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Coordination of selenium to molybdenum in formate dehydrogenase H from *Escherichia coli*

[selenocysteine/selenoenzyme/Mo(V) electron paramagnetic resonance signal/molybdopterin]

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ABSTRACT **Formate dehydrogenase H from *Escherichia coli* contains multiple redox centers, which include a molybdopterin cofactor, an iron–sulfur center, and a selenocysteine residue (SeCys-140 in the polypeptide chain) that is essential for catalytic activity. Here we show that addition of formate to the native enzyme induces a signal typical of Mo(V) species. This signal is detected by electron paramagnetic resonance (EPR) spectroscopy. Substitution of ^{77}Se for natural isotope abundance Se leads to transformation of this signal, indicating a direct coordination of Se with Mo. Mutant enzyme with cysteine substituted at position 140 for the selenocysteine residue has decreased catalytic activity and exhibits a different EPR signal. Since determination of the Se content of wild-type enzyme indicates ≈1 gram atom per mol, we conclude that it is the Se atom of the SeCys-140 residue in the protein that is coordinated directly with Mo. The amino acid sequence flanking the selenocysteine residue in formate dehydrogenase H is similar to a conserved sequence found in several other prokaryotic molybdopterin-dependent enzymes. In most of these other enzymes a cysteine residue, or in a few cases a serine or a selenocysteine residue, occurs in the position corresponding to SeCys-140 of formate dehydrogenase H. By analogy with formate dehydrogenase H in these other enzymes, at least one of the ligands to Mo should be provided by an amino acid residue of the protein. This ligand could be the Se of a selenocysteine residue, sulfur of a cysteine residue, or, in the case of a serine residue, oxygen.**

Molybdenum-containing enzymes are widely distributed in nature and play crucial roles in living organisms and in biogeochemical cycles (1–4). Among these enzymes are those that have a Mo atom coordinated to a member of a family of pterins called molybdopterins (5–7).

The coordination sphere of Mo has been extensively studied by electron paramagnetic resonance (EPR) spectroscopy and x-ray absorption spectroscopy (3, 4, 8). Most of the enzymes studied have been shown to have dioxo- or oxosulfido ligands to Mo. In addition, a few sulfo ligands were found but whether these were sulfo groups present in the pterin moiety, in cysteine (Cys) residues of the protein, or in additional cofactors is unknown. Because of the apparent lack of homology between potential Mo-binding domains of eukaryotic and prokaryotic molybdopterin-containing enzymes (9), it is unclear at present how Mo and molybdopterins are bound to proteins.

Formate dehydrogenase H ([Se]FDH_H), an 80-kDa enzyme from *Escherichia coli*, contains a single selenocysteine residue (SeCys) encoded by UGA (10, 11) and in addition is a molybdopterin guanine dinucleotide and iron–sulfur-containing enzyme (12–15). A mutant form of this enzyme, [S]FDH_H, that contains Cys instead of SeCys was obtained by replacing the TGA codon with TGC (16). This substitution of sulfur for Se led to a considerable decrease in catalytic activity (k_{cat} = 2800 sec^{-1} for [Se]FDH_H and k_{cat} = 9 sec^{-1} for [S]FDH_H; ref. 14). A mutant form of the enzyme containing serine (Ser) in place of SeCys was catalytically inactive (16). From a number of studies, it is evident that the SeCys-140 residue is essential for fully active [Se]FDH_H (14) and its location in the vicinity of the Mo center should be important.

Recently, we have shown that Mo in nicotinic acid hydroxylase from *Clostridium barkeri* is directly coordinated with Se (17). In this enzyme, Se is present in an unidentified labile form instead of in SeCys (17, 18). This Se is involved in the catalytic cycle of nicotinate hydroxylation but is not essential for the NADPH oxidase activity of the enzyme (17–19). In the present report, we show that Mo in [Se]FDH_H also is directly coordinated with Se, but in this case it is the Se of the SeCys residue in the protein that is the ligand to Mo.

