The Molybdenum Cofactor of *Escherichia coli* Nitrate Reductase A (NarGHI)

EFFECT OF A *mobAB* MUTATION AND INTERACTIONS WITH [Fe-S] CLUSTERS*

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We have studied the effect of a *mobAB* mutation and tungstate on molybdo-molybdopterin-guanine dinucleotide (Mo-MGD) insertion into *Escherichia coli* nitrate reductase (NarGHI). Preparation of fluorescent oxidized derivatives of MGD (Form A and Form B) indicates that in a *mobAB* mutant there is essentially no detectable cofactor present in either the membrane-bound (NarGHI) or purified soluble (NarGH) forms of the enzyme. Electron paramagnetic resonance characterization of membrane-bound cofactor-deficient NarGH suggests that it has altered electrochemistry with respect to the dithionite reducibility of the [Fe-S] clusters of NarH. Potentiometric titrations of membrane-bound NarGHI indicate that the NarH [Fe-S] clusters have midpoint potentials at pH 8.0 (E_{m,n,o} values) of +180 mV (3Fe-4S) cluster, +130, −55, and −420 mV ([4Fe-4S] clusters) in a wild-type background and +180, +80, −35, and −420 mV in a *mobAB* mutant background. These data support the following conclusions: (i) a model for Mo-MGD biosynthesis and assembly into NarGHI in which both metal chelation and nucleotide addition to molybdopterin preceed cofactor insertion; and (ii) the absence of Mo-MGD significantly affects E_{m,n,o} of the highest potential [4Fe-4S] cluster.

*Escherichia coli*, when grown anaerobically with nitrate as respiratory oxidant, develops a respiratory chain terminated by a membrane-bound quinol:nitrate oxidoreductase (NarGHI)1 (1–3). This enzyme is a heterotrimeric complex iron-sulfur molybdenzyme comprising a molybdenum cofactor-containing catalytic subunit (NarG; 139 kDa), an [Fe-S] cluster-containing electron transfer subunit (NarH; 58 kDa), and a heme-containing membrane anchor subunit (NarI; 26 kDa). NarGHI is an excellent example of a family of bacterial iron-sulfur molybdoenzymes, which includes *E. coli* Me₆SO reductase (DmsABC (4)), formate dehydrogenase N (FdnGHI (5)), and *Wolinella succinogenes* polysulfide reductase (PsrABC (6)). Sequence comparisons between NarG and a large number of bacterial molybdenozymes indicate that this subunit binds the molybdenum cofactor and is the site of nitrate reduction (3, 4, 7).

The structures of a number of bacterial molybdenozymes have recently been determined. These include Me₆SO reductases from *Rhodobacter sphaeroides* (8) and *Rhodobacter capsulatus* (9), and formate dehydrogenase H from *E. coli* (10). In each case, a molybdo-bis(molybdopterin guanine dinucleotide) (Mo-bisMGD) at the active site. Given the significant sequence similarity between these enzymes and that of the catalytic subunit of NarGHI (NarG), it is likely that NarG also contains a Mo-bisMGD cofactor at its active site. However, because the cofactor has not been unequivocally identified as a Mo-bisMGD, it will be referred to herein as Mo-MGD. One of the final steps in the biosynthesis of Mo-MGD (11) is the addition of a guanine nucleotide to Mo-molybdopterin (Mo-MPT) to form Mo-MGD (12). In a *mobAB* mutant, this step does not take place. The *mob* locus comprises two open reading frames, *mobA* and *mobB*. The gene product of *mobA*, MobA, has been shown to be responsible for nucleotide addition (13), whereas the gene product of *mobB*, MobB, enhances nucleotide addition but is not essential for it (14). The effect of *mobAB* mutations on *E. coli* NarGHI and DmsABC has been studied in detail (15, 16). Purified soluble NarGHI dimer prepared from a *mobAB* strain can be incubated in the presence of MobA and GTP, generating enzyme that is able to reduce nitrate with benzyl viologen (BV⁻) as electron donor (13). This reconstitution presumably occurs as a result of the conversion of Mo-MPT to Mo-MGD. However, its extent corresponds to only 5–10% of the expected level. In these reconstitution experiments, it has been suggested that the source of Mo-MPT is the purified NarGHI dimer (13, 15). These results contrast with those reported for DmsABC, in which no MoPT or molybdenum was detected in the enzyme studied in a *mobAB* background (16). However, in this latter case, the enzyme studied was the membrane-bound heterotrimer rather than the soluble DmsABC dimer. It would therefore be interesting to determine the effect of the *mobAB* mutation on the membrane-bound NarGHI holoenzyme.

