the normal FeMoco biosynthetic pathway[13]. A significant progress in determining the protein components involved in the biosynthesis of the FeMoco ensued from the development of an *in vitro* assay for FeMoco biosynthesis[14]. This assay utilized a mixture of extracts prepared from mutant strains that presumably had complementary defects in FeMoco biosynthesis, so as to attempt reconstitution of the MoFe protein activity. This application proved immensely useful in recognizing NifH, NifEN and NifV as some of the key players in the FeMoco biosynthetic pathway and helped in the purification of an apo-MoFe protein[13-17]. As understood from research conducted thus far, a stepwise

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overview of the events involved in the biosynthesis and assembly of the FeMoco includes: (i) the formation of an Fe/S core of the FeMoco (designated NifB-co) by NifB[18,19] (ii) transfer of the NifB-co to the $\alpha_2\beta_2$ tetrameric scaffold NifEN protein[20,21] (iii) maturation of the FeMoco and apo-MoFe protein by the combined action of the Fe protein and MgATP[17,22,23] and further processing of the NifB-co on NifEN by an unknown mechanism, forming the completed FeMoco (iv) the final transfer of the fully formed FeMoco to a *nafY*-encoded protein, called χwhich is a FeMoco carrier that aids in the insertion of the FeMoco into the apo-MoFe[24,25]. In addition to these major events, a few other important steps are also involved in the entire process.The NifU and NifS proteins participate in the initial Fe/S mobilization[26,27], the NifQ protein is involved in an early stage of the metal core formation and found to be essential in Mo-limiting conditions[28], the NifV protein contributes the homocitrate component of the FeMoco[29] and the NifX protein probably functions as an 'escort' protein that delivers the FeMoco or its precursors from one assembly site to another or plays a role in specifying the organic acid moiety of FeMoco[30,31].

Although a wealth of data is available to support the functions of the NifU, NifS, NifB, NifEN, NifH and NifV, more research is required to explain the role of the NifX protein. The *nifX* gene is located downstream of the *nifEN* genes, such that *nifENX* are transcribed as a single transcription unit. The NifX protein is comprised of 158 amino acids and is a ~17 kDa protein[32]. The small molecule binding domain (SMBD) of NifX has widespread phyletic distribution and therefore indicates an ancient origin or extensive dissemination by horizontal transfer during evolution, or both[33]. Eventhough a deletion in the *nifX* gene did not show a significant effect on the nitrogenase activity *in vivo*, the purified NifX protein was shown to be important in the stimulation of FeMoco synthesis in an *in vitro* FeMoco synthesis reaction[10,34]. The NifX, NifB, NifY and NafY are most likely related proteins, owing to their overall sequence similarity and the presence of a FeMoco binding 'core domain' in all of them[35]. This conserved family of proteins has a β_1–β_2–β_3–α_1–α_2–β_4–α_3–β_5–α_4–α_5 fold in its core domain, a polarized surface charge distribution and shows considerable similarity with the ribonuclease H family[35]. In NifB, the NIFX SMBD is fused with a biotin-synthase-like, metal-dependent catalytic domain and in the archaeal MTH1172 protein, it is fused to a cation transporter[33]. Previous studies in *Azotobacter vinelandii* have identified certain important molecular interactions of the NifX protein with the other Nif proteins involved in the FeMoco biosynthesis/transport pathway; mainly it was shown that NifX binds to the NifB-co and also a FeMoco precursor from the NifEN complex[10,30].

Clearly, a protein-protein interaction based study of NifX with other FeMoco maturation proteins would enhance our insight into the role of NifX and may help to solve unanswered questions regarding its position in the FeMoco biosynthetic pathway. We therefore studied the interactions of NifX with some of the other relevant proteins participating in FeMoco biosynthesis /assembly/transport /insertion, so as to deduce the entire sequence of events involved. We determined the specific interactions of NifX with NifB, NifH, NifN, NifD, NifK, NifDK (fusion) and NafY using the BacterioMatch Two-Hybrid System™[36,37]. Accordingly, a translationally fused construct of NifX with the N-terminal α-RNAP of the pTRG target vector was made and its interaction was tested with the above proteins, each translationally fused to the λCI of the pBT vector. The strength of the interactions, as determined by measuring the β-galactosidase activity, demonstrated that direct protein-protein interaction exists between (i) NifB and NifX (ii) NafY and NifX and (iii) NifDK (fusion) and NifX (iv) NifK and NifX. The extent of interaction between NifK and NifX proteins was much higher than between NifD and NifX, when individually tested; also, unsubstantial interaction was found between NifH and NifX and NifN and NifX. Our results present a new dimension to the FeMoco biosynthesis pathway with reference to the role played by NifX. We propose that the NifX performs overlapping functions in the FeMoco biosynthetic pathway wherein it may either function as a transporter of the FeMoco precursor to the NafY protein or directly assist in the insertion of the completed FeMoco into the apo-MoFe protein.

