Functional Expression of a Fusion-dimeric MoFe Protein of Nitrogenase in Azotobacter vinelandii*

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The MoFe protein component of the complex metalloenzyme nitrogenase is an $\alpha_2\beta_2$ tetramer encoded by the nifD and the nifK genes. In nitrogen fixing organisms, the $\alpha$ and $\beta$ subunits are translated as separate polypeptides and then assembled into tetrameric MoFe protein complex that includes two types of metal centers, the P cluster and the FeMo cofactor. In Azotobacter vinelandii, the NifEN complex, the site for biosynthesis of the FeMo cofactor, is an $\alpha_2\beta_2$ tetramer that is structurally similar to the MoFe protein and encoded as two separate polypeptides by the nifE and the nifN genes. In Anabaena variabilis it was shown that a NifE-N fusion protein encoded by translationally fused nifE and nifN genes can support biological nitrogen fixation. The structural similarity between the MoFe protein and the NifEN complex prompted us to test whether the MoFe protein could also be functional when synthesized as a single protein encoded by nifD-K translational fusion. Here we report that the NiFD-K fusion protein encoded by nifD-K translational fusion in A. vinelandii is a large protein (as determined by Western blot analysis) and is capable of supporting biological nitrogen fixation. These results imply that the MoFe protein is flexible in that it can accommodate major structural changes and remain functional.

The nitrogenase enzyme, which is responsible for conversion of atmospheric nitrogen to ammonia, is found in all diazotrophs. It is actually composed of two separately purified, oxygen-labile, metalloproteins designated the Fe protein and the MoFe protein (1–7). The Fe protein is a homodimer of two identical subunits encoded by the nifH. Both subunits are bridged by one 4Fe-4S metal center and contain two nucleotide (MgATP or MgADP)-binding sites (5, 8–10). The Fe protein serves as the obligate electron donor to MoFe protein during catalysis in a MgATP- and reductant-dependent process (11, 12). The MoFe protein exists as an $\alpha_2\beta_2$ tetramer of about 240 kDa in size, encoded by nifD and nifK genes, respectively (13, 14). Each $\alpha$ heterodimer subunit binds two unique metal clusters, the FeMo cofactor and the P cluster. The FeMo cofactor that serves as the site of dinitrogen binding and reduction by the enzyme is located in the $\alpha$ subunit, and the P cluster, which is positioned at the interface of $\alpha$ and $\beta$ subunits of the heterodimer, is believed to mediate electron transfer from the Fe protein to the FeMo cofactor (15–17). During catalysis, the Fe protein forms a complex with the MoFe protein and transfers one electron to the MoFe protein with concomitant hydrolysis of two ATPs per Fe-MoFe protein interaction. It is accepted that the electron flow is from the Fe protein cluster to the MoFe protein P cluster and then to the FeMo cofactor, which is the substrate-binding and reduction site (13, 18–20).

Molecular evolutionary history of the nifDK, based on comparative analysis of the amino acid sequence of NifD, NifK, NifE, and NifN peptides, suggested that the genes encoding the NifDK and NifEN constitute a novel paralogous gene family (21). The NifEN complex, which is required for the biosynthesis of the FeMo cofactor of nitrogenase, is structurally analogous to the MoFe protein and provides an assembly site for FeMo cofactor biosynthesis (22, 23). Like the MoFe protein, the NifEN complex is also an $\alpha_2\beta_2$ heterotetramer of about 200 kDa in size and contains unique metal clusters that are similar to the P cluster of the MoFe protein. According to the model proposed by Goodwin et al. (24), each NifEN complex (4Fe-4S) cluster is bridged between a NifE-N heterodimer subunit interface at a position analogous to that occupied by the P clusters of the MoFe protein. The NiFD is homologous to the NifE, and NifK is homologous to the NifN as shown in Fig. 1. In fact homology modeling studies showed that the predicted structure of the NifE is very similar to the crystallographic model of NifD, and the predicted structure of the NifN is very similar to the crystallographic model of NifK (data not shown). The organization of these genes (nifD followed by nifK and nifE followed by nifN) is also conserved among diazotrophs (21, 25). However, in Anabaena variabilis the NifEN complex is encoded by a fused nifE-N like gene instead of two separate nifE and nifN genes (26). In A. variabilis there are two nif clusters, the nif1 and the nif2. Whereas the nif1 cluster carries two separate nifE and nifN genes, the nif2 cluster has the nifE-N gene fusion. A comparison of the nucleotide sequences of the naturally fused NifE-N complex to that of the NifEN complex synthesized by translating two separate genes in this organism showed that nifE-N junction of the translationally fused nifE-N was different from the junction of the nifE and nifN genes of the nif1 cluster. This difference resulted in changes in the amino acid sequence of NifE-N fusion protein encoded by the nif2 cluster when compared with the NifE and the NifN encoded by the nif1 cluster. In Azotobacter vinelandii, the NifEN complex is encoded by the nifE and nifN genes that are translated separately. Comparison of the amino acid sequences of the NifEN complexes from A. variabilis to that of A. vinelandii showed that the NifEN complex encoded by the nif1 cluster of A. variabilis is very similar to that of the A. vinelandii. The observation that in A. variabilis the NifE-N fusion protein could function in nitrogen fixation in a comparable manner to that of the wild type NifEN complex implied that this complex