The operon encoding NarGHI (*narGHI*) has a fourth open reading frame (*narJ*) whose product is not part of the holoen-

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* The abbreviations used are: NarGHI, nitrate reductase holoenzyme; BV⁻, reduced benzyl viologen; DmsABC, Me₆SO reductase; EPR, electron paramagnetic resonance; ICPE, inductively coupled plasma emission spectroscopy; IPTG, isopropyl-β-1-thiogalactopyranoside; MGD, molybdopterin guanine dinucleotide; Mo-MGD, molybdo-bis(molybdopterin guanine dinucleotide); Mo-MGD, molybdo-MGD; MPT, molybdopterin; Mo-MPT, molybdopterin-MPT; NarGHI, nitrate reductase soluble dimer; FdnGHI, formate dehydrogenase N; FdhF, formate dehydrogenase H; MOPS, 4-morpholinopropanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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zyme (2, 17). NarJ is a system-specific chaperone that is produced in substoichiometric amounts compared with the other subunits encoded by the operon (18). It appears to hold cofactor-deficient NarGH in a cofactor-insertion competent conformation in E. coli strains that are unable to synthesize cofactor (19). The NarGH dimer appears to be unstable and is devoid of Mo-MGD in a narJ mutant background.

The [Fe-S] clusters of NarGHI have been investigated in considerable detail using a combination of site-directed mutagenesis of cluster-ligating Cys residues and EPR spectroscopy (20-24). These studies have provided convincing evidence for the presence of one [3Fe-4S] cluster and three [4Fe-4S] clusters located in the NarH subunit of purified preparations of the NarGH dimer. These clusters appear to be ligated by four ferredoxin-like Cys groups (I-IV), and the presence of these groups identifies NarH as being a member of an emerging family of four cluster proteins. This family includes E. coli DmsB (of DmsABC), FdnH (of the formate dehydrogenase, FdnGHI), HycB (of formate-hydrogen lyase), and NarY (of the alternative nitrate reductase, NarZV), as well as many other proteins from other organisms (3, 4). It has recently been suggested on the basis of sequence alignments that the enzyme contains a fifth cluster, a [4Fe-4S] cluster, ligated by a cluster of Cys residues located toward the N terminus of NarG (3, 25). In the structure of formate dehydrogenase II (FdhF) (10), an N-terminal Cys group ligates a [4Fe-4S] cluster that is in close proximity to one of the pterins of the Mo-bisMGD cofactor. In the Thiosphaera pantotropha periplasmic nitrate reductase (NapA), a cluster was detected by EPR spectroscopy (10) which can also be assigned to the N-terminal Cys group. In both these enzymes, a role for the cluster can be envisioned in electron transfer to or from the Mo-MGD cofactor. Although many of the bacterial molybdoenzymes have an N-terminal Cys group in their catalytic subunit, there are important subclasses with different spacings between the putative cluster ligands (26). In both NarGHI and DmsABC, there is no evidence for the presence of a cluster within the catalytic subunit (22, 26), and the enzymes both belong to a specific subclass.

In this paper, we have investigated the effect of a mobAB mutation on the assembly of Mo-MGD into the soluble NarGH dimer and the membrane-bound NarGHI holoenzyme. We have compared the effect of the mobAB mutation with the effect of the molybdenum antagonist tungsten and have investigated the effect of the absence of Mo-MGD on the midpoint potentials of the NarH [Fe-S] clusters.

MATERIALS AND METHODS

E. coli LCB79 (araD139 Δ(lacPOZYA-argF) rpsL thi Δ79(nar-lac) StrepB) (27) is a wild-type with respect to mobAB and was used to express fully functional NarGHI, E. coli TP1000 (F ΔlacU169 araD139 rpsL510 relAI ptoF rbsR fbbR ΔmobAB) StrepB (17) is a mobAB deletion mutant. NarGH was overexpressed from pVAT700 (23) transformed into either LCB79 or TP1000. For quantification of the molybdenum content of wild-type NarGHI, E. coli LC24048 (thi-1 thr-1 leu-6 lacY1 supE44 rpsL175 Δnar25(narG-narH) Δ(narU-narZ) Δ1 kan8 Spe8 StrepB (28)) transformed with pVAT700 was used.