MATERIALS AND METHODS

Strains, plasmids and growth conditions: The bacterial strains and plasmids used in this study are described in Table 1. *E. coli* strains were normally grown at 37°C in 2YT media[38]. Ampicillin, chloramphenicol and tetracycline were used to a final concentration of 50, 34 and 5 μg mL^{-1}, respectively, wherever the selection was made.

General molecular techniques: Restriction enzymes were purchased from Promega (Madison, WI). DNA sub-cloning, plasmid DNA isolations, restriction

Table 1: Bacterial Strains and Plasmids used in this study

Strain/plasmid	Relevant characteristics and description	Source/reference
Escherichia coli TOP10	F¯*mcr*A Δ (*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen, CA
Escherichia coli XL-1 Blue	*MRF' K, Δ(mcrA) 183Δ(mcr CB-hsdSMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relAl lac (F' pro AB lacI^q Z Δ M15 Tn5 (Kan)^r]*	Stratagene, CA
PCR 2.1 TOPO	*Amp^r, Kan^r,* (3.9kb), used for direct cloning of PCR products, *lacZα* fragment, *MCS, M13*	Invitrogen, CA
pBT	*Cm^R*, 52 bp *MCS*, 3.2 kb size, *MCS, p15A* origin of replication, *lac-UV5* , λ cl ORF	Stratagene, CA
pTRG	*Tet^R*, 60 bp *MCS*, 4.4 kb size, MCS, *lac-UV5* promoter, *CoE1* origin of replication, *RNAPα ORF*	Stratagene, CA
pBG1866	Derivative of pBT in which 879 bp *nifH* fragment was cloned into *BamHI* site of pBT to generate an in-frame λCI:NifH translation fusion	[39]
pBG1713	Derivative of pBT in which 3062 bp *orf* corresponding to *nifD-K* fusion gene from *XhoI-EcoRV* digested pBG1712 was cloned into *SmaI-XhoI* site of pBT bait vector to generate an in-frame λCI:NifD-K translation fusion	[40]
pBG1716	Derivative of pTRG in which 2335 bp nifKTY fragment was cloned into the *BamHI* site of pTRG to generate an in-frame α-RNAP: NifX translation fusion	[40]
pBG1718	Derivative of pBT in which 2335 bp *nifKTY* fragment released from *BamHI* digested pBG1715 was cloned into *BamHI* site of pBT to generate an in-frame λCI NifKTY translation fusion	[40]
pBG1777	Derivative of PCR 2.1 TOPO in which 1377 bp *nifN* fragment was cloned directly after PCR using appropriate olignonucleotides designed with flanking *EcoRI-BamHI* sites	This study
pBG1778	Derivative of pBT in which 1377 bp *nifN* fragment released from *EcoRI-Bam*HI digested pBG1777 was cloned into the *EcoRI-BamHI* site of pBT to generate an in-frame λCI:NifN translation fusion	This study
pMH5014	Derivative of PCR 2.1 TOPO in which 1-300 bp *nifX* fragment was cloned directly after PCR using appropriate olignonucleotides designed with flanking *BamHI-EcoRI* sites	This study
pBG1786	Derivative of pTRG in which 1-300 bp *nifX* fragment released from *BamHI-EcoRI* digested pMH5014 was cloned into the *BamHI-EcoRI* site of pTRG to generate an in-frame α-RNAP:N1NifX translation fusion	This study
pBG1782	Derivative of PCR 2.1 TOPO in which 150-477 bp *nifX* fragment was cloned directly after PCR using appropriate olignonucleotides designed with flanking *BamHI-EcoRI* sites	This study
pBG1787	Derivative of pTRG in which 150-477 bp *nifX* fragment released from *BamHI-EcoRI* digested pBG1782 was cloned into the *BamHI-EcoRI* site of pTRG to generate an in-frame α-RNAP:C2NifX translation fusion	This study
pMH5000	Derivative of PCR 2.1 TOPO in which 477 bp *nifX* fragment was cloned directly after PCR using appropriate olignonucleotides designed with flanking *BamHI-EcoRI* sites	This study
pMH5002	Derivative of pTRG in which 477 bp *nifX* fragment released from *BamHI-EcoRI* digested pMH5000 was cloned into the *BamHI-EcoRI* site of pTRG to generate an in-frame α-RNAP:NifX translation fusion	This study
pMH6000	Derivative of PCR 2.1 TOPO in which 1509 bp *nifB* fragment was cloned directly after PCR using appropriate olignonucleotides designed with flanking *EcoRI-BamHI* sites	This study
pMH6002	Derivative of pBT in which 1509 bp *nifB* fragment released from *EcoRI-BamHI* digested pMH6000 was cloned into the *EcoRI-BamHI* site of pBT to generate an in-frame λCI:NifB translation fusion	This study
pBG1785	Derivative of PCR 2.1 TOPO in which 732 bp *nafY* fragment was cloned directly after PCR using appropriate olignonucleotides designed with flanking *EcoRI-BamHI* sites	This study
pBG1790	Derivative of pBT in which 732 bp *nafY* fragment released from *EcoRI-BamHI* digested pBG1785 was cloned into the *EcoRI-BamHI* site of pBT to generate an in-frame λCI:NafY translation fusion	This study

