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Structure of precursor bound NifEN: a nitrogenase FeMo cofactor maturase/insertase*

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Abstract

NifEN plays an essential role in the biosynthesis of the nitrogenase FeMo cofactor (M-cluster). It is an $\alpha_2\beta_2$ tetramer that is homologous to the catalytic MoFe protein (NifDK) component of nitrogenase. NifEN serves as a scaffold for the conversion of an iron-only precursor to a matured form of the M-cluster before delivering the latter to its target location within NifDK. Here, we present the structure of the precursor-bound NifEN of *Azotobacter vinelandii* at 2.6 Å resolution. From a structural comparison of NifEN with des-M-cluster NifDK and holo NifDK, we propose similar pathways of cluster insertion for the homologous NifEN and NifDK proteins.

Nitrogenase is a complex metalloenzyme that catalyzes a key step in the global nitrogen cycle: the reduction of atmospheric dinitrogen to bioavailable ammonia. The Mo-dependent nitrogenase is a two-component system, in which the Fe protein (NifH) mediates the ATP-dependent transfer of electrons to the catalytic MoFe protein (NifDK) during substrate turnover (1). The MoFe protein is an $\alpha_2\beta_2$ -tetramer that contains two unusual metalloclusters per $\alpha\beta$ -dimer, the P-cluster and the M-cluster. The P-cluster is an [8Fe-7S] cluster at the α/β -subunit interface, coordinated by three Cys ligands from the α -subunit and three Cys ligands from the β -subunit. The M-cluster (or FeMo cofactor) is a [Mo-7Fe-9S-X-homocitrate] cluster (where X = C, N or O) located within the α -subunit, coordinated by a His ligand at the Mo end and a Cys ligand at the opposite Fe atom, with a Lys residue binding to the homocitrate entity (2, 3). During catalysis, the P-cluster is thought to mediate the electron flow from the Fe protein to the M-cluster, where substrate reduction occurs.

NifEN is an essential player in M-cluster biosynthesis (4–6). It presumably receives a precursor form of the M-cluster from NifB and hosts the conversion of this precursor to a mature M-cluster before delivering the latter to the MoFe protein (4, 5). A role for NifEN in FeMoco biosynthesis was initially hypothesized based on a considerable degree of similarity between the primary sequences of NifEN and MoFe protein, suggesting that NifEN may contain P- and M-like clusters (6). Subsequently, the NifEN-associated clusters were identified through the biochemical and spectroscopic analyses of three forms of NifEN. The

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first, apo NifEN, is free of any cofactor species and contains a [4Fe-4S] cluster in place of the [8Fe-7S] P-cluster (7). The second, NifEN, contains, in addition to the [4Fe-4S] cluster, an all-iron precursor that closely resembles the Fe/S core of the M-cluster (8). The third, holo NifEN, contains a mature M-cluster and the [4Fe-4S] cluster (9). NifEN could be readily converted to holo NifEN by Fe protein-mediated insertion of Mo and homocitrate into the precursor (10), and holo NifEN could directly serve as a cofactor donor for the apo MoFe protein (11). These observations not only establish the role of NifEN in cofactor biosynthesis, but also illustrate the dynamic nature of the cofactor site in NifEN during the assembly process. However, the mechanistic details of the biosynthetic events on NifEN have remained unclear without structural information on this protein. In this study, the structure of the precursor-bound NifEN of *Azotobacter vinelandii* has been solved to 2.6 Å resolution (12). From a structural comparison of NifEN with des-M-cluster MoFe protein (apo NifDK) (11) and holo MoFe protein (NifDK) (2, 3), we propose similar pathways of cluster insertion for the homologous NifEN and NifDK proteins.