From a comparison of the deduced amino acid sequences for several prokaryotic molybdopterin-containing enzymes with the sequence of [Se]FDH_H (10, 11), it is concluded that there is a conserved Mo-binding amino acid residue that can be either SeCys, Cys, or Ser.

MATERIALS AND METHODS

Cell Culture. For EPR studies *E. coli* strain FM911 containing plasmid pFM20 (12, 16) served as the source of wild-type [Se]FDH_H and *E. coli* mutant strain WL31153 containing plasmid pFM201 (14, 16) was the source of mutant formate dehydrogenase H, [S]FDH_H. These strains were cultured as described (12, 16). For estimation of the extent of Se incorporation in [Se]FDH_H and [S]FDH_H *E. coli* strain FM911 containing plasmid pFM20 and strain FM911 containing plasmid pFM201 were cultured in medium containing 0.5 μM [^{75}Se]selenite (0.8 mCi/μmol; 1 Ci = 37 GBq; University of Missouri Research Reactor Facility). Plasmid pFM20 contains the wild-type [Se]FDH_H gene and plasmid pFM201 contains the mutant gene with TGC in place of TGA. *E. coli* strain FM911 is deleted for the [Se]FDH_H gene but can incorporate Se specifically into proteins, whereas strain WL31153 cannot because it lacks SeCys synthase required for selenocystyl-tRNA formation and the cognate elongation factor (selB gene product) required for SeCys insertion at UGA (16, 20). [Se]FDH_H enriched with ^{77}Se was produced by growth of strain FM911 containing plasmid pFM20 in a medium containing 1 μM [^{77}Se]selenite. The [^{77}Se]selenite

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Abbreviations: [Se]FDH_H, selenocysteine-140 containing formate dehydrogenase H from *E. coli*; [S]FDH_H, cysteine-140 containing mutant formate dehydrogenase H from *E. coli*; SeCys, selenocysteine residue; EPR, electron paramagnetic resonance.

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was prepared by oxidation of elemental ^{77}Se (94.75% from Oak Ridge National Laboratories) with a small amount of concentrated nitric acid. To avoid induction of nitrate reductase-linked formate dehydrogenase N, FDH_N (21), the nitrate content of the medium was maintained below 15 μM.

Purification of Enzymes. [Se]FDH_H and [S]FDH_H were isolated as described by Axley *et al.* (12). Because of the extreme oxygen sensitivity of [Se]FDH_H and [S]FDH_H, all isolation and experimental procedures (except PAGE analyses) were performed in a nitrogen atmosphere in the National Institutes of Health Anaerobic Laboratory (22) operating at 0–1 ppm of oxygen. To remove the last traces of contaminating oxygen, all solutions were sparged for 15 min with oxygen-free argon prior to use.

Other Methods. Electronic absorption spectra of enzymes were recorded with a Hewlett–Packard model 8452A spectrophotometer. Protein concentrations were estimated from absorptivity measurements at 280 nm, assuming a solution containing 1 mg of FDH_H per ml gives an A_{280} value of 2.5 (13). Benzylviologen-linked formate dehydrogenase activities of [Se]FDH_H and [S]FDH_H were monitored as described (12). SDS/PAGE analyses of enzyme preparations were carried out in air on 12% gels (NOVEX, San Diego). Proteins were stained with Coomassie blue. For ^{75}Se detection in gels, a PhosphorImager (Molecular Dynamics) was used. The ^{75}Se content of protein fractions was determined with a Beckman 5000 gamma counter.

Preparation of EPR Samples. Enzyme solutions in 20 mM Mes/50 mM potassium phosphate buffer, pH 6.5/2 mM dithiothreitol/3 mM sodium azide were concentrated to ≈10 mg of protein per ml using 50-ml Amicon concentrating cells and Centricon-30 microconcentrators. The ability of azide to protect enzyme from inactivation by oxygen (12) was confirmed in the present study. Prior to the EPR measurements, enzyme solutions were frozen in liquid nitrogen. Formate-treated enzyme samples were prepared by incubating the enzyme for 1 min with 20 mM sodium formate at room temperature.