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The metalloprotein is flexible and could accommodate major structural changes and differences in the pattern of biosynthesis and assembly (being translated as a single protein versus being translated as two separate proteins and then assembled as a heterodimer subunit) as long as its catalytic center is not affected seriously by the changes. Because the MoFe protein shares structural similarity with the NifEN complex in *A. vinelandii*, we were interested in testing whether the MoFe protein could be synthesized as a fusion protein by translationally fusing the *nifD* and *nifK* genes. To test this idea, we generated an *A. vinelandii* strain that encodes NifD-K fusion protein encoded by translationally fused *nifD* and *nifK* genes. Here we report the structural and functional properties of the NifD-K fusion protein.

**MATERIALS AND METHODS**

*Bacterial Strains and Growth Conditions*—*A. vinelandii* strains were grown at 30 °C in modified Burk’s nitrogen-free (BN/H11002) medium whenever nitrogen-deficient medium was needed, and when nitrogen was required BN− medium supplemented with 400 μg/ml of ammonium acetate was used. The *Escherichia coli* strains were grown at 37 °C in Luria broth or 2YT (28). Ampicillin and kanamycin were used to a final concentration of 50 and 20 μg/ml, respectively, wherever the selection was made. The bacterial strains and plasmids used in this study are listed in Table I.

**Construction of Plasmids Carrying *A. vinelandii* nifD-K Gene Fusion**—The strategy used for the construction of plasmids carrying *A. vinelandii* nifD-K gene fusion is shown in Fig. 2. Initially we constructed the plasmid pBG1404 that carries the *A. vinelandii* nifD-K gene fusion as shown in step 1. The chromosome of the wild type *A. vinelandii* was isolated and was used as template to amplify the *nifD* and the *nifK* genes by PCR amplification (29). The primers used to amplify the *nifD* gene were D5-oligo and BAMD3-oligo (Fig. 2, step 1).

The D5-oligo, 5'-GACCGGTATGTCGCGCGAAGAGGTTGAAT-3', was the 5'-primer starting from the 3rd nucleotide of the *nifD* gene. The BAMD3-oligo, 5'-GGATCCGATTGCTCTGCGATCCGGCGCTGGCGGC-3', was the 3'-primer and contained a BamHI site. This sequence corresponded to the region of *nifD* that spans the codons 488 to the end of the ORF1 and another 21 nucleotides. The two stop codons that follow the *nifD* ORF were changed to sense codons (TGA to GGA and TAG to TCG), and the remaining 15 nucleotides corresponded to five sense codons (CAG, AGC, AAT, CGC, and AGC). Thus, the DNA fragment amplified by using these primers corresponded to region of the NifD from the second codon to the end of the open reading frame, and an extra seven sense codon extending the ORF. Creating a BamHI site at