enzyme digestions, agarose gel electrophoresis, ligations and *E. coli* transformation were carried out as described in the laboratory manual or according to the manufacturer's protocol[37,38]. Oligonucleotides used for PCR amplification were purchased from Integrated DNA Technologies (Coralville, IA). Following are the primers used to PCR amplify required gene fragments from *A. vinelandii* cells for cloning into pTRG or pBT vectors of the BacterioMatch Two Hybrid System^{TM}: (1) *nifX* (pTRG) (i) 5' GGA TCC ATG TCC AGC CCG ACC GGC 3'. (2) *nifB* (pBT) (i) 5' GAA TTC AGA ACT GAG CGT ACT TGG GCA AAA CAA T 3' and (ii) 5'GGA TCC TCA GGC CTT GGC CTG CAG CAG GGC T 3' (3) *nifN* (pBT) (i) 5'GAA TTC ATG GCC GAG ATC ATC AAT CGC AAC AAG GCC 3' and (ii) 5' GGA TCC TCA GTG CCT CCA TTG CGG CTG TTC GGT TGC CGG 3' (4) *nafY* (pBT) (i) 5'GAA TTC AGT AAC CCC CGT GAA

CAT GAG TCG CGA 3' (ii) 5' GGA TCC TCA TGC CCT GGC CGC CTC GTC CTC GTC 3' (5) First 300 bp of *nifX* encoding N-terminal NifX (pTRG) (i) 5' GGA TCC ATG TCC AGC CCG ACC CGA CAA TTG CAG GTA 3' (ii) 5' GAA TCC GGC CAT CAA CTG GCG CAC CGC CGA GGC GCC 3' (6) Last 324 bp of *nifX* encoding C-terminal NifX (pTRG) (i) 5'GGA TCC CGC TCC CAG CTC CTC TCG GTC GTC GAG TTC 3' (ii) GAA TTC CTA TTC GTC CCA GCC TTC GGC GGC CAT GGC 3'. In all the above oligonucleotides, the underlined sequences represent restriction enzyme recognition sites that were introduced during primer design so as to facilitate cloning of the DNA fragments into unique sites of the pBT or pTRG vectors. 'GGA TCC' is the *BamHI* recognition sequence and 'GAA TTC' is the *EcoRI* recognition sequence. The bacterial two-hybrid vectors pBT bait and pTRG target, containing the λCI and α-RNAP domains, respectively, were the starting plasmids used for cloning the various gene fragments. Each gene fragment was PCR amplified using specific oligonucleotides as listed above and cloned into PCR 2.1 TOPO (Invitrogen, Carlsbad, CA) by ligation and transformation. These plasmids were then digested with *EcoRI* and *BamHI* and the resulting fragments were purified and ligated to the *EcoRI-BamHI* digested pBT or pTRG vectors. The ligated mixtures were used to transform *E. coli* XL1-Blue cells and desired clones were obtained and verified from the subsequent transformants by DNA isolation and restriction digestion analysis. The verified clones were designated as pMH5002 (α-RNAP + NifX), pBG1786 (α-RNAP +N-terminal (N1) NifX), pBG1787 (α-RNAP +C-terminal (C2) NifX), pMH6002 (λCI + NifB), pBG1778 (λCI + NifN) and pBG1790 (λCI + NafY). The construction of the plasmids pBG1716 (α-RNAP + NifK), pBG1718 (λCI + NifK) and pBG1866 (λCI + NifH) has been described elsewhere[39,40]. Subsequently, desired combinations of plasmids (e.g. pTRG-NifX + pBT-NafY) were used for cotransformation of *E. coli* XL1-Blue cells containing the reporter cassette. The resultant cotransformants were inoculated for performing β-galactosidase activity assays.