The crystal structure determination of NifEN is summarized in Tables S1 and S2. Like the $\alpha_2\beta_2$ -tetrameric NifDK, NifEN consists of a pair of $\alpha\beta$ -dimers that are related by a molecular twofold rotation axis (Fig. 1A). The α - and β -subunits of NifEN, like those of NifDK and apo NifDK, are composed of three domains each— α I, α II, and α III, and β I, β II, and β III, respectively. All domains of NifEN, as for NifDK and apo NifDK, are organized around a common core of a four-stranded, parallel β -sheet flanked with α -helices and additional β -strands (13). Moreover, NifEN contains two types of clusters that correspond to the P- and M-clusters in NifDK: one, termed the O-cluster, is a [4Fe-4S] cluster that is coordinated by Cys α 37, Cys α 62, Cys α 124 and Cys β 44 at the same site in the α/β -subunit interface as the P-cluster; the other, termed the L-cluster, is an iron-only precursor form of the M-cluster that is at least ligated by Cys α 25 at one end (Fig. 1B) (14). Although the electron density is not sufficiently well resolved to unambiguously establish the structure of the L-cluster, the shape and extent of the density is compatible with the core geometry of the M-cluster and, therefore, consistent with the previously proposed 8Fe-model (8) of this M-cluster precursor (Fig. 1B).

Strikingly, although the L- and M-clusters are both positioned at the junction between the α I-, α II- and α III-domains of their respective proteins, the L-cluster of NifEN is nearly surface exposed, possibly shielded from solvent by only a small stretch of disordered polypeptide between residues α 14 and α 24. In contrast, the M-cluster of NifDK occupies a buried site ~10 Å below the surface of the protein (2). Given the homology between NifEN and NifDK, it is possible that, following maturation, the M-cluster is inserted into a site in NifEN that is analogous to its binding site in NifDK. Significantly, the α -subunit of NifEN assumes a conformation that is more “open” than that of NifDK (presumably corresponding to holo NifEN) yet less “open” than that of apo NifDK (presumably corresponding to apo NifEN), suggesting that the α -subunit undergoes major structural rearrangements upon the maturation of the L-cluster on NifEN (Fig. 1C). Consistent with this proposal, a comparison of the molecular surfaces of these proteins reveals the presence of a positively charged funnel in apo NifDK, which is closed in NifDK containing a buried M-cluster (Fig. S2). There is a similar accumulation of positive charges on the surface of NifEN; however, an insertion funnel could not be clearly defined, perhaps due to a partial closure of the entrance by docking of the L-cluster (Fig. S2). In contrast, the β -subunits of the three proteins are similar in structure, except for the presence of an extended loop and an extra helix-turn-helix structure in the holo and apo NifDK (Fig. S3).

The α I- and α II-domains of apo NifDK do not show significant C_α -deviations from those of NifDK, except for a short loop (α 49–52) that follows a disordered region (α 1–48) at the N-terminus of the protein (Fig. S4A, upper; Fig. 2A, left). A similar loop (α 24–27) at the N-

terminus of NifEN aligns more closely with the corresponding region in NifDK (Fig. S4A, lower; Fig. 2A, right). Cys α 25, which ligates one of the terminal Fe atoms of the L-cluster, is located near the end of this loop. Thus, this loop is likely oriented by docking of the L-cluster on Cys α 25. Apart from this feature, NifEN contains an extra sequence (α 187–192), which forms a larger external loop between the α I (α helix I^I)- and α II (β strand 1^{II})-domains (Fig. S4A and 2A). At the site that would contain a buried M-cluster in NifDK, the area surrounding the potential Cys α 250 ligand (α 248–252) features an α -helix (C^{II}) that is shorter toward the N-terminus, with an extended loop in its place that could add flexibility to the dynamic M-cluster site in NifEN (Fig. S4A and 2A).