1 The abbreviation used is: ORF, open reading frame.
## TABLE I

| Strain or plasmid | Relevant characteristics and description | Source or Ref. |
|-------------------|------------------------------------------|---------------|
| *E. coli* INVaF’ | F’ endA1 recA1 hsdR17 (rK- mK-) x2F- supE44 thi-1 gyr96 relA1 d(lacZΔM15 del(lacZYA-argF)U169 deorK) | Invitrogen |
| *A. vinelandii* Trans | Wild type, NifE’, soil bacterium | Laboratory stock |
| *A. vinelandii* BG1065 | Nif’; nifDK::kan (nifD/k-anamycin-nif/K) kanamycin-resistant, generated by integration on kan cassette via nifDK homology using the plasmid pBG1021 | This work |
| *A. vinelandii* BG1304 pCR/2.1 | Nif’, defined nifD/K fusion, generated by using pBG1404 into *A. vinelandii* BG1065 | This work |
| *A. vinelandii* BG1404 | NifK, nifDK, and the following seven codons were omitted) to the end of the ORF. | Invitrogen |
| pUC19 | BamHI fragment for creating the translational fusion of NifK | This work |
| pBG1021 | Derivative of pUC19 in which a 157-bp of HindIII-Smal DNA fragment corresponding to the site region of the nifD gene, a 1252-bp of HindIII DNA fragment corresponding to the kanamycin cassette, and 806 bp of *Smal*-EcoRI DNA fragment corresponding to the nifK region, was ligated. | This work |
| pBG1285 | Derivative of pCR/2.1 in which a 1496-bp DNA fragment that encodes the NifD open reading frame from *A. vinelandii* Trans was cloned. This fragment was generated by PCR amplification using the 5’ primer starting from the 3rd nucleotide of nifD and 3’ primer containing two site-directed mutagenesis to remove the two stop codons. | This work |
| pBG1279 | Derivative of pCR/2.1 in which a 1546-bp DNA fragment that encodes the NifK open reading frame from *A. vinelandii* Trans was cloned. This fragment was generated by PCR amplification using the 5’ primer containing BamHI site on DNA corresponding to the 3’ end of nifD. | This work |
| pBG1404 | Derivative of pCR/2.1 in which a 1706-bp DNA fragment that encodes the NifK open reading frame from *A. vinelandii* Trans was cloned. This fragment was generated by PCR amplification using the 5’ primer containing BamHI site on DNA corresponding to the 3’ end of nifD. | This work |
| pBG1404 | Derivative of pCR/2.1 in which a 1510 bp of EcoRV-BamHI DNA fragment from pBG1285 that corresponding to the nifD gene and 1552 bp of BamHI-EcoRI DNA fragment from pBG1279 that corresponding to the nifK gene was ligated. As a result this plasmid has a unique BamHI site at the nifK gene. | This work |

### RESULTS AND DISCUSSION

Transformation of *A. vinelandii*, Growth Curve, and Western Blotting—Because the plasmids pBG1404 and pBG1021 are derivatives of the pUC18, they are not capable of replicating in *A. vinelandii*. However, the very high recombination efficiency of *A. vinelandii* allows rescuing of the regions that are homologous to the chromosome from non-replicative plasmids. Therefore, these plasmids could be used to deliver the nifD-K fusion or the disrupted nifD-K (due to the insertion of the kanamycin resistance gene) to the chromosome of wild type *A. vinelandii* via homologous recombination. The transformation of *A. vinelandii* Trans was carried out as described previously (31–33).

The resulting transformants were selected either on BN medium containing 5 μg/ml kanamycin or on BN– medium. Growth characteristics of the transformants were determined by growth curve analysis as described previously (34). ECL Western blotting analysis system (Amersham Biosciences) was used to determine the presence of the NifD-K fusion protein in the cell lysates of *A. vinelandii* strains carrying the wild type nifDK operon and the nifD-K translational fusion. Anti-MoFe protein antibody was a gift from Prof. Barbara K. Burgess, Department of Molecular Biology and Biochemistry, University of California, Irvine.

It was shown previously that the NifE-N fusion protein in *A. variabilis* is functional in biological nitrogen fixation (26, 34). As mentioned above, the NifEN complex is a metalloprotein that resembles the MoFe protein in many aspects. For example, both the MoFe protein and the EN complex are seen as 2β2 tetramers. It was also shown that the NifEN has two identical [4Fe-4S]5+ clusters similar to the P clusters found in the MoFe protein. The extent of amino acid sequence homology between the components of these two metalloproteins (between NifD and NifK as well as between NifK and NifN) is also significant (Fig. 1). The observation that the NifE-N fusion protein could support biological nitrogen fixation as efficiently as the wild type NifEN complex implies that this metalloprotein could accommodate major structural changes and differences in the pattern of their biosynthesis (either translated as
homologous recombination with identical regions of the nifD and nifK on the A. vinelandii chromosome and cause rescuing of the nifD-K gene fusion or the kanamycin resistance gene onto the chromosome.