β-Galactosidase assay: The β-galactosidase activity assay was performed as described under 'Molecular cloning'[38]. Briefly, single *E. coli* transformants were inoculated into 5 mL of 2YT media supplemented with 34 μg mL^{-1} of chloramphenicol and 5 μg mL^{-1} of tetracycline. The cells were then incubated overnight at 37 °C with shaking at 250 rpm. Then 200 μL of the overnight culture was diluted into 5 mL of the same media and incubated at 37 °C with shaking, until OD_{600} was between 0.3 and 0.6 (initial OD_{600} was 0.05). Cells from 1.5 mL of the culture were collected by centrifugation and resuspended in 500 μL Z-buffer[38]. The resuspended cells were lysed by adding 50 μL of chloroform and 25 μL of 0.1% SDS and kept at 28 °C with shaking for 5–10 min. To measure the β-galactosidase activity from the cell lysate, 900 μL of Z-buffer-β-mercaptoethanol solution (0.27 mL of β-mercaptoethanol per 100 mL of Z-buffer) was added to 100 μL culture followed by 200 μL Z-buffer/ONPG (*o*-nitrophenyl-β-D-galactopyranoside—4 mg ONPG per mL of Z-buffer). The time of ONPG addition was recorded and the tubes were incubated at 37 °C. When yellow color was visible, 500 μL of 1 M Na_2CO_3 was added to each tube to terminate the reaction and the time was recorded. The optical densities at 420 nm as well as 550 nm were recorded. The β-galactosidase units were defined as the amount of enzyme which hydrolyzes 1 μmol of ONPG to o-nitrophenol and D-galactose per minute. β-Galactosidase activity in Miller Units was calculated as $1000 \times [OD_{420} - (1.75 \times OD_{550})/(t \times V \times OD_{600})]$, where t is the elapsed time (in min) of incubation, V is the 0.1 mL × concentration factor and OD_{600} is the absorbance of 1 mL of culture at 600 nm.

Structural homology modeling: The Swiss PDB software (freely available online)[41] was used for homology modeling and 3D representation of the derived NifX protein structure. The NafY structure (PDB ID: 1P90) was used as the template for superimposing the raw NifX amino acid sequence and deriving its 3D structure.

RESULTS AND DISCUSSION

Interaction of NifB with NifX. The BacterioMatch™ Two-Hybrid System was used to determine which proteins in the FeMoco maturation pathway directly interacted with NifX. The NifB protein provides a Fe-S precursor, termed NifB-co, for FeMoco assembly[18,19]. Since the *nifB* gene product was found to be essential for the functioning of alternative nitrogenases (Mo-independent) also, therefore it is thought that the NifB-co represents the Fe-S primary cluster for both, the synthesis of FeMoco and the cofactors of alternative nitrogenases[18,42]. It is believed that the NifB-co is transferred from NifB to NifEN, which acts as a site for rearrangement of the primary [Fe-S] to form the [Fe-S] core of the FeMoco, probably after a structural contact between NifEN and

NifH[19]. The NifB protein sequence comprises of two different domains: an N-terminal SAM radical domain and a C-terminal NifX-like domain, that is a domain present in certain FeMoco binding proteins[43]. The presence of the C-terminal NifX-like domain could indicate that this region in NifB is either involved in binding NifB-co precursors and forming the NifB-co or binding a preformed NifB-co for transfer to another protein in the FeMoco biosynthetic pathway[44].

Although it was evident from previous reports that NifX is capable of binding to NifB-co in one of the stages during FeMoco biosynthesis[30], a direct protein-protein interaction between the NifB and NifX proteins had not been demonstrated before. Our results support the idea that there is an interaction between NifB and NifX as found by measuring the β-galactosidase activity of *E. coli* cotransformants harboring the plasmids pMH6002 (λCI:NifB) and pMH5002 (RNAP:NifX). As compared to the β-galactosidase activity obtained from *E. coli* cotransformants harboring only the pBT and pTRG plasmids (negative control--22.93 $\pm$ 6.12 Miller Units), the activity obtained from cells harboring pMH6002 and pMH5002 was much higher (49.7 $\pm$ 9.11 Miller Units), indicating a protein-protein interaction between NifB and NifX (Table 2). Since the NifB and NafY proteins share many similarities between their amino acid sequences and since NafY was shown to bind to the apodinitrogenase[35], we also examined the ability of NifB and NifK to interact with each other and found that the β-galactosidase activity of *E. coli* cotransformants harboring the plasmids pMH6002 (λCI:NifB) and pBG1716 (RNAP:NifK) was 20.47 $\pm$ 4.78 Miller Units, suggesting lack of interaction between NifB and NifK.

Interaction of NafY with NifX: A new family of iron and molybdenum (or vanadium) cluster-binding proteins comprising of NifY, NifX, VnfX and NafY was described by Rubio *et. al* in 2002[25]. The *A. vinelandii* NafY protein (nitrogenase accessory factor Y, also known as γ, was reported to be a molecular chaperone that assisted in maintaining the apodinitrogenase in a conformation that facilitated the insertion of the FeMoco by acting as a metallo-insertase[25]. Mutational analysis of the strains containing mutations in both *nafY* and *nifX* showed that these were severely affected in diazotrophic growth, indicating their physiological importance in the stabilization of the apodinitrogenase[25]. Subsequently, the 3-dimensional structure of the NafY protein from *A. vinelandii* revealed two distinct domains of this protein: an N-terminal (Met1 to Leu98) domain and a C-terminal (Glu99 to Ser232) 'core' domain[35]. The NafY core domain was shown to be capable of binding the FeMoco of nitrogenase but unable to bind to apodinitrogenase in the absence of the N-terminal domain[35].