The α III-domain of apo NifDK shows significant C_{α} -deviations from that of NifDK in four distinct areas: a “lid loop” (α 353– α 364), a disordered region (α 381– α 407), and regions surrounding the homocitrate anchor (α 421– α 428) and the histidine ligand (α 436– α 446) of the M-cluster, respectively (Fig. S4B, upper). By comparison, the α III-domain of NifEN shows C_{α} -deviations from NifDK that are reminiscent of, yet much less significant than, those of apo NifDK from NifDK (Fig. S4B, lower). The β -strands (1^{III}, 2^{III} and 4^{III}) and α -helices (A^{III}, C^{III}, and D^{III}) of apo NifDK are significantly shortened and displaced from those of NifDK (Fig. 2B, left). These changes result in a significant relocation of the lid loop and the adjacent (disordered) region in apo NifDK, which completely opens up the M-cluster site for cluster insertion (11). The same β -strands (1^{III}, 2^{III} and 4^{III}) and α -helices (A^{III}, C^{III}, and D^{III}) in NifEN are shorter than those in NifDK; however, they are more extended and less displaced than those in apo NifDK, resulting in a conformation of NifEN intermediate between those of apo and holo NifDK (Fig. 2B, right). The changes of these structural elements, along with the structuring of α helix B^{III}, lead to the formation of an ordered, yet partially closed M-cluster site in NifEN (Fig. 2B, right). In particular, the downwardly bent β -strand 1^{III} anchors the lid loop in a position close to that in NifDK; whereas β -strand 2^{III}, together with α -helix B^{III}, extend toward the L-cluster at the surface of the protein (Fig. 2B, right). Two β -strands (3^{III} and 5^{III}) and one α -helix (E^{III}) are missing from the structures of both NifEN and apo NifDK (Fig. 2B). The presence of irregular loops in places of β -strand 3^{III} (which “hinges” the α -helix B^{III} at the L-cluster site) and β -strand 5^{III}/ α -helix E^{III} (which flank the Asn ligand at the M-cluster site) may contribute to the conformational flexibility of the polypeptides surrounding the L- and M-cluster sites of NifEN, which could facilitate the transfer of the cluster from the L-cluster site to the M-cluster site upon maturation.

A closer examination of the L- and M-cluster sites in the three proteins provides additional insights into the pathway of M-cluster assembly. In all cases, an “L-cluster site” can be identified at the surface of the structure, located directly above the M-cluster site within the protein. The “L-cluster site” in apo NifDK is wide open, with a disordered region (α 381–389) and a loop (α 49–65) adjacent to Cys α 45 shifted away from those in holo NifDK (Fig. 3A and S5A). The corresponding regions in NifEN (α 357–365 and α 24–40) are ordered, suggesting a concerted action between the two regions upon the ligation of the L-cluster to Cys α 25 at the tip of the loop. The arrangement of the L-cluster site in NifEN is similar to that of the “L-cluster site” in NifDK; however, NifDK contains amino acids with bulky side chains (e.g., Phe α 381, His α 383, and Tyr α 276) that close up the M-cluster site (located below the “L-cluster site”), whereas NifEN contains amino acids with small side chains (e.g., Ala α 253 and Ser α 251) that may provide the cluster with more freedom to move between the L- and M-sites during cluster assembly and delivery. A similar pattern is observed when the M-cluster sites of the three proteins are compared (Fig. 3B and S5B). The M-cluster site of apo NifDK is opened by a significant displacement of the lid loop (α 355–359), while the corresponding loop in NifEN (α 333–337) is positioned similarly to that in NifDK. Likewise, the likely ligands (Asn α 418 and Cys α 250) and homocitrate anchor (Arg α 402) of the M-cluster in NifEN occupy positions that are similar to those of

the M-cluster (His $\alpha 442$, Cys $\alpha 275$, and Lys $\alpha 426$) in NifDK. Despite these similarities, Tyr $\alpha 444$, which switches positions with His $\alpha 442$ and locks the M-cluster in place in NifDK through its bulky side chain (4), is not present in NifEN. This could facilitate the eventual release of the cluster from the M-cluster site of NifEN and its subsequent delivery to the homologous M-cluster site in NifDK. In contrast, the O-cluster site in NifEN assumes a nearly identical conformation to the P-cluster sites in apo and holo NifDK, except for the presence of an incomplete complement of Cys ligands that only accommodates a [4Fe-4S] cluster (Fig. 3C).

