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January 1994

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Nicotinic acid hydroxylase from *Clostridium barkeri*: Electron paramagnetic resonance studies show that selenium is coordinated with molybdenum in the catalytically active selenium-dependent enzyme

[selenoenzyme/Mo(V) electron paramagnetic resonance signal/molybdopterin/FeS clusters/labile selenium]

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ABSTRACT   Nicotinic acid hydroxylase from *Clostridium barkeri* contains selenium in an unidentified form that is dissociated as a low molecular weight compound upon denaturation of the enzyme. Other cofactors of this enzyme are molybdopterin, FAD, and iron–sulfur clusters. In the current study, we show that the enzyme, as isolated, exhibits a stable Mo(V) electron paramagnetic resonance (EPR) signal ("resting" signal) and that this signal is correlated with the selenium content and nicotinate hydroxylase activity of the enzyme. Substitution of ^{77}Se for normal selenium isotope abundance results in splitting of the Mo(V) EPR signal of the native protein without affecting the iron signals of the FeS clusters. The Mo(V) EPR signal and nicotinic acid hydroxylase activity of enzyme isolated from cells grown in selenium-deficient medium are barely detectable. In contrast, the EPR signals of the FeS clusters, the electronic absorption spectrum, the NADPH oxidase activity, and the chromatographic behavior are changed little and are typical of active selenium-containing enzyme. An EPR signal indicative of the presence of molybdenum in the selenium-deficient enzyme also is exhibited. From these results, we conclude that a dissociable selenium moiety is coordinated directly with molybdenum in the molybdopterin cofactor and, moreover, this selenium is essential for nicotinic acid hydroxylase activity.

Selenium is present in several enzymes as a highly specific component that is essential for catalytic activity. In some of these enzymes selenium occurs in the polypeptide in the form of selenocysteine inserted cotranslationally as directed by the UGA codon (1–3). A few other selenium-dependent enzymes [i.e., nicotinic acid hydroxylase and xanthine dehydrogenase present in certain anaerobic bacteria (4, 5)] contain selenium in a dissociable form that has not been identified (6). These enzymes also contain iron–sulfur clusters, a molybdopterin cofactor, and FAD, but because of their marked oxygen sensitivity, they have not been studied in detail. Nicotinic acid hydroxylase exhibits two other catalytic activities in addition to conversion of nicotinic acid to 6-hydroxynicotinic acid with concomitant reduction of NADP$^+$ (7).
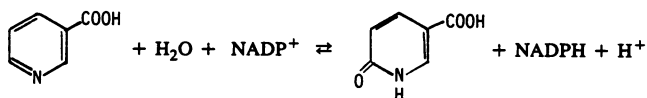
With NADPH as substrate, the enzyme exhibits NADPH oxidase activity in the presence of oxygen or diaphorase activity with various dyes as electron acceptor.

It was observed earlier (4, 6) that growth of *Clostridium barkeri* in a selenium-supplemented medium resulted in ele-

vated levels of nicotinic acid hydroxylase activity in cell extracts. The enzyme was labeled with ^{75}Se when cells were grown in the presence of [^{75}Se]selenite (6), but unlike several other selenoenzymes, the radiolabel was not present in the polypeptide chain. Instead, when the labeled protein was heat-denatured or treated with chaotropic agents, the ^{75}Se was released from the enzyme in the form of a low molecular mass compound (6, 8). When the ^{75}Se-labeled native protein was inactivated by treatment with various alkylating agents, the selenium was recovered almost quantitatively in the form of the corresponding dialkylselenides, suggesting its presence as a cofactor component. Among the structures that would be consistent with these chemical properties are the selenium analog of an iron–sulfur cluster, selenium as an outer ligand of molybdenum in molybdopterin, a selenotrisulfide (RS–Se–SR) complex, or RS–Se$^-$. Although such selenium compounds have been prepared *in vitro*, their specific occurrence in native proteins is not documented. Results of the study reported here clearly show that selenium is present in the enzyme as a ligand of molybdenum and, moreover, it is essential for the nicotinic acid hydroxylase activity of the enzyme.

MATERIALS AND METHODS

C. barkeri was cultured anaerobically in a nicotinic acid-mineral salts medium supplemented with 0.6–1% Difco yeast extract and 1 μM selenite as described (6). Cells grown in the same medium without added selenite were used as the source of low selenium enzyme. Selenium-deficient enzyme was purified from cells cultured in the absence of selenite in a low sulfur medium containing an autolysate prepared from selenium-deficient Torula yeast (ICN) instead of the usual Difco yeast extract. Growth of *C. barkeri* and consumption of nicotinate were slow in this medium and a lower than normal yield of purified enzyme was obtained from the cells. For ^{77}Se-enriched enzyme, bacteria were cultured in a medium containing 1 μM [^{77}Se]selenite in place of normal Se isotope abundance selenite. ^{77}Se (94.75%) from Oak Ridge National Laboratories was oxidized to selenite with 71% (vol/vol) nitric acid and added directly to the culture medium. The resulting nitrate concentration (50–100 μM) in the medium did not inhibit growth of *C. barkeri*. For enzyme enriched with ^{95}Mo, molybdate in the medium was replaced with [^{95}Mo]molybdate prepared by oxidation of 96.47% ^{95}Mo (a gift from W. Orme-Johnson, Massachusetts Institute of Technology). Two-liter cultures containing 1 μM [^{75}Se]selenite

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Abbreviation: EPR, electron paramagnetic resonance.
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(0.5 mCi; 1 Ci = 37 GBq; Research Reactor Facility, University of Missouri) were the source of radioactive cells for preparation of ^{75}Se-labeled enzyme.