In a study that involved utilization of ^{99}Mo and a purified *in vitro* FeMo-co biosynthesis system for investigating the incorporation of the ^{99}Mo radiolabel into proteins involved in the biosynthesis of FeMo-co, it was found that there was transfer of the ^{99}Mo label from NifH and NifX to NafY or apodinitrogenase[31]. In light of the earlier experiments, our data strengthens the role of NifX as a transporter of a fully formed FeMoco cluster to NafY. Our work helped in detecting a direct protein-protein interaction between NifX and NafY, as evident from the β-galactosidase activity (42.49 $\pm$ 8.62 Miller Units) of *E. coli* cells harboring the plasmids pMH5002 (RNAP:NifX) and pBG1790 (λCI:NafY) (Table 2). Through earlier studies, it was predicted that the NifX was involved in the latter part of the biosynthetic pathway of FeMoco formation[31]. Our results confirmed the above prediction, because we were able to identify an interaction between NifX with NafY but neither between NifX and NifN or NifX and NifH, both of which are involved in the initial steps of the pathway. The β-galactosidase activity corresponding to the interaction between NifH and NifX was 32.98 $\pm$ 6.67 Miller Units and between NifN and NifX was 31.32 $\pm$ 6.05 Miller Units, indicating absence of any interaction between these pairs of proteins, when compared to the negative and positive controls (22.93 $\pm$ 6.12 and 115.59 $\pm$ 25.01 Miller Units respectively) (Table 2). We also tested the validity of the results obtained through the BacterioMatch™ Two Hybrid system by determining whether the NafY and NifK proteins, that have been reported to associate with each other based on immunoblot analyses in earlier studies[35], showed evidence of interaction with each other by this method. Our results established that a strong interaction exists between the NafY and NifK proteins (55.71 $\pm$ 9.30 Miller Units) and that this bacterial two-hybrid method was indeed reliable for our experimental objectives (Table 2).

Interaction of NifDK (fusion) and NifK with NifX: A role for NifX in the insertion process of the FeMoco into apodinitrogenase has been suggested earlier[31]. In that study, the *in vitro* FeMoco biosynthesis assay was performed and the incorporation of ^{99}Mo radiolabel into apodinitrogenase was examined in reactions including and excluding NifX. It was found that the minus NifX

Table 2: Results of the liquid β-galactosidase assay with ONPG as substrate to demonstrate protein-protein interaction

Plasmid to which λCI was translationally fused	Plasmid to which α-RNAP was translationally fused	β-Galactosidase activity (Miller units)[a]	Interacting Peptides
pBG1713 (λCI: NifD-K fusion)	pMH5002 (RNAP: NifX)	45.24 ± 8.72	NifD-K and Nif X
pMH5007 (λCI: NifD)	pMH5002 (RNAP: NifX)	36.95 ± 3.74	None
pBG1718 (λCI: NifK)	pMH5002 (RNAP: NifX)	46.34 ± 8.81	NifK and NifX
pBG1718 (λCI: NifK)	pBG1786 (RNAP:N1NifX)	33.93 ± 8.74	None
pBG1718 (λCI: NifK)	pBG1787 (RNAP:C2NifX)	35.50 ± 7.81	None
pBG1778 (λCI: NifN)	pMH5002 (RNAP: NifX)	31.32 ± 6.05	None
PBG1866 (λCI: NifH)	pMH5002 (RNAP: NifX)	32.98 ± 6.67	None
pMH6002 (λCI: NifB)	pMH5002 (RNAP: NifX)	49.7 ± 9.11	NifB and NifX
pMH6002 (λCI: NifB)	pBG1716 (RNAP: NifK)	20.47 ± 4.78	None
pBG1790 (λCI: NafY)	pMH5002 (RNAP: NifX)	42.49 ± 8.62	NafY and NifX
pBG1790 (λCI: NafY)	pBG1716 (RNAP: NifK)	55.71 ± 9.30	NafY and NifK
pBT	pMH5002 (RNAP: NifX)	29.87 ± 1.63	None
pBT	PTRG	22.93 ± 6.12	None
pBT-LGF2	pTRG-Gal11^{P}	115.59 ± 25.01	Mutant form of Gal1 protein with Gal11^{P}

[a]Each assay was performed a minimum of four times for accuracy and the β-galactosidase activity units shown are an average of three independent observations.