Similar pathways of cluster insertion can be proposed for NifEN and NifDK on the basis of these structural analyses (Fig. 4). It involves the formation of an apo protein that contains an open cluster insertion funnel, the docking of the cluster at (or near) the entrance of the funnel, and the subsequent insertion of the cluster into the funnel. In the case of NifEN, the L-cluster is delivered from NifB to the surface of apo NifEN, where it is poised to be transformed into a mature M-cluster upon the insertion of Mo and homocitrate by NifH (Fig. 4A). The M-cluster is then relocated from the surface to the binding site within NifEN. Interactions between NifEN and NifDK may cause conformational changes that release the M-cluster and facilitate its movement back to the surface of NifEN. Subsequently, the M-cluster is transferred to and incorporated into NifDK in a manner similar to the insertion of M-cluster into NifEN; only the M-cluster is securely locked at its binding site in this case (Fig. 4B). The coordination of the various cluster intermediates to cysteines located in loops (Cys $\alpha 25$ of NifEN and, potentially, the corresponding Cys $\alpha 45$ of NifDK) may serve to tether and facilitate efficient intermolecular transfer of these species without them escaping to the surrounding environment. Mechanistic parallels in this respect are evident between the functions of NifEN and metallochaperones for copper and other transition metals (15).

The presence of similar insertion funnels in these homologous proteins is further supported by the recently solved crystal structure of protochlorophyllide reductase (BchNB) (16, 17). Responsible for the reduction of a specific double bond of protochlorophyllide (Pchl_{id}), BchNB is an $\alpha_2\beta_2$ tetramer that assumes a conformation highly homologous to those of NifEN and NifDK (Fig. S6). Moreover, it contains one [4Fe-4S] cluster and one Pchl_{id} at locations similar to those of the O/P-clusters and L/M-clusters in NifEN and NifDK. Interestingly, based on their locations in the respective homologous proteins, the L-cluster, M-cluster and Pchl_{id} can be sequentially placed along the insertion funnel in apo NifDK (Fig. S7). This observation supports the hypothesis that a common ancestral protein—which appears to have adapted to accommodate different ligands—gave rise to all of these homologous proteins (18).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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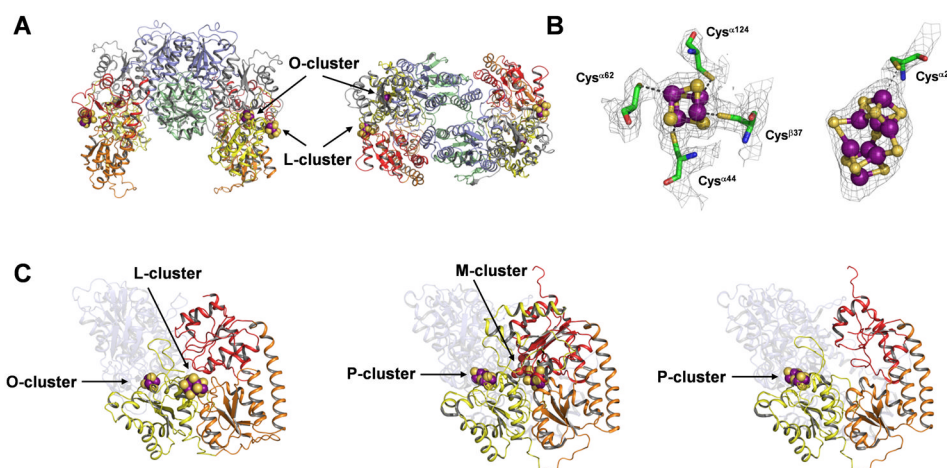