Growth of Cells and Preparation of Membrane Vesicles

Cells were grown microaerobically in 2-liter batch cultures of Terrific Broth (29) at 30 °C in the presence of 100 μg ml⁻¹ streptomycin and ampicillin. Where appropriate, the growth medium was supplemented with 15 mM sodium tungstate. A 10% inoculum was used, and NarGH overexpression was achieved by addition of 0.2 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). Following addition of the inoculum and the IPTG, cells were grown overnight with gentle shaking at 30 °C. Cells were harvested by centrifugation, washed in 100 mM MOPS and 5 mM EDTA (pH 7). Membrane vesicles were prepared as described previously (30).

Preparation of Purified NarGH Dimer

Purified NarGH was prepared from wild-type and mobAB genetic backgrounds as described previously (20). Lyophilized NarGH was re-suspended in 100 mM Tris and 5 mM EDTA (pH 8.3).

Preparation of Fluorescent MPT Derivatives

Membrane Vesicles—The presence of MPT in membrane vesicles was assayed by acid denaturation followed by I₃ and KIO₄ to produce an extract that contained the Form A fluorescent derivative of MPT that will be referred herein to as a “Form A extract” (12). 20 mg of membrane protein was used as starting material. Prior to the recording of fluorescence spectra using a Perkin-Elmer LS-50B luminescence spectrometer, small aliquots (100–200 μl) of the acid-denatured iodine-oxidized extract were added to 3 ml of 1 M NH₄OH in a fluorescence cuvette.

Purified NarGH—The presence of MPT in purified NarGH was assayed by preparation of an extract containing the Form B fluorescent derivative of MPT that will be referred herein to as a “Form B extract” (31, 32). Protein samples containing 2 mg of NarGH were diluted with 1 ml of 100 mM Tris (pH 7.5) and were incubated at 100 °C for 25 min. Insoluble material was removed by centrifugation at 13,000 rpm for 30 min. Aliquots of the supernatant fraction (200 μl) were added to 3 ml of 1 M NH₄OH in a fluorescence cuvette. Excitation and emission spectra were recorded as described in the legend to Fig. 1.

Quantitation of Molybdenum

The amount of molybdenum present in membrane fractions was determined by wet ashing the samples and performing inductively coupled plasma emission spectroscopy (ICPES). The molybdenum content of membrane vesicles from TP1000/pVA700 (mobAB mutant) and LC24048/pVA700 (wild-type) was analyzed by ICPES. The NarGH content of the membranes used in the ICPES studies was determined by rocket immunoelectrophoresis as described previously (20).

Protein Assays

Protein concentrations were assayed by the Lowry method, modified by the inclusion of 1% (w/v) sodium dodecyl sulfate in the incubation mixture to solubilize membrane proteins (33).

Preparation of EPR Samples

Membrane vesicles were suspended at a protein concentration of 30 mg ml⁻¹ in 100 mM MOPS and 5 mM EDTA (pH 7.0). Dithionite-reduced (5 mM) samples were incubated under argon at 23 °C for 5 min. Oxidized samples were prepared by incubating membranes in the presence of 0.2 mM potassium ferricyanide for 2 min. EPR spectra were recorded using a Bruker Spectrospin ESP-300 spectrometer equipped with an Oxford Instruments ESR-900 flowing helium cryostat. Instrument conditions and temperatures were as described in the individual figure legends.

Redox Potentiometry

Redox titrations were carried out under argon at pH 8.0 in 100 mM Tricine and 5 mM EDTA as described previously (34). Membranes were prepared in 100 mM MOPS and 5 mM EDTA (pH 7) (see above) were pelleted by ultracentrifugation and resuspended in the pH 8.0 buffer. The following redox mediators were used at a concentration of 50 μM: quinidine, 2,6-dichlorophenindophenol, 1,2-naphthoquinone, toluidine blue, phenazine methosulfate, thionine, duroquinone, methylene blue, resorufin, indigotrisulfonate, indigodisulfonate, anthraquinone-2-sulfonic acid, phenosafranine, benzyl viologen, and methyl viologen. All samples were prepared in 3-mm internal diameter quartz EPR tubes, were rapidly frozen in liquid nitrogen-chilled ethanol, and were stored under liquid nitrogen until use.