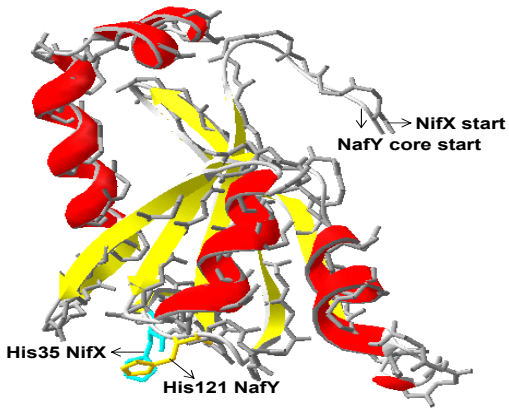

Fig. 1: Homology modeling of NifX using NafY core structure. The NafY core domain structure (PDB ID: 1P90) was used as a template for homology modeling of NifX. The NafY structure is represented in ribbons and the NifX structure in backbone. The NafY core domain start (Glu99) and NifX start (Met 1) are indicated by arrows. The His121 ligand of NafY that is known to be important for FeMoco binding (11) is shown to coincide with the His35 residue of NifX

reaction showed lower levels of ^{99}Mo label incorporated into dinitrogenase in comparison to the NifX containing reaction[31]. Evidence obtained through our two-hybrid data clearly indicates the presence of protein-protein interaction between the NifDK protein encoding a single fused unit of dinitrogenase[45] and NifX and between the NifK component of the dinitrogenase and NifX. To further analyze if the NifX protein possessed specific or separate domains that helped in its interaction with 300 bp of *nifX*, encoding the N-terminal half of the NifX protein (N1X) and the region spanning 216-477 bp of *nifX*, encoding the C-terminal half of NifX fusion) and pMH5002 (α-RNAP:NifX) was 45.24 ± 8.72 Miller Units and that of pBG1718 (λCI: NifK) and pMH5002 (α-RNAP:NifX) was 46.34 ± 8.81. The β-galactosidase activity of the positive control that included cotransformed plasmids encoding two known interacting proteins – a mutant form of Gal1 protein and the Gal11^{P}, fused to λCI of pBT and α-RNAP of pTRG respectively, was 115.59 $\pm$ 25.01 Miller Units and value for the negative control that included cotransformed plasmids pBT and pTRG only was 22.93 $\pm$ 6.12 Miller Units. The β-galactosidase activity corresponding to interaction between NifD and NifX was 36.95 ± 3.74 Miller Units, suggesting that there was much lesser or no interaction between the two proteins (Table 2). To further analyze if the NifX protein possessed specific or separate domains that helped in its interaction with 300 bp of *nifX*, encoding the N-terminal half of the NifX protein (N1X) and the region spanning 216-477 bp of *nifX*, encoding the C-terminal half of NifX the NifK protein, we cloned the region spanning 1-300 bp of *nifX*, encoding the N-terminal half of the NifX protein (N1X) and the region spanning 216-477 bp of *nifX*, encoding the C-terminal half of NifX (C2X), into the pTRG plasmid. The β-galactosidase activity indicating the extent of interaction between NifK and NifN1X was 33.93 ± 8.74 Miller Units and between NifK and NifC2X was 35.50 ± 7.81 Miller Units (Table 2). Thus, the N-terminal and C-terminal NifX domains were unable to retain the ability to interact with the NifK protein individually, indicating that either the entire NifX protein is essential for interaction with NifK or a domain comprising an overlapping region from the N-