EPR Spectroscopy. EPR spectra were recorded with a Bruker X-band ESP300 spectrometer (17) operating with a microwave rectangular TE102 cavity (ER 4102 ST) and equipped with a 5352B microwave frequency counter (Hewlett–Packard). The EPR spectra of Mo(V) species were accumulated at 130 K, 2.5 G modulation amplitude, 0.41 ms time constant, 42 s sweep time, 700 G sweep width, 9.46 GHz microwave frequency, and 15.2 mW microwave power.

RESULTS

The frozen solution of "as-isolated" [Se]FDH_H is EPR silent at 40–130 K. At 130 K, formate-treated [Se]FDH_H exhibits the EPR signal presented in Fig. 1, spectrum a. The signal has an intense but poorly resolved component in the $g = 2$ region and a much weaker component with g_z of 2.094. At higher gain a number of satellites are observed (Fig. 1, spectrum b). The hyperfine features are characteristic of $^{95,97}Mo(V)$ paramagnetic species (3). The formate-induced signal of [Se]-FDH_H is called the 2.094 signal. Comparison of the g values of the 2.094 signal with those determined for other Mo-containing enzymes reveals that the unusually high g_z value of 2.094 is not characteristic of known Mo-containing enzymes (23). However, the high g_z value of 2.067 was observed in nicotinic acid hydroxylase, which contains Se coordinated to Mo (17). To determine whether the unusually high g_z value