RESULTS

**Effect of the mobAB Mutation on the Presence of Mo-MGD in NarGH—**We have previously shown that in a mobAB mutant, no detectable Mo-MPT is assembled in the DmsABC holoenzyme (16). This result contrasts with reports that the NarGH dimer purified from a mobAB mutant contains Mo-MPT (13, 15). To reconcile these results, we subjected membrane-bound NarGHI holoenzyme to acid denaturation followed by I₃ and KIO₄ oxidation to produce the fluorescent Form A derivative of MPT (12). As a negative control for these experiments, we also studied Form A extracts from membranes from tungstate-grown E. coli.
and mutant background (c) NarGHI to ICPES analysis. Wild-type enzyme contains approximately 390 and 445 nm in the excitation and emission spectra, being the Form A derivative of Mo-MPT (12). Fig. 1A shows equivalent spectra obtained from membranes prepared from wild-type cells grown in the presence of 15 mM tungstate.

Fig. 1B shows fluorescent spectra of Form B extracts from wild-type (Fig. 1B, a and c) and mobAB mutant (Fig. 1B, b and d) NarGHI. In spectra of the wild-type sample, intense peaks were observed in the excitation spectrum at approximately 290 and 410 nm. In the emission spectrum, an intense peak was observed at 480 nm. These wavelengths are consistent with the species released from the wild-type NarGHI being the Form B derivative of Mo-MGD. In fluorescence spectra of the mobAB mutant NarGHI extract (Fig. 1B, b and d), the features of the fluorescence spectra are diminished, with a diffuse peak in the excitation spectrum at 365 nm and a small peak at approximately 475 nm in the emission spectrum. These results suggest that, in a mobAB mutant, Mo-MPT is either absent from NarGHI or is present at very low occupancy (see “Discussion”).

Effect of the Absence of Cofactor on EPR Spectra of the NarH [Fe-S] Clusters—To investigate the functional relationship between Mo-MGD cofactor of NarG and the NarH [Fe-S] clusters, we recorded EPR spectra at 12K of dithionite-reduced and ferricyanide-oxidized membrane samples containing wild-type and mobAB mutant NarGHI. Fig. 2a shows the spectrum of dithionite-reduced wild-type NarGHI in membranes at pH 7.0. We have previously demonstrated that in the absence of redox mediators, the $E_{m,n,s} = -400$ mV cluster remains oxidized in the presence of excess dithionite (24), and the spectrum in Fig. 2a corresponds to this partially reduced form of the enzyme. Fig. 2b shows an equivalent spectrum of mobAB mutant NarGHI. This spectrum corresponds to that of the fully reduced enzyme reported by Guigliarelli et al. (22). The spectrum of NarGHI in membranes from tungstate-grown cells is essentially identical to that of mobAB mutant NarGHI (data not shown), indicating that the cofactor-deficient form of the enzyme may have significantly altered [Fe-S] cluster midpoint potentials. Fig. 2, c and d, shows spectra of ferricyanide-oxidized wild-type and mobAB mutant NarGHI in membranes, showing the spectrum of the [3Fe-4S] cluster of NarH. Only minor differences are apparent between the two spectra, indicating that the EPR lineshape of the [3Fe-4S] cluster is not significantly altered by the absence of Mo-MGD.

Effect of the Absence of Cofactor on the Redox Potentiometry of the NarH [Fe-S] Clusters—Three observations prompted us to examine the effect of the absence of Mo-MGD on the redox potentiometry of the NarH [Fe-S] clusters: (i) the dithionite-reducibility of the lowest potential [4Fe-4S] cluster in cofactor-deficient NarGHI (Fig. 2); (ii) the presence of a [4Fe-4S] cluster in close proximity to the Mo-bisMGD of FdhF of E. coli (10); and (iii) the presence of a [4Fe-4S] cluster in NapA of T. pantotropha (25).

Fig. 3 shows representative EPR spectra of redox-poised samples at pH 8.0 for NarGHI-enriched membranes from a wild-type strain. Between +332 and +243 mV, the spectrum is dominated by the oxidized [3Fe-4S] cluster. The EPR spectrum of this cluster in membrane-bound NarGHI has a slightly axial line shape, with a peak at $g = 2.02 (g_z)$, and a peak-trough at $g = 1.99 - 1.96 (g_x)$, and $g = 1.99 (g_y)$. Between +243 and +129 mV, the spectrum of the [3Fe-4S] cluster diminishes and is substantially replaced.
by that of the highest potential [4Fe-4S] cluster of NarGHI. As reported by Guigliarelli et al. (22), the spectrum of this species appears to arise from two conformations, characterized by features at approximately $g = 2.05, 1.95, 1.87$ and $g = 2.01, 1.89, 1.87$, respectively (22). The spectrum is also complicated by an overlapping Mo(V) signal at approximately $g = 1.98$.