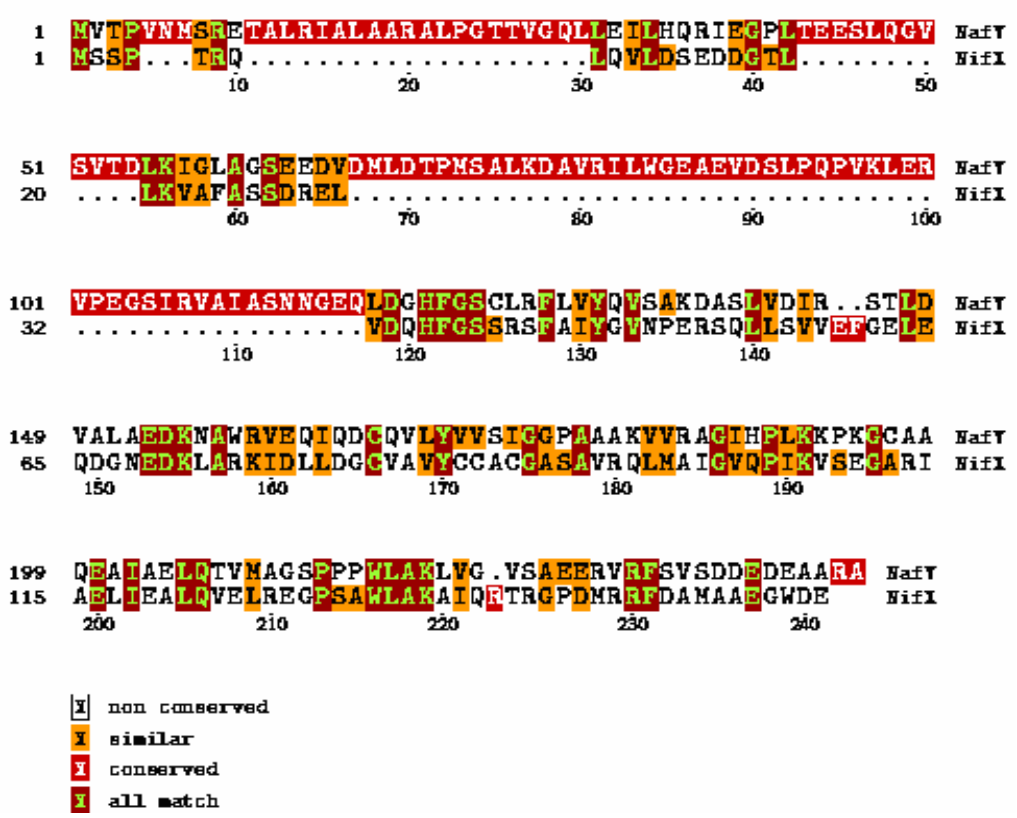

Fig. 2: ClustalW alignment of NifX and NafY protein sequences from *A. vinelandii*

terminal and C-terminal domains contains the necessary stretch of residues for interacting with NifK.

The NifX family of proteins comprises of proteins like NifB, NifY, NafY, VnfX and NifX and is based on the presence of a FeMoco-cluster binding domain. As seen in Fig. 1., the NifX protein is largely similar to the C-terminal domain of the NafY protein. The C-terminal NafY protein known as the 'core' domain, was shown to be capable of binding to the FeMoco but unable to bind to the apodinitrogenase in the absence of the first domain, indicating that the N-terminal region of NafY was important in interacting with dinitrogenase[35]. Our studies have demonstrated that the NifX is capable of interacting with the NifK protein. The fact that the NifX does not have a domain corresponding to the N-terminal region of NafY and yet is able to interact with NifK could be explained as a possible conservation of a few residues at the beginning of the NifX protein that are important in maintaining an interaction with NifK (Fig. 2). It could also be that whereas NafY interacts with a different region of the dinitrogenase protein, the NifX acts upon yet another separate region of the same protein and therefore the NifX protein which could actually be considered as only the C-terminal half of NafY, may be involved in the dual role of FeMoco binding and dinitrogenase binding but awaits specific evidence regarding the residues involved.

Through this work, utilizing a simple two-hybrid approach, we have been able to reveal certain important interactions between the NifX protein and some other proteins related to the FeMoco biosynthetic pathway of nitrogenase. Mainly, the following interactions were found: (i) NifB and NifX (ii) NafY and NifX (iii) NifDK(fusion) and NifX (iv) NifK and NifX. Further protein-protein interaction based studies involving NifU, NifS, NifQ and NifV with NifX would certainly prove useful in better deciphering the role of NifX and the events of the FeMoco biosynthetic pathway as a whole. However, in light of our current findings and several previous insights into the FeMoco biosynthetic pathway, we propose a model that best explains the order of events that occurs during the FeMoco biosynthesis, assembly and insertion. It should be noted that there exist several different views of the exact