Nicotinic acid hydroxylase was isolated as described by Dilworth (6) except HPLC columns were used and a final hydroxyapatite HPLC step was added to obtain pure enzyme. Nicotinate-dependent reduction of NADP$^+$ was monitored in oxygen-free solutions as described by Holcenberg and Stadtman (7). This assay was used to follow purification of enzyme from selenium-supplemented cells and also for low selenium enzyme. The nicotinic acid hydroxylase activity of selenium-deficient enzyme from cells grown in the Torula yeast extract medium was too low to measure. Therefore, purification was monitored by measuring NADPH oxidase activity and by spectral analysis of fractions from HPLC profiles. The radioactivity of fractions containing ^{75}Se-labeled enzyme was determined in a Beckman 5000 γ counter. Native and SDS/PAGE analyses of enzyme preparations were carried out using 12% gels (Novex, San Diego). For ^{75}Se detection in gels a PhosphorImager, Molecular Dynamics, was used. Prior to electron paramagnetic resonance (EPR) measurements, enzyme samples were placed in EPR tubes with an internal diameter of 3.8 mm and frozen in liquid N$_2$. EPR spectra were recorded on a Bruker (Billerica, MA) ESP-300 spectrometer operating on X-band and equipped with a 5352B microwave frequency counter (Hewlett-Packard). Magnetic field calibration was made with a diphenylpicrylhydrazyl standard. Low-temperature measurements (<77 K) were performed with an Oxford Instruments ESR910 cryostat. For detection of spectra at 130 K, a Bruker variable-temperature unit, ER4111 VT, continuous-flow liquid nitrogen cryostat was used. A modulation frequency of 100 kHz, a microwave frequency of 9.45 GHz, and modulation amplitudes of 2.5 G and 5 G (1 G = 0.1 mT), for detection of Mo(V) and FeS signals, respectively, were used.

RESULTS AND DISCUSSION

Levels of nicotinic acid hydroxylase in crude cell extracts were significantly elevated when bacteria were grown in selenium-supplemented culture medium (Table 1). Dependency on selenium was particularly marked when an extract prepared from selenium-deficient Torula yeast was used as the source of required unidentified nutrients (9). Activities of purified enzymes isolated from these extracts (Table 1) clearly show that selenium is essential for nicotinic acid hydroxylase activity but not for NADPH oxidase activity. These results extend those of studies reported previously (4, 6) showing a correlation of increased nicotinate hydroxylase activity with selenium supplementation of culture media.

Table 1. Effect of selenium supplementation of growth medium on nicotinic acid hydroxylase activity of enzyme preparations

| Growth medium component(s) | Enzyme activity | | | |
| | Crude extract | | Pure enzyme | |
	Hydroxylase	Oxidase	Hydroxylase	Oxidase
Difco yeast + Se	0.20*	0.24	20*	7*
	0.32†	0.24†		
Difco yeast − Se	0.03	0.25	4.4	2.8
	0.02†	0.12†		
Torula yeast + Se	0.29	0.12	—	—
Torula yeast − Se	0.003	0.16	0.18	3.5

Hydroxylase activity (nicotinic acid-dependent reduction of NADP$^+$), μmol NADPH per min per mg of protein; oxidase activity (NADPH oxidation by oxygen), μmol per min per mg of protein (6). Selenium was added as 0.5–1.0 μM selenite.
*Average value of several preparations.
†Data are from Dilworth (6).

PAGE analysis of the native protein (Fig. 1) shows comigration of ^{75}Se and protein. A molecular weight of ≈160,000 is estimated by comparison with the migrations of protein standards. SDS/PAGE analysis (Fig. 1) shows that the enzyme is a complex of four dissimilar subunits with estimated molecular weights of 50,000, 37,000, 33,000, and 23,000. Of particular importance is the fact that ^{75}Se is completely dissociated from protein in the SDS gel and instead migrates near the tracking dye. Quantitative removal of ^{75}Se from nicotinic acid hydroxylase by SDS treatment and migration of radioactivity ahead of bromophenol blue tracking dye on SDS/PAGE analysis also were observed by Dilworth (6). This property and the fact that all of the selenium was recovered as the corresponding symmetrical dialkylselenides after inactivation of enzyme by treatment with alkylating agents (6) clearly show that selenium occurs in nicotinic acid hydroxylase in an unusual cofactor form.

The occurrence of selenium in nicotinic acid hydroxylase as a dissociable cofactor, instead of as a UGA-directed selenocysteine residue in a polypeptide, affords an explanation of the selenium-independent synthesis of a form of the enzyme that is indistinguishable in many of its properties from the active hydroxylase. The two protein forms show similar chromatographic behavior at every isolation step, their electronic absorption spectra indicate similar contents of FAD and iron–sulfur centers (data not shown), both contain molybdenum (see later), and both oxidize NADPH with oxygen or artificial dyes as electron acceptors. Attempts so far to regenerate the active hydroxylase form of the enzyme by addition of potential selenium sources to "apoenzyme" under various conditions have not been successful.

The actual selenium content of fully active nicotinic acid hydroxylase is difficult to determine in view of the tendency of the enzyme to lose variable amounts of selenium as well as other cofactors during isolation and storage. A calculated molar ratio of selenium to enzyme of 0.4–0.8 was estimated from data for ^{75}Se-labeled preparations in the present study. Dilworth (6) reported a range of 0.1–0.5 equivalent of selenium per mol of enzyme calculated on the basis of a molecular weight of 300,000 determined earlier by ultracentrifugational