As the $E_h$ is further reduced from $1129$ to $23$ mV, the Mo(V) signal disappears, and the composite spectrum of the highest potential [4Fe-4S] cluster remains, revealing an additional peak at $g = 2.01$. Between $-83$ and $-268$ mV, there is a subtle increase in the intensity of the $g = 2.05$ peak and $g = 1.89 - 1.87$ peak-trough and a slight decrease in the intensity of the $g = 1.95$ peak-trough. Between $-371$ and $-540$ mV, the spectrum is extensively broadened by the interaction of the lowest potential [4Fe-4S] cluster with the other reduced clusters. This broadening is accompanied by the appearance of a deep trough at $g = 1.92$.

Similar redox-poised spectra are obtained from TP1000/pVA700 (mobAB) membranes (Fig. 4), the notable exceptions being: (i) the absence of a Mo(V) signal at $g = 1.98$ in any of the spectra recorded; and (ii) at approximately $+132$ mV, there is an absence of the spectrum of the reduced highest potential [4Fe-4S] cluster, and a significant amount of oxidized [3Fe-4S] cluster remains. These results suggest that the absence of Mo-MGD in the mobAB mutant NarGHI affects the midpoint potentials of the two highest potential clusters of NarH. Redox-poised membranes from LCB79/pVA700 grown in the presence
of 15 mM sodium tungstate to prevent Mo-MGD assembly yield essentially similar spectra to those reported for membranes from TP1000/pVA700 membranes (data not shown).

Plots of the intensity of the $g = 2.02$ peak of the oxidized [3Fe-4S] cluster versus redox potential ($E_m$) (Fig. 5A) indicate that in the wild-type and mobAB mutant enzymes, this center has two major subpopulations with different midpoint potentials at pH 8.0 ($E_{m,0}$). The data from the wild-type strain can be fitted to two components, a major one at $E_{m,0} = +180$ mV (69%) and a minor one at $E_{m,0} = +100$ mV (31%). In the mobAB mutant enzyme, the high potential component is altered ($E_{m,0} = +180$ mV (61%)), but there is a significant negative shift in the low potential component from $+100$ mV to $+55$ mV (39%).

An essentially identical shift of the low potential [3Fe-4S] component is observed in the tungstate-grown LCB79/pVA700 membranes. In this case, the high potential component has an $E_{m,0}$ of $+170$ mV (62%), and the low potential component has an $E_{m,0}$ of $+55$ mV (37%). These potentiometric results are summarized in Table I. Comparison of the $E_m = +132$ mV and $E_m = +327$ mV spectra of Fig. 4 indicates that the line shapes of the major and minor subpopulations of the [3Fe-4S] cluster are essentially identical and consistent with them both arising from overexpressed NarGHI in the mobAB membranes used herein.

Determination of the midpoint potentials of the [4Fe-4S] clusters of NarGHI is complicated by the interactions between these clusters as they become reduced (22). Plots of the intensity of the $g = 1.89$ peak versus $E_m$ appear to be representative of the redox state of the clusters at each potential (Fig. 5B). For the wild-type, mobAB mutant (Fig. 5B), and tungstate-grown membranes, these plots have two positive phases, corresponding to the reduction of the two highest potential [4Fe-4S] clusters, and one negative phase, corresponding to the reduction of the lowest potential [4Fe-4S] cluster. In the wild-type enzyme, the positive phases have $E_{m,0}$ values of $+130$ and $-55$ mV, and the negative phase has an $E_{m,0}$ of $-420$ mV. In the mobAB mutant membranes, the positive phases have $E_{m,0}$ values of $+80$ and $-35$ mV, whereas in the tungstate-grown membranes, the positive phases have $E_{m,0}$ values of $+80$ and $-30$ mV. The negative phases of these plots have $E_{m,0}$ values of $-420$, $-420$, and $-410$ mV for the wild-type, mobAB mutant, and tungstate-grown membranes, respectively (Table I). Thus, the most significant shifts elicited by the absence of Mo-MGD appear to be that of the $E_{m,0}$ of the highest potential [4Fe-4S] cluster from $+130$ to $+60$ mV and that of the minor [3Fe-4S] cluster component from $+100$ to $+55$ mV.