Construction of the A. vinelandii strain BG1065 in which the kanamycin resistance gene interrupted the nifDK expression on the chromosome was described previously (30). We observed that the crude extracts from A. vinelandii strain BG1065 contained only the NifD, when these extracts were subjected to SDS-PAGE, Western blotting, and probing with anti-MoFe protein antibody. This strain could grow on BN^- agar containing 5 μg/ml kanamycin (Fig. 4) but could not grow on BN^- agar, because this strain was unable to produce any functional MoFe protein (Fig. 4). This strain was then transformed with pBG1404 that carries the uninterrupted nifD-K gene fusion. The resulting transformants were selected on BN^- agar. These transformants could grow on BN^- agar only when the nifD-K translational fusion junction disrupted by the kanamycin resistance gene present on the chromosome of the A. vinelandii strain BG1065 was replaced by the nifD-K gene fusion junction present on the pBG1404. Homologous recombination that would lead to this event would also cause the loss of the kanamycin resistance. One of these transformants that could grow on BN^- agar, but was unable to grow on BN^- agar containing 5 μg/ml kanamycin, was designated A. vinelandii strain BG1304 (Fig. 4). This result confirmed that the nifD-K gene fusion junction present on the pBG1404 was rescued on to the chromosome of A. vinelandii strain BG1304 by homologous recombination and also that the kanamycin resistance gene was lost during this process. Nucleotide sequence of the nifD-K junction present on the chromosome of A. vinelandii strain BG1304 was further verified by PCR amplification and nucleotide sequencing of the DNA specifying nifD-K junction. The sequence of the nifD-K junction present on the chromosome of A. vinelandii BG1304 is shown in Fig. 5.

The NifD-K Fusion Could Support Biological Nitrogen Fixation—The observation that the A. vinelandii strain BG1064 could grow on BN^- agar plates indicated that the MoFe protein encoded by the nifD-K translational fusion is functional in biological nitrogen fixation. To analyze further whether the MoFe protein encoded by the nifD-K gene fusion is comparable with the wild type MoFe protein in its ability to participate in biological nitrogen fixation, we compared the growth rates of A. vinelandii Trans (which expresses the wild type MoFe protein encoded by separate nifD and nifK genes) with that of the A. vinelandii strain BG1304 (which expresses the MoFe protein encoded by the nifD-K gene fusion) on nitrogen-deficient medium. Single colonies from each strain were inoculated into BN^-medium, and growth rate was assessed using a Klett-Summer Colorimeter (Klett Manufacturing Co., Inc., New York) as described previously. These experimental results showed that compared with A. vinelandii Trans, A. vinelandii strain BG1304 was a slow-growing strain on nitrogen-deficient medium (Fig. 6). These results suggested that although the MoFe protein encoded by the nifD-K gene fusion junction is capable of supporting the biological nitrogen fixation, its efficiency is not as good as that of the wild type MoFe protein.

The MoFe Protein of A. vinelandii strain BG1304 Is a 120-kDa Protein—SDS-PAGE analysis followed by Western blotting and probing with anti-MoFe protein-antibody was employed to determine the molecular size of the MoFe protein components encoded by the A. vinelandii strain BG1304. The wild type MoFe protein is made of two separately purified proteins, the α subunit or the NifD with a molecular mass of 55 kDa and the β subunit or the nifK with a molecular mass of 59 kDa. When the nifD and nifK were fused translationally (as in A. vinelandii
FIG. 3. Structural changes at the translationally fused junction of ORFs of nifD and nifK in the plasmid pBG1404. NifDK1 represents the junction in the chromosome of wild type A. vinelandii where nifD and nifK are translated separately. NifD-K represents the junction of translationally fused nifD and nifK present in plasmid pBG1404. Asterisk represents the stop codon of nifD on the chromosome. The following M is the translation initiation codon for nifK. The dashes correspond to the missing amino acids when nifD and nifK were translationally fused by ligating the BamHI ends of the DNA fragments specifying the nifD and the nifK. During this construction, the first eight amino acids of the NifK were removed, and the ORF of NifD was extended by seven amino acids by converting the stop codons to sense codons. These changes combined with loss of nucleotides during the ligation of the BamHI ends of the DNA fragments specifying the nifD and the nifK, and two nucleotide changes that happened during the PCR amplification, resulted in a total loss of three amino acids and seven mismatches as shown in the figure.