Fig. 1. (A) The structure of the NifEN tetramer with the molecular twofold axis oriented vertically (left) and along the viewing direction (right). The domains of the α -subunits are colored yellow (α I), orange (α II), and red (α III); and the domains of β -subunits are colored gray (β I), blue (β II), and green (β III). The L-cluster (precursor of M-cluster) and O-cluster ([4Fe-4S] cluster) are illustrated as space-filling models, with atoms colored as follows: Fe, purple; S, yellow. (B) Structures and ligands of O-cluster (left) and L-cluster (right) overlaid with electron density maps. Both clusters are illustrated as ball-and-stick models, whereas the ligands are shown in stick presentation. Note that additional L-cluster ligands may be present (14). The atoms are colored as follows: Fe, purple; S, yellow; O, red; C, green; N, blue. The structure of L-cluster overlaid with anomalous difference electron density map is shown in Fig. S1. (C) Structures of the $\alpha\beta$ -pairs of NifEN (left), NifDK (middle, PDB entry 1M1N) and apo NifDK (right, PDB entry 1L5H). The α -subunits are presented in the foreground, and the β -subunits are rendered transparent in the background. The domains of the subunits in all three proteins are colored as in A. All clusters are illustrated as space-filling models, with atoms colored as follows: Fe, purple; S, yellow; O, red; C, gray. The Mo atom and the interstitial ligand of the M-cluster are not visible.