Midpoint Potentials of the Mo(V/VI) and Mo(V/IV) Couples of Wild-type NarGHI at pH 8.0—Fig. 6 shows a plot of the intensity of the $g = 1.98$ peak of the Mo(V) spectrum of NarGHI from LCB79/pVA700 membranes versus $E_m$. The EPR spectrum of the Mo(V) signal at 75K (data not shown) is essentially identical to the “high pH” form previously reported (22, 35, 36), and the potentiometric data can be fitted to two $E_{m,0}$ values of $+190$ mV for the Mo(VI) couple and $+95$ mV for the Mo(V/IV), in agreement with previously reported values (22). A more detailed study of the electrochemistry of the Mo-MGD cofactor of NarGHI and the effects of pH and NarG site-directed mutants will appear elsewhere.2

**DISCUSSION**

We have demonstrated herein that the effects of the mobAB mutation of E. coli TP1000 and growth of the wild-type strain

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2 A. Magalon, M. Asso, B. Guigliarelli, R. A. Rothery, G. Giordano, P. Bertrand, and F. Blasco, submitted for publication.

**TABLE I**

| Enzyme                | $E_{m,0}$ (mV) |
|-----------------------|---------------|
|                       | [3Fe–4S]$^a$ | [4Fe–4S]$^b$ | [4Fe–4S]$^b$ | [4Fe–4S]$^b$ |
| Wild-type (LCB79/pVA700)  | 100 (31%)     | 80 (35%)    | 35 (39%)    | 420          |
| mobAB (TP1000/pVA700)   | 180 (61%)     | 80 (35%)    | 35 (39%)    | 420          |
| Tungstate-grown (LCB79/pVA700) | 55 (39%)     | 80 (30%)    | 30 (31%)    | 410          |

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$^a$ The [3Fe–4S] $E_{m,0}$ values are derived from fits to the Nernst equation of the intensity of the $g = 2.02$ peak versus $E_m$ (Fig. 5A). Numbers in parentheses are the percentage contributions of the two components.

$^b$ The $E_{m,0}$ values of the two highest potential [4Fe–4S] clusters were determined from the positive phases of plots of the $g = 1.89$ peak trough versus $E_m$ (Fig. 5B).

$^c$ The $E_{m,0}$ of the lowest-potential [4Fe–4S] cluster was determined from the negative phase of plots of the $g = 1.89$ peak trough versus $E_m$ (Fig. 5B).

**FIG. 5.** Potentiometric redox titration of the NarGHI [Fe–S] clusters in the membranes prepared from wild-type [black square] and mobAB mutant (black triangle) cells. A, potentiometric titration of the [3Fe–4S] cluster. The $g = 2.02$ signal amplitude was plotted versus $E_m$. The wild-type titration [black square] could be fitted to two components with $E_{m,0}$ values of $+180$ mV (69%) and $+100$ mV (31%). The mobAB mutant titration [black triangle] could also be fitted to two components with $E_{m,0}$ values of $+180$ mV (61%) and $+55$ mV (39%). B, potentiometric titration of the [4Fe–4S] clusters measured by plotting the intensity of the $g = 1.89$ to $g = 1.87$ peak-trough. The $g = 1.89–1.87$ signal amplitude was plotted versus $E_m$. The wild-type titration [black square] was fitted to three components with $E_{m,0}$ values of $+130$ mV (76%), $-55$ mV (24%), and $-420$ mV (10%). The mobAB mutant titration was also fitted to three components with $E_{m,0}$ values of $+80$ mV (51%), $-35$ mV (49%), and $-420$ mV (10%).

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**TABLE I** NarGHI [Fe–S] cluster midpoint potentials in situ in LCB79 and TP1000 membranes