FIG. 4. Translationally fused NifD-K can support growth of A. vinelandii on nitrogen deficient medium. The chromosome of A. vinelandii BG1065 carries the kanamycin resistance gene that disrupts synthesis of the MoFe protein. This strain can grow on BN+ agar supplemented with kanamycin but cannot grow on nitrogen-deficient BN− agar. Neither A. vinelandii Trans (wild type) nor A. vinelandii BG1304 that carries the translationally fused nifD-K are able to grow on BN− agar supplemented with kanamycin because they do not have kanamycin resistance gene. However, both strains are capable of growing on BN− agar. These results indicated that the A. vinelandii BG1304, a derivative of A. vinelandii BG1065, had lost the kanamycin marker that was originally present on the chromosome of A. vinelandii BG1065 during homologous recombination between the nifD and nifK regions on the chromosome and the plasmid pBG1404 and gained the nifD-K gene fusion.

FIG. 5. Nucleotide sequence of the junction of the translationally fused nifD and nifK genes in the chromosome of A. vinelandii BG1304. PCR amplification of the nifD-K junction present on the chromosome of A. vinelandii BG1304 was carried out using internal primers corresponding to middle regions of the nifD and NifK. Nucleotide sequence was determined using dideoxy nucleotide sequencing technique. The location of C-terminal region of the nifD and N-terminal region of the nifK is labeled. The BamHI site at the junction of the NifD-K fusion is shown in b0ldface.

Using anti-MoFe antibody as probe. In the sample A. vinelandii BG1304, a band corresponding to the molecular mass of about 120 kDa was visible suggesting that the MoFe protein of this strain is composed of translationally fused NifD and NifK proteins. This is the new 120-kDa protein band that was also found in the SDS-polyacrylamide gel lane carrying the sample from A. vinelandii BG1304 after Coomassie Blue staining (Fig. 7A). The Western blotting analysis using anti-MoFe antibody as probe showed a band of ~60 kDa that corresponded to the NifD and NifK proteins (which could co-migrate on the gel under these experimental conditions) in the lane containing the sample from of A. vinelandii Trans. This was expected because the NifD and NifK are synthesized separately in this strain. Taken together, these results confirmed that the MoFe protein of A. vinelandii BG1304 is indeed a NifD-K fusion protein.

Next we verified whether the nifD-K gene fusion was integrated downstream to the nifH promoter in such a way that the
Fig. 6. The *A. vinelandii* BG1304 is a slow growing strain on nitrogen-deficient medium. The growth rate of the *A. vinelandii* (AV) BG1304 was compared with that of the wild type strain *A. vinelandii* AV Trans and also with the *A. vinelandii* BG1065 which the MoFe protein synthesis is disrupted by the insertion of a kanamycin resistance gene. All the strains were grown from single colonies, and the medium used was nitrogen-deficient BN- liquid medium. The *A. vinelandii* BG1065 did not grow on BN- liquid medium. *A. vinelandii* BG1304 was capable of growing on BN- liquid medium, although it took longer time to reach stationary phase suggesting that its growth rate was slower than that of the *A. vinelandii* AV Trans.

![Image](image_url)

**FIG. 6.** The MoFe protein synthesized by translationally fused nifD-K in *A. vinelandii* BG1304 is a large protein. A shows the Coomassie Blue-stained SDS-polyacrylamide gel. Each lane contained 0.5 mg of total protein. A band corresponding to 120 kDa is visible in the lane 2, which was loaded with cell lysate from *A. vinelandii* BG1304. This molecular size corresponds to the size of translationally fused NifD-K. This band is missing in the lane 1, which was loaded with cell lysate from *A. vinelandii* AV Trans. B shows the results of Western blot analysis after being probed with anti-MoFe antibody. *A. vinelandii* BG1304 shows a band of about 60 kDa molecular size (lane 3) that corresponds to co-migrating α and β subunits (or NifD and NifK) of the MoFe protein. *A. vinelandii* BG1304 shows a band of about 120 kDa molecular size (lane 4) that corresponds to the NifD-K fusion protein.