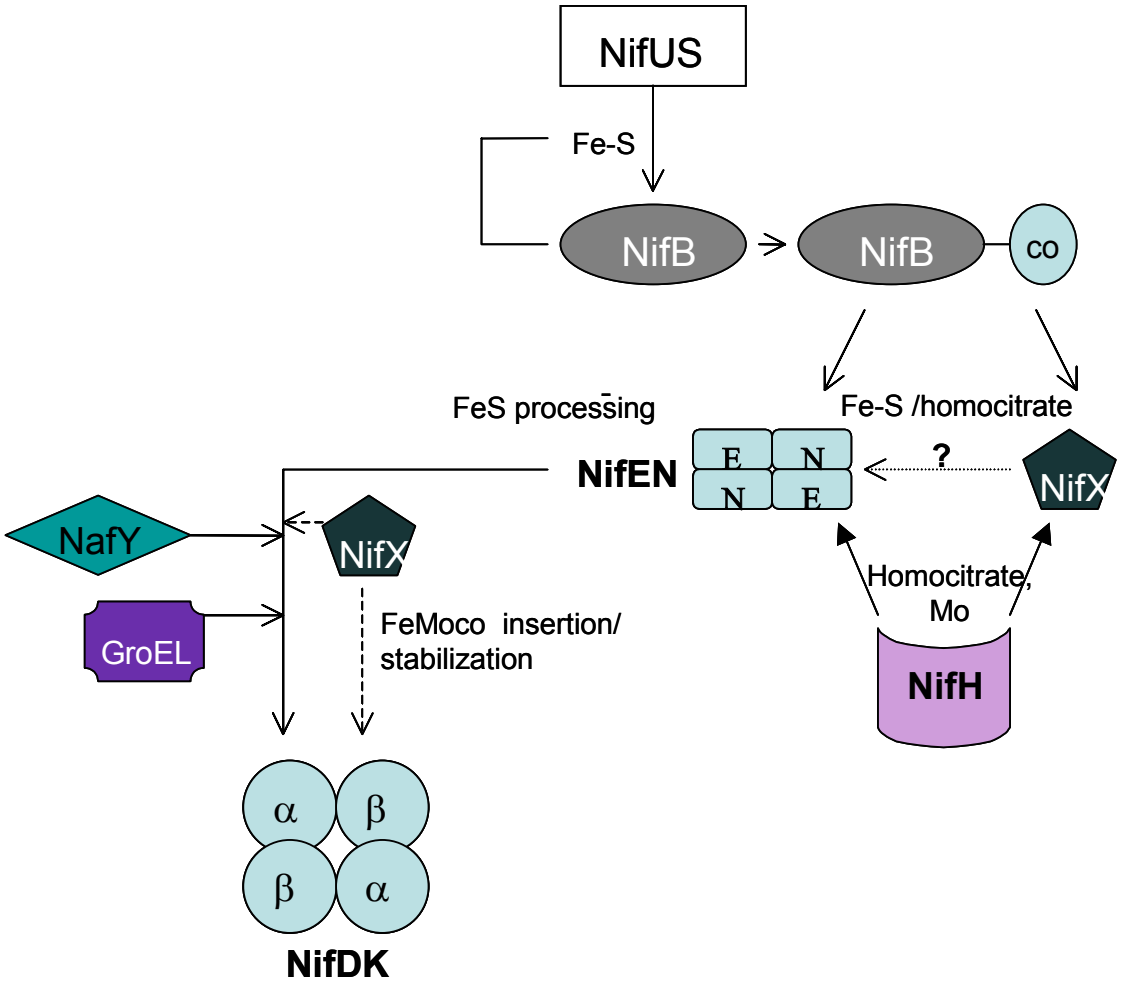

Fig. 3: Proposed model for the role of NifX in FeMoco biosynthesis/insertion pathway. The NifU and NifS proteins participate in the initial Fe/S mobilization; this precursor Fe-S moiety is then transferred to the NifB protein, which is then termed as NifB-co. The NifX protein may play a role as a molybdenum (Mo) carrier from the NifB to the NifEN complex or it may provide the homocitrate moiety to NifEN. This processed FeMoco is inserted and stabilized in the NifDK (MoFe) protein and this activity is assisted by NifX, NafY and GroEL

sequence of events in the FeMoco biosynthetic pathway due to the complexity of the system and possible redundant functions of the involved proteins, causing difficulty in assigning confirmed roles. As seen in our suggested model in Fig. 3, the FeMoco biosynthetic pathway starts with the initial mobilization and assembly of the Fe-S fragments and is carried out by the NifUS complex[26]; This Fe-S fragment is then delivered to NifB and a Fe-S core is formed on it[18]. Following this, the core is transferred from NifB to the NifEN complex. Based on previous studies and the detection of an interaction between NifB and NifX in our study, it could be possible that NifX facilitates the transfer of the core from NifB to NifEN[30]. It is possible that the addition of homocitrate takes place at this step, since one previous study has shown that the NifX may be involved in specifying the organic acid moiety of the FeMoco precursor[31]. Following this, multiple FeMoco processing steps take place on the NifEN complex, which acts as a scaffold for this purpose[7,8]. Very recently, Corbett *et. al* showed through x-ray absorption spectroscopy studies that the first isolatable FeMoco precursor on NifEN was a molybdenum-free analog of FeMoco and that the molybdenum was probably incorporated afterwards at the position previously occupied by the non-cysteine ligated terminal iron atom[46]. As found in other recent studies, the NifH may perform the role of facilitating the insertion of Mo and homocitrate into the FeMoco, probably after MgATP hydrolysis[47]. The GroEL

protein is also required for the final assembly of the MoFe protein; It functions as a molecular chaperone for insertion of FeMoco into the MoFe protein[48]. It could be possible that the MgATP hydrolysis is actually necessary for the function of GroEL in the final assembly reaction and has no significant link with the NifH protein[48]. As found in our studies, the interaction of NifX with NafY indicates that the NifX may again function as an escort protein at this step and participate in the transfer of the intermediate or completed FeMoco cluster to the NafY protein. In the following steps, the assembled FeMoco that has been delivered to the NafY protein is carried to the apo-dinitrogenase. It has been suggested that the NafY protein dissociates from the MoFe protein after the final insertion process in completed. Since we have detected an interaction between the NifX and NifK proteins in this work, we propose that the NifX may perform a similar role to that of NafY and the final insertion of the FeMoco into the apo-dinitrogenase may involve the participation of NifX. It may also be possible that the NifX stabilizes the apo-dinitrogenase in a conformation suitable for FeMoco insertion through its interaction with NifK. There have been earlier speculations concerning the overlapping or redundant roles of the proteins involved in the FeMoco biosynthetic pathway, for example, the role of NifY was found to be similar to that of NafY in studies involving the FeMoco biosynthetic pathway in *K. pneumoniae* and *A. vinelandii*[49]. However, this occurrence has not been yet explainable and future studies that investigate the FeMoco biosynthetic pathway in detail may lead to meaningful answers.

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