| Enzyme                | $E_{m,0}$ (mV) |
|-----------------------|---------------|
|                       | [3Fe–4S]$^a$ | [4Fe–4S]$^b$ | [4Fe–4S]$^b$ | [4Fe–4S]$^b$ |
| Wild-type (LCB79/pVA700)  | 100 (31%)     | 80 (35%)    | 35 (39%)    | 420          |
| mobAB (TP1000/pVA700)   | 180 (61%)     | 80 (35%)    | 35 (39%)    | 420          |
| Tungstate-grown (LCB79/pVA700) | 55 (39%)     | 80 (30%)    | 30 (31%)    | 410          |
amplitude of 6 Gpp. Other EPR conditions were as described for Fig. 2. The intensity of the LCB79/pVA700 membranes. In the absence of cofactor has a significant effect on the midpoint reductase, DmsABC (16). Furthermore, we have shown that the occupancy of Mo-MPT in NarGHI and NarGH from a mutan background. In these studies, it was assumed that NarGH was the source of Mo-MPT (13, 15). The absence of cofactor in NarGH and NarGHI from the mobAB mutant strain has to be reconciled with previous studies that have demonstrated reconstitution of BV:ni trate oxi doreductase activity by addition of Moba and GTP to NarGH isolated from a mobAB mutant background. In these studies, it was assumed that NarGH was the source of Mo-MPT (13, 15). The data presented herein and previous mobAB mutant reconst itution results can be reconciled if the observed turnover numbers in the reconstitution experiments correspond to a small fraction of the turnover number of the Mo-MGD-containing enzyme derived from a wild-type strain. It is also possible that the occupancy of Mo-MPT in NarGHI and NarGH from a mobAB mutant strain is different (cf. Fig. 1, A and B), with there being more cofactor present in the soluble dimer, as suggested by the increased relative fluorescence of samples derived from the mobAB mutant soluble dimer compared with those derived from NarGH in mobAB mutant membranes. The suggestion that there is a low occupancy of Mo-MGD in the mobAB mutant enzyme appears to be the simplest explanation that can reconcile the lack of detectable cofactor and the observed reconstitution of activity. Recent findings on the function of NarJ suggest that it functions to hold cofactor-deficient NarGH in a cofactor-binding conformation (19). NarJ is found to bind specifically to cofactor-deficient NarGH from a mobAB mutant (data not shown), and it is possible that the previously observed reconstitution in mobAB mutant NarGH arose from Mo-MPT provided by a Mo-MPT carrier protein associated with a NarGH-NarJ complex.

The absence of Mo-MPT from the mobAB mutant NarGH holoenzyme and NarGH dimer has implications for the final stages of cofactor biosynthesis and insertion into E. coli molybdenum. MPT is converted to Mo-MPT by the addition of molybdenum. Molybdenum is bound and transported into the cytoplasm of E. coli by the products of the modABCD operon (37, 38) and subsequently chelated by MPT. The presence of tungsten (as tungstate) prevents both molybdenum ligation by MPT and insertion of the organic component of the cofactor into NarGH. Previous studies have suggested that in the presence of tungstate, cofactor accumulates in the cytoplasm either as an "empty" MPT or as a tungsten-ligated form (39). Neither of these two forms appear to be able to assemble into NarGH (Fig. 1). In contrast to the conclusions drawn from earlier studies, our results with a mobAB mutant indicate that nucleotide attachment and metal chelation must precede cofactor insertion into NarGH. Since both the soluble (NarGH) (20, 22) and membrane-bound (NarGHI) (24, 40) forms of the enzyme can readily be studied and appear to contain Mo-MGD in a wild-type background, cofactor insertion most likely occurs prior to attachment of the NarGH dimer to the membrane anchor subunit (NarI). In the presence of tungstate or in a mobAB mutant background, it is clear that attachment of cofactor-deficient NarGH to the membrane-bound NarI...
is still able to occur, thus bypassing the blockage in Mo-MGD biosynthesis and insertion. Overall, the final stages of cofactor biosynthesis and insertion into NarGHI appear to be very similar to those proposed for its biosynthesis and insertion into DmsABC (16), except that in the latter case no system-specific chaperone has so far been identified.

We have evaluated the midpoint potentials of the NarH [Fe-S] clusters in the NarGHI holoenzyme at pH 8.0 in Tricine buffer. In previous studies of the soluble NarGH dimer (20–23), the midpoint potentials of the [Fe-S] clusters of NarH in the soluble NarGH holoenzyme have been determined to be +80 mV ([4Fe-4S] cluster), +60 mV ([3Fe-4S] cluster), −200 mV, and −400 mV ([4Fe-4S] clusters) in a Tris buffer at pH 8.3. Clearly, the data presented herein (Fig. 5) suggest that the \( E_m \) of the three highest-potential clusters appear to be significantly higher in membrane preparations of NarGHI compared with the soluble NarGH dimer. Three possible explanations exist for these differences: (i) the conformation of the NarH subunit is altered by the presence of the NarI membrane anchor subunit, resulting in higher \( E_m \) for the three highest potential clusters; (ii) a lack of solvent accessibility to the clusters in the membrane-bound enzyme causes the higher \( E_m \) values; and (iii) freezing-induced pH changes occur in samples prepared in the Tris buffer, as has been previously demonstrated by Williams-Smith et al. (41). To generate the potentiometric data presented herein, a switzeniron buffer was used (Tricine) instead of the anionic Tris buffer used in previous potentiometric studies of the NarGH dimer [Fe-S] clusters. Freezing of Tris buffers can result in an apparent pH of approximately +2.3 (41), which could result in an alkalization of the Tris buffer previously used in studies of NarGH to a pH of approximately 9.6.