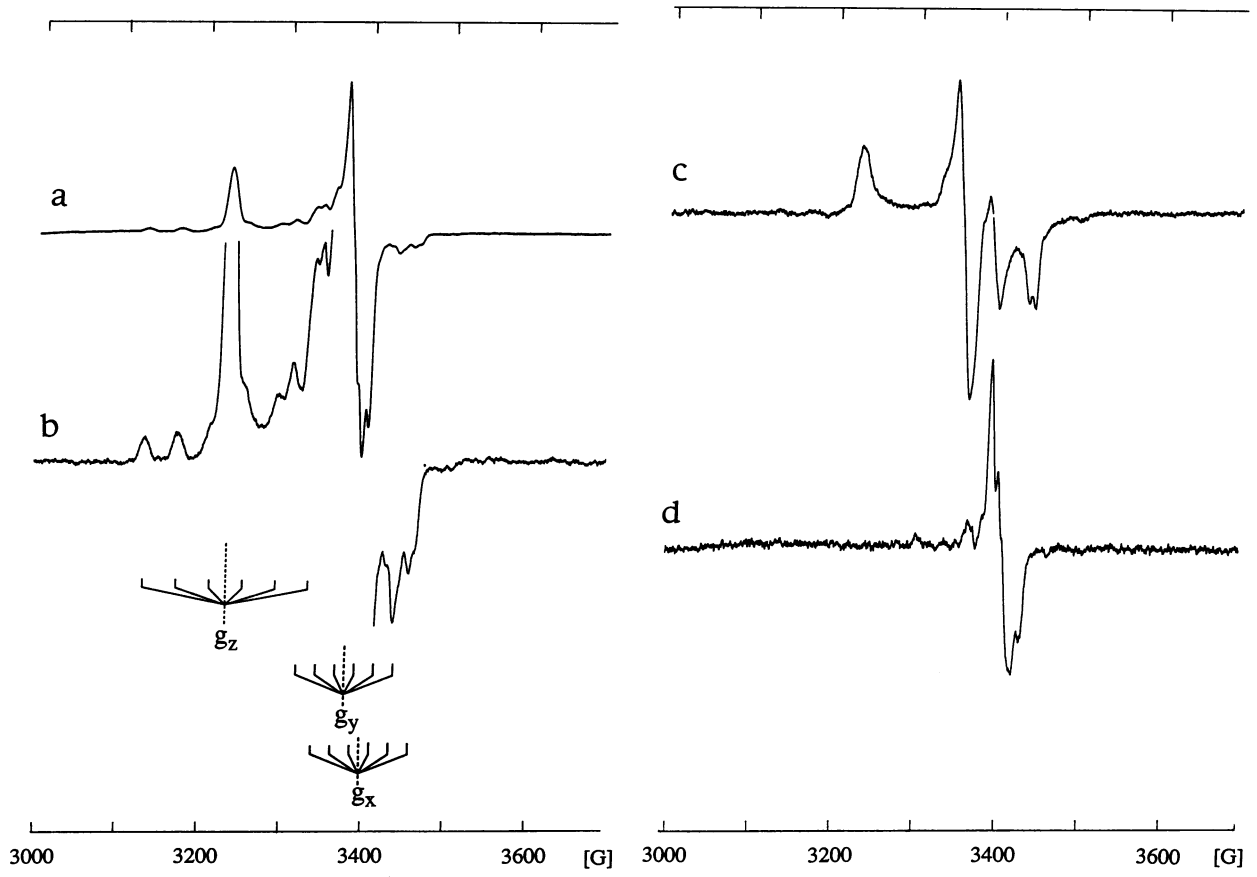

FIG. 1. EPR spectra of [Se]FDH_H and [S]FDH_H. Spectrum a, [Se]FDH_H in the presence of 20 mM sodium formate. Spectrum b, same as spectrum a but at higher gain (×8). Dashed lines indicate g_x, g_y, and g_z components of Mo(V) species with $I = 0$ ($g_x = 1.989$, $g_y = 2.001$, $g_z = 2.094$). Short sticks indicate hyperfine EPR features from Mo(V) isotopes with $I = 5/2$. Spectrum c, ^{77}Se-enriched [Se]FDH_H in the presence of 20 mM sodium formate. Spectrum d, mutant [S]FDH_H in the presence of 20 mM sodium formate.

Table 1. Sequence domains of conserved amino acid residues involved in binding to Mo

Enzyme	Location of domains	Conserved domain	Conserved amino acid residue	Ref.
FDHH *E.c.*	129–147	GTNNVDCCARV**U**HGPSVAG	SeCys-140	10
FDHN *E.c.*	185–203	GMLAVDNQARL**U**HGPTVAS	SeCys-196	24
FDH *W.s.*	175–193	GTNNLDTIARI**C**HAPTVAG	Cys-186	25
FDH *M.f.*	121–139	GTHNIDHCARL**C**HGPTVAG	Cys-132	28
NRG *E.c.*	211–229	GGTCLSFYDWY**C**DLPPASP	Cys-222	9
NRZ *E.c.*	211–229	GGTCLSFYDWY**C**DLPPASP	Cys-222	9
NR *K.p.*	117–135	GAANIDTNSRL**C**MSSAVTG	Cys-128	26
NR *A.e.*	170–188	RSNNIDPNARH**C**MASAAAG	Cys-181	27
DMSR *E.c.*	166–183	GG-YLNHYGDY**S**SAQIAEG	Ser-176	29
BSR *E.c.*	100–117	GG-YTGHLGDY**S**TGAAQAI	Ser-110	30

Prokaryotic enzymes dependent on a molybdopterin cofactor are as follows: FDHH *E.c.*, formate dehydrogenase H from *E. coli*; FDHN *E.c.*, α subunit of formate dehydrogenase N from *E. coli*; FDH *W.s.*, α subunit of formate dehydrogenase from *Wolinella succinogenes*; FDH *M.f.*, α subunit of formate dehydrogenase from *Methanobacterium formicicum*; NRG *E.c.*, α subunit of respiratory nitrate reductase from *E. coli*; NRZ *E.c.*, second nitrate reductase from *E. coli*; NR *K.p.*, α subunit of nitrate reductase from *Klebsiella pneumoniae* M5a1; NR *A.e.*, α subunit of nitrate reductase from *Alcaligenes eutrophus* H16; DMSR *E.c.*, α subunit of dimethyl sulfoxide reductase from *E. coli*; BSR *E.c.*, α subunit of biotin sulfoxide reductase from *E. coli*; U, SeCys residue. This table is a summary of the sequence analyses presented in refs. 9 and 24–27.