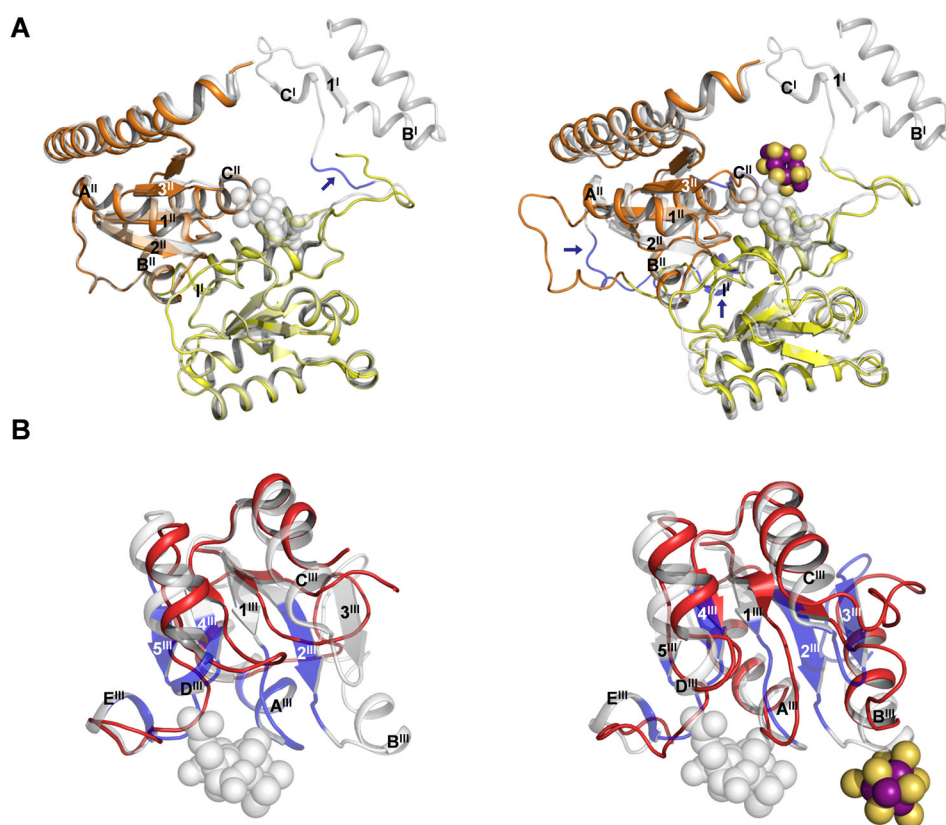


Fig. 2. (A) Superposition of α I- and α II-domains of apo NifDK and NifDK (left) and those of NifEN and NifDK (right). The domains of apo NifDK and NifEN are color coded as in Fig. 1. NifDK is shown in gray (transparent). Regions with significant shifts in C_{α} -positions are colored blue and indicated by arrows, and the secondary structural elements of these regions are labeled according to those in Fig. S4A. The L-cluster is illustrated as in Fig. 1. The M-cluster is shown as a space-filling model in gray (transparent). PYMOL was used to prepare the figure (18). (B) Superposition of the α III-domains of apo NifDK and NifDK (left) and those of NifEN and NifDK (right). Apo NifDK and NifEN are colored red; whereas NifDK is shown in gray (transparent). Regions with significant shifts in C_{α} -positions are colored blue. The secondary structural elements are labeled according to those shown in Fig. S4A. The L-cluster is illustrated as in Fig. 1. The M-cluster is shown as a space-filling model in gray (transparent). PYMOL was used to prepare the figure (19).

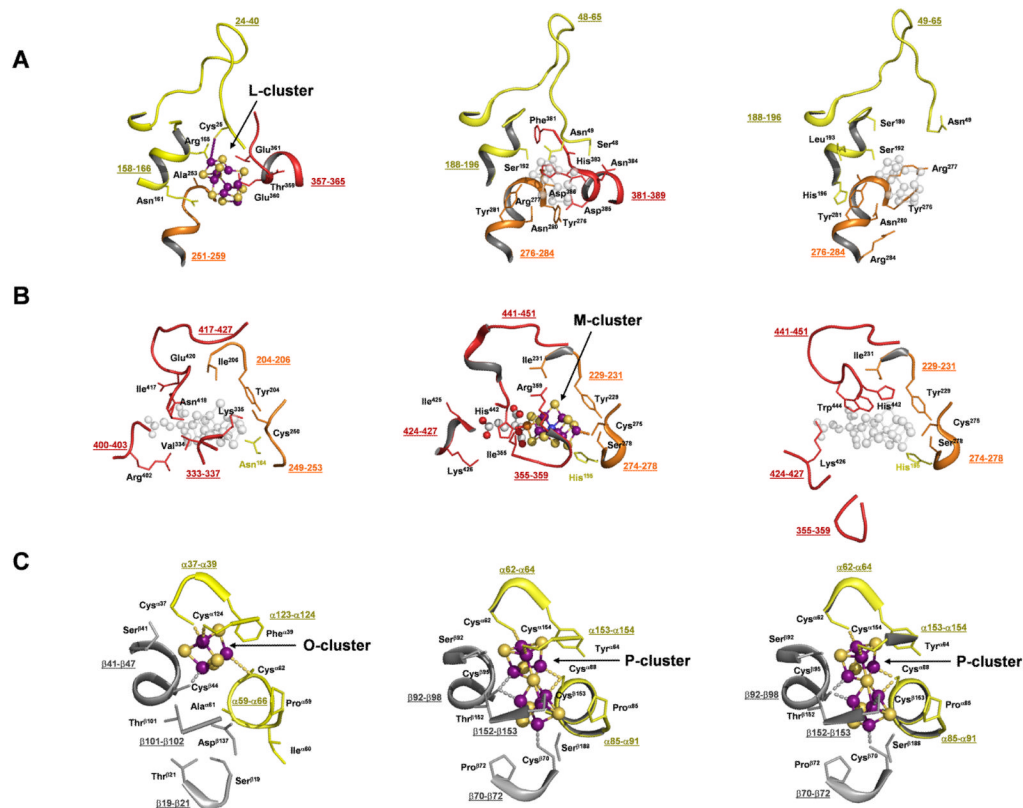


Fig. 3. Close-up of the protein environment surrounding the L-cluster (**A**), M-cluster (**B**) and O/P-cluster (**C**) of NifEN (left), NifDK (middle) and apo NifDK (right). Shown are parts of the backbones (with numbers of residues underlined) and some side chain residues in the close vicinity of the clusters. The domains of the α - and β -subunits are colored as in Fig. 1A. All clusters are illustrated as ball-and-stick models, with atoms colored as follows: Fe, purple; S, yellow; O, red; C, gray; Mo, orange; X, blue. The superimposed positions of the L-cluster in NifDK and apo NifDK (A, middle and right), and of the M-cluster in NifEN and apo NifDK (B, left and right), are indicated in gray (transparent). PYMOL was used to prepare the figure (19).

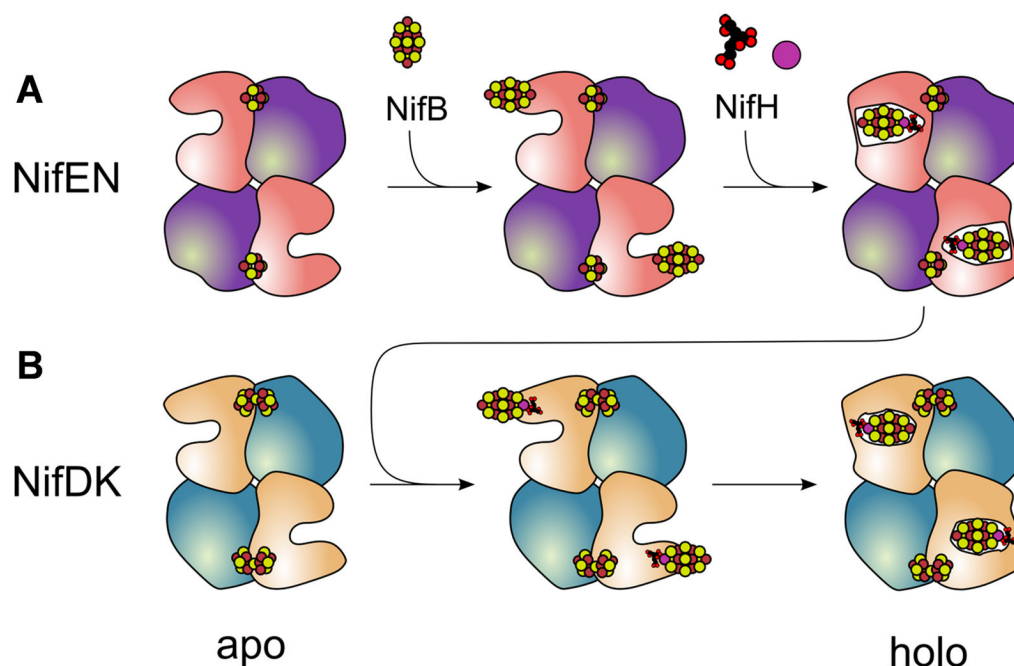


Fig. 4. Schematic presentation of the proposed biosynthesis events that occur on NifEN (**A**) and NifDK (**B**). (**A**) Following the synthesis of an O-cluster containing, yet L-cluster deficient apo NifEN (left), the L-cluster is delivered from NifB to the entrance of the cluster insertion funnel in NifEN (middle). Subsequently, the Fe protein (NifH) inserts Mo and homocitrate into the L-cluster, resulting in a matured form of the M-cluster that is transferred to its binding site in NifEN (right). (**B**) NifDK first appears as a P-cluster containing, yet M-cluster deficient apo form (left). The mature M-cluster is then transferred from NifEN to the entrance of the cluster insertion funnel in NifDK upon direct protein-protein interactions (middle). Subsequently, the M-cluster is inserted into its binding site in NifDK, leading to the formation of holo NifDK (right). The proposed biosynthetic events on NifEN parallel those on NifDK, which is consistent with the close similarities between the overall structure and the cluster topology of the two proteins. The presence of O-cluster ([4Fe-4S]) and P-cluster ([8Fe-7S]) in apo NifEN and apo NifDK, respectively, may further imply an evolutionary connection between the two proteins. It can be hypothesized that the P-cluster site evolved from the O-cluster site upon the acquisition of missing ligands, thereby accommodating a second [4Fe-4S] cluster at this site for the subsequent coupling of two [4Fe-4S] modules into a mature P-cluster (20).