The effect of the mobAB mutation on the \( E_m \) of the highest-potential [4Fe-4S] cluster suggests that this cluster may be located in close proximity to the Mo-MGD cofactor of NarGHI. The absence of Mo-MGD causes a \( \Delta E_m \) of −50 mV for this cluster. This cluster is ligated by the first three Cys residues of Cys group I of NarH (21) and the last Cys residue of group IV (23), and its absence in NarH mutants dramatically inhibits, but does not abolish, electron transfer through NarGHI to nitrate. It is therefore possible that it functions in a similar manner to the [4Fe-4S] cluster of FdhF (10), acting as the direct electron donor to the Mo-MGD. The effect of the absence of cofactor on the \( E_m \) of the highest potential [4Fe-4S] cluster is somewhat surprising in light of its effect on the dithionite-reducibility of the lowest potential [4Fe-4S] cluster (23). We anticipated that loss of cofactor would raise the \( E_m \) of this low potential center. A previous study (24) has demonstrated that the presence of redox mediators such as those routinely used in potentiometric titrations allows the \( E_m \) of −420 mV to become accessible to reducing equivalents derived from dithionite. However, it appears from the results presented herein that the dithionite accessibility of the \( E_m \) is related to the absence of Mo-MGD, suggesting that this might be achieved via the empty cofactor-binding pocket. This would place the \( E_m \) of −420 mV in the vicinity of this pocket in close proximity to the highest potential [4Fe-4S] cluster. This suggestion is in agreement with the model for [Fe-S] cluster ligation proposed by Guigliarelli et al. (23) in which the Cys groups I and IV provide ligands to the highest and lowest potential [4Fe-4S] clusters, respectively. It is also in agreement with the broadening of the features of the spectrum of the highest potential cluster upon reduction of the lowest potential cluster, which suggests that these two clusters might form an eight-iron ferredoxin-like pair within NarGHI (23).

The observed shift in the \( E_m \) of the low potential component of the [3Fe-4S] cluster from 100 mV in the wild-type enzyme to 55 mV in the mobAB mutant and tungstate-grown enzyme is probably a consequence of the proximity of the [3Fe-4S] cluster to the highest potential [4Fe-4S] cluster in the enzyme. This phenomenon may be a manifestation of the negative redox cooperativity observed between the highest potential [4Fe-4S] cluster and the [3Fe-4S] cluster in previous studies of the NarGH dimer (22). This is supported by the similar magnitudes of the observed shifts for the [4Fe-4S] cluster and subpopulation of the [3Fe-4S] cluster (23). This is also in agreement with the broadening of the features of the spectrum of the highest potential cluster upon reduction of the lowest potential cluster, which suggests that these two clusters might form an eight-iron ferredoxin-like pair within NarGHI (23).

Our investigation of the [Fe-S] cluster \( E_m \) values of the wild-type and mobAB mutant NarGHI in situ in membrane vesicles was prompted by the dithionite reducibility of the \( E_m \) of −420 mV cluster in cofactor-deficient NarGHI in the absence of redox mediators at pH 7.0. Potentiometric studies were carried out at pH 8.0, as this is close to the pH used in previous EPR determinations of the NarH [Fe-S] cluster. Although differences in the \( E_m \) values were observed between the wild-type and cofactor-deficient enzymes, it should be noted that the EPR spectra of wild-type and mobAB mutant NarGHI in their various redox states are nearly identical (cf. Figs. 3 and 4), indicating that neither the structure of the [Fe-S] centers nor their relative arrangement is modified by the absence of Mo-MGD.

Overall, the data presented herein indicate that the final stages of Mo-MGD biosynthesis and insertion into NarGHI are similar to those proposed for DmsABC (16), except that DmsABC appears to accept Mo-MGD without the aid of a Nar-like chaperone. The absence of Mo-MGD from NarGHI has a significant effect on the \( E_m \) values of the highest potential [4Fe-4S] cluster and on a subpopulation of the [3Fe-4S] cluster. These results represent an important step in delineating (i) the pathway of Mo-MGD cofactor biosynthesis and insertion and (ii) the electron transfer pathway to the cofactor through the [Fe-S] clusters of NarH.

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The Molybdenum Cofactor of Escherichia coli Nitrate Reductase A (NarGHI): EFFECT OF A mobAB MUTATION AND INTERACTIONS WITH [Fe-S] CLUSTERS

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