of 2.094 is indicative of coordination of Se with Mo(V) ion in formate dehydrogenase, the ^{77}Se-enriched [Se]FDH$_H$ was studied. As shown in Fig. 1, spectrum c, substitution of ^{77}Se (I = 1/2) for the natural isotope abundance Se leads to a splitting of the high-field components of the 2.094 signal, providing evidence of direct coordination of Se with Mo. Similar experiments with the mutant [S]FDH$_H$ containing Cys in place of SeCys showed that the "as-isolated" form of this enzyme exhibited an EPR signal different from the 2.094 signal (Fig. 1, spectrum d). Formate addition did not affect the signal of this mutant enzyme. These data support our conclusion concerning the involvement of Se as a ligand to Mo.

If Se coordination with Mo requires a form analogous to the labile Se species present in nicotinic acid hydroxylase, then there might be another Se compound present in [Se]FDH$_H$ in addition to the SeCys residue in the polypeptide. To determine the Se content and chemical form(s) of Se in formate dehydrogenase, [Se]FDH$_H$ and [S]FDH$_H$ were isolated from ^{75}Se-labeled cells. Based on a comparison of the ^{75}Se content of the selenite in the culture medium and the amount of radioactivity in the isolated proteins, the calculated molar ratio of Se to protein was ≈1 for [Se]FDH$_H$ and ≈0.1 for [S]FDH$_H$. SDS/PAGE analysis of the purified proteins did not reveal the presence of any dissociable ^{75}Se-containing moiety in either wild-type or mutant enzyme (data not shown), whereas under the same conditions a radioactive Se compound that migrates near the tracking dye dissociates from ^{75}Se-labeled nicotinic acid hydroxylase during SDS/PAGE analysis (17).

From these experiments, it is clear that only about one Se atom per enzyme molecule is present in formate dehydrogenase H and this is equivalent to the amount of Se present in the single SeCys residue in the protein. Thus, we conclude that in this enzyme Mo is coordinated with the Se atom of SeCys.

DISCUSSION

Our data provide strong evidence that a direct coordination of the Se atom of SeCys-140 with Mo gives rise to the 2.094 EPR species detected in formate dehydrogenase H of *E. coli*. It will be interesting if this unusual type of coordination of Mo with an amino acid residue in a molybdopterin-dependent enzyme also occurs in other enzymes of this class.

Recently, Wootton *et al.* (9) showed that in the deduced primary sequences of six Mo-containing enzymes from prokaryotes there is a region of conserved amino acid sequence in which SeCys, Cys, or Ser occurs in an invariant position. The gene sequences of additional molybdopterin-containing enzymes from prokaryotes (24–27) also fit this pattern (Table 1). Based on our finding that Se in the SeCys-140 residue of formate dehydrogenase H of *E. coli* is coordinated with Mo, we predict that the corresponding amino acid residue in these other enzymes also supplies a ligand to Mo. Thus, in formate dehydrogenase H from *E. coli* (24) Se of SeCys-196 should be the ligand to Mo, whereas in two other formate dehydrogenases that contain Cys in place of SeCys (25, 28) the ligand should be sulfur. Likewise, sulfur of the Cys residues present in nitrate reductases from *E. coli* and other bacteria (9, 26, 27, 31) (Table 1) can be predicted to serve as the ligand to Mo. Although the similarity of domains of dimethyl sulfoxide reductase (29) and biotin sulfoxide reductase (30) to those of the SeCys or Cys-containing enzymes is weaker, it is likely that in these enzymes the ligand to Mo is the oxygen atom of a Ser residue (9). Structurally, the three amino acids in question—SeCys, Cys, and Ser—are similar and differ only by the one atom involved in the coordination to Mo. Although a number of amino acids were suggested to be involved in Mo binding in eukaryotic molybdopterin-dependent enzymes (32), similar predictions cannot be made for these enzymes because of lack of identification of any similar conserved domain (9).

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