transcription of the fusion gene was regulated by the nifH promoter, as seen in the case of wild type *A. vinelandii*. A PCR amplification of the nifD-K fusion gene using the forward primer corresponding to the C-terminal sequence of the nifH and the reverse primer corresponding to the nifK C-terminal sequence, and chromosomal DNA of *A. vinelandii* BG1304 as template, resulted in generating a DNA band corresponding to 2.6 kb, the molecular size expected only if nifD-K fusion gene was integrated downstream to the nifH. This PCR product was subjected to nucleotide sequence analysis, and the location of integration and presence of the nifD-K fusion junction was further confirmed. This result suggested that the nifD-K fusion gene was integrated downstream to the nifH promoter. To confirm that the expression of the nifD-K fusion gene was under the transcriptional control of the nifH promoter, we grew *A. vinelandii* BG1304 in Burk’s medium supplemented with nitrogen. *A. vinelandii* AV Trans grown under similar conditions was used as the control. Crude extracts from *A. vinelandii* BG1304 and *A. vinelandii* AV Trans were then subjected to SDS-PAGE, Western blotting, and probing with anti-MoFe protein antibody. No bands corresponding to the MoFe protein were detected either in the crude extracts from *A. vinelandii* BG1304 or in the crude extracts from *A. vinelandii* AV Trans. These results supported the idea that the nifD-K fusion gene in the chromosome of *A. vinelandii* BG1304 is under the transcriptional control of the nifH promoter.

The MoFe protein encoded by *A. vinelandii* BG1304 (or the NifD-K fusion protein) has many structural differences when compared with the wild type MoFe protein encoded by *A. vinelandii* AV Trans. The wild type MoFe protein is visualized by using the RasMol program in Fig. 8A. This figure shows one α and β subunit of the MoFe protein depicted as ribbon diagram and the other α and β subunit of the MoFe protein depicted as line diagram. The Ala-481 at the C terminus of the NifD and the Pro-13 at the N terminus of the NifK are shown. In the NifD-K fusion protein, both these residues were connected with the amino acid sequence that included α481–α491, and nine newly inserted residues (GSQSNRIQL). The Fig. 8B shows the NifD-K fusion protein with these newly inserted residues that join the C terminus of NifD to the N terminus of NifK. The structural changes include the following: (a) conversion of two stop codons that terminate translation of the NifD into sense codons and thus extend the NifD by seven new amino acids; (b) removal of the first eight amino acids of the NifK resulting in removing the translation initiation codon of the NifK, where translation is normally initiated to synthesize NifK; (c) conversion of the 9th and 10th amino acids of NifK from Lys and Ala to Gly and Ser, respectively; and (d) a change in the ORF of the NifD-K fusion junction caused by joining the BamHI ends of the DNA fragments encoding nifD and nifK along with two nucleotide changes introduced during PCR amplification. As shown in Fig. 3, these changes resulted in an overall loss of three amino acids and seven mismatches at the fusion junction of the NifD and the NifK, generating a NifD-K fusion protein that was smaller in size when compared with the wild type MoFe protein. Moreover, the biosynthesis and assembly of the MoFe protein encoded by *A. vinelandii* BG1304 is also significantly different from that of the wild type MoFe protein encoded by *A. vinelandii* AV Trans, because in the first case a single large NifD-K fusion protein is synthesized, and in the second case the smaller NifD and NifK are synthesized separately and then assembled together. The extent of amino acid changes that occurred during the construction of NifD-K fusion protein is a total of 10 amino acids. It is interesting to note that the NifD-K fusion protein was functional despite all these amino acid changes and could support biological nitrogen fixation. The reduced efficiency of the NifD-K fusion protein to support
growth on nitrogen-deficient medium when compared with the wild type MoFe protein is not surprising considering the major structural changes that were introduced during the construction of the fusion protein. Nevertheless, the fact that the NifD-K fusion protein is functional points out that the MoFe protein is also flexible, similar to the NifE-N (34) regarding the structural changes that were introduced during the construction of the fusion protein. Nevertheless, the fact that the NifD-K fusion protein is functional points out that the MoFe protein is also flexible, similar to the NifE-N (34) regarding the structural changes that were introduced during the construction of the fusion protein. Nevertheless, the fact that the MoFe protein is also flexible, similar to the NifE-N (34) regarding the structural changes that were introduced during the construction of the fusion protein.

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