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Uncoupling Nitrogenase: Catalytic Reduction of Hydrazine to Ammonia by a MoFe Protein in the Absence of Fe Protein-ATP

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Reduction of N_2 to ammonia by the Mo-dependent nitrogenase requires the action of two component proteins called the Fe protein and the MoFe protein.[1] The MoFe protein contains the multimetallic FeMo-cofactor catalytic cluster [7Fe-9S-Mo-X-*R*-homocitrate] at which substrates bind, and the auxiliary P-cluster [8Fe-7S] that is proposed to mediate electron transfer from the Fe protein (Figure 1).[2] The Fe protein contains a [4Fe-4S] cluster and two MgATP binding sites.[3] During catalysis by nitrogenase, the Fe protein transiently associates with the MoFe protein and a single electron is transferred from the Fe protein to the MoFe protein in conjunction with the hydrolysis of two MgATP molecules.[4] The Fe protein-2MgADP then dissociates from the MoFe protein, allowing another reduced Fe protein-2MgATP to bind to the MoFe protein. This cycle is repeated to accumulate electrons in the MoFe protein to support substrate reduction. The Fe protein with bound ATP is the only known reductant of the MoFe protein that supports reduction of N_2 or any of a number of other small substrates.[1] Although several small electron transfer proteins (e.g. flavodoxin or ferredoxin) or electron mediators can donate electrons to oxidized metal clusters in the MoFe protein, none of these molecules can support substrate reduction.

These observations show that the Fe protein is more than a simple reductant of the MoFe protein, suggesting instead a specific task such as inducing conformational changes within the MoFe protein that allow substrate binding and/or electron transfer to FeMo-cofactor. This is supported by a recent report that electron transfer between the Fe protein and MoFe protein is conformationally gated, involving protein conformational changes.[5] A number of X-ray structures of Fe protein-MoFe protein complexes have been solved in various nucleotide-bound states,[6] yet no structural changes have been observed within the MoFe protein that might explain the role of the Fe protein/ATP.

In recent years, we have probed MoFe protein function by substituting specific amino acids for different amino acids and then characterizing the properties of the resulting MoFe protein variants.[4] We reasoned that it might be possible to explore the putative Fe protein conformational change within the MoFe protein by means of amino acid substitution of residues near the P cluster, FeMo-cofactor, or in between these two clusters (Figure 1). Here, we report that when the β-98^{Tyr} residue, located within the MoFe protein between the P-cluster and FeMo-cofactor, is substituted by histidine, the resulting MoFe protein (β-98^{His}) can reduce the nitrogenous substrate hydrazine (N_2H_4) to ammonia in the absence

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of the Fe protein and ATP, with electrons being supplied instead by a Eu^{II}-polyaminocarboxylate complex.

When electrochemically generated Eu^{2+} (reduction potential −0.36 V versus the standard hydrogen electrode (SHE) at pH 7.0) is mixed with a polyaminocarboxylate ligand (L) such as EGTA (ethylene glycol-bis(2-aminoethyl)-N,N,N′,N′-tetraacetate-(4-)) or DTPA (diethylenetriamine-N,N,N′,N″,N″-pentaacetate(5-)), it instantly forms a strong 1:1 complex that is a powerful and reactive one-electron reductant; the $Eu^{III/II}$-EGTA and $Eu^{III/II}$-DTPA redox couples have reduction potentials of −0.88 V and −1.14 V, respectively (pH 8.0).[7]

When the wild-type MoFe protein without Fe protein/ATP is mixed with Eu^{II}-EGTA or Eu^{II}-DTPA, no significant reduction of N_2 or hydrazine to ammonia is detected (Figure 2, panel A).[8] We also examined a number of MoFe proteins with amino acid substitutions near the P cluster or FeMo-cofactor: α-$70^{Val→Ala}$, α-$195^{His→Gln}$, β-$188^{Ser→Cys}$, and α-$70^{Val→Ala}$/α-$96^{Arg→His}$ (Figure 1). Each of these residues in the MoFe protein has been previously implicated in some aspect of electron transfer or substrate reduction by the MoFe protein.[4] When any one of these MoFe protein variants was mixed with Eu^{II}-DTPA, no significant reduction of hydrazine (Figure 2, panel A) or N_2 to ammonia was detected.

A prior study of a MoFe protein with β-98^{Tyr} substituted by histidine, which is located between the P cluster and the *R*-homocitrate end of FeMo-cofactor (Figure 1), implicated the tyrosine in electron transfer between the clusters.[9] When the β-98^{His} MoFe protein was mixed with Eu^{II}-DTPA and hydrazine, a time-dependent production of ammonia was observed (Figure 2, panel A). The production of ammonia was dependent on the presence of the β-98^{His} MoFe protein, Eu^{2+}, L, and hydrazine (Figure 2, panel B). After one minute of assay, a specific activity for hydrazine reduction of 170 ± 8 nmols NH_3/min/mg MoFe protein was determined. This rate is 94% of the rate measured for a fully functioning wild-type or β-98^{His} MoFe protein with Fe protein, MgATP and a MgATP regeneration system, and the reductant dithionite under the same conditions. The reduction of hydrazine by the β-98^{His} MoFe protein without Fe protein/ATP was found to continue over extended times (tested to 25 min), although the specific activity at longer times was lower (50 nmol NH_3/min/mg MoFe protein at 20 min) than at earlier times, suggesting that a component of the reaction was being depleted. Addition of more Eu^{II}-DTPA to an assay that had run for 20 min reactivated the reaction to near the initial rates, indicating that depletion of the reductant was the cause of declining rates over time. The rate of reduction of hydrazine depended on the concentration of Eu^{II}-DTPA and hydrazine, in each case showing saturation kinetics, with maximal rates observed at 1 mM Eu^{II}-DTPA and 50 mM hydrazine. Doubling the amount of MoFe protein doubled the rate of hydrazine reduction. The milder reductant Eu^{II}-EGTA was also examined and was found to support hydrazine reduction, albeit at lower rates (17 nmol NH_3/min/mg MoFe protein) when compared to Eu^{II}-DTPA.

Reduction of N_2 to ammonia by the β-98^{His} MoFe protein was not detected with either reductant even after extended reaction times. The Eu^{II}-DTPA and Eu^{II}-EGTA complexes catalyze H^+ and acetylene reduction in the absence of MoFe protein, so it was not possible to test these substrates in this reaction.

The reduction of N_2 by nitrogenase is a 6 e^-/6 H^+ reaction that is likely to involve metal-bound partially reduced intermediates at the level of reduction of diazene (N_2H_2) and hydrazine.10 Hydrazine is reduced to ammonia by the fully functioning nitrogenase,[11] so it is reasonable to conclude that hydrazine joins late in the N_2 reduction pathway, requiring only $2e^-/2H^+$ for reduction.[12] Our observation of hydrazine reduction by the β-98^{His} MoFe protein, but not N_2 reduction, is consistent with the fact that hydrazine is easier to reduce than N_2.[13–14] The hydrazine reduction observed here cannot be catalyzed by FeMo-cofactor

that has been released from the protein because isolated FeMo-cofactor does not catalyze this reaction[15] and the β-98^{His} MoFe protein is stable.[9] Further, we have determined that the β-98^{His} MoFe protein retains normal acetylene reduction activity[16] when combined with Fe protein and ATP following removal of the Eu^{II}-L by gel filtration chromatography.

The results presented here demonstrate the catalytic reduction of hydrazine to ammonia by the β-98^{His} MoFe protein uncoupled from electron delivery by the Fe protein and ATP, instead using only a simple, but powerful, electron donor. How substitution of β-98^{Tyr} by histidine has activated the MoFe protein for hydrazine reduction without the Fe protein and ATP hydrolysis has yet to be established, but the results indicate that amino acid substitutions within the MoFe protein can partially mimic some of the conformational changes induced by the Fe protein/ATP that either activate electron transfer from the P-cluster to FeMo-cofactor or allow substrate binding. The specific roles of the P-cluster and FeMo-cofactor in the Eu^{II}-L-dependent reduction of hydrazine observed here are under active investigation.

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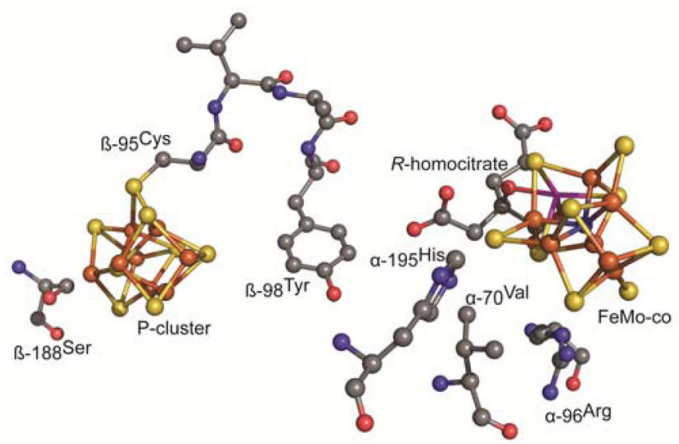

Figure 1.

MoFe protein metal clusters and key amino acid residues are shown. The Fe protein association site is to the left outside of the view. Colors are Mo in magenta, Fe in rust, S in yellow, C in gray, O in red, and N in blue. Based on the protein data base file 1M1N.pdb.[2]

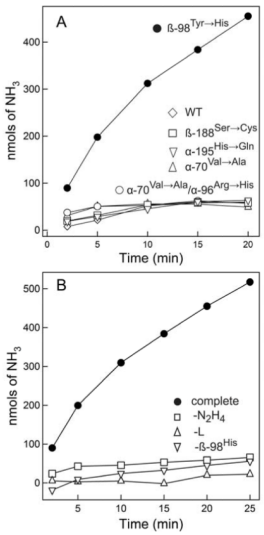

Figure 2.

Hydrazine reduction to ammonia is shown as a function of time for different MoFe proteins (panel A) and for the β-98^{His} MoFe omitting each component of the assay (panel B). No Fe protein or ATP is added. Omission of Eu^{2+} resulted in no detection of ammonia. The concentration of hydrazine is 50 mM, the quantity of MoFe protein is 0.7 mg (2.8 nmol), and the concentration of Eu^{II}-DTPA is 0.5 mM (panel A) and 1 mM (panel B), with the pH = 7.0 and temperature = 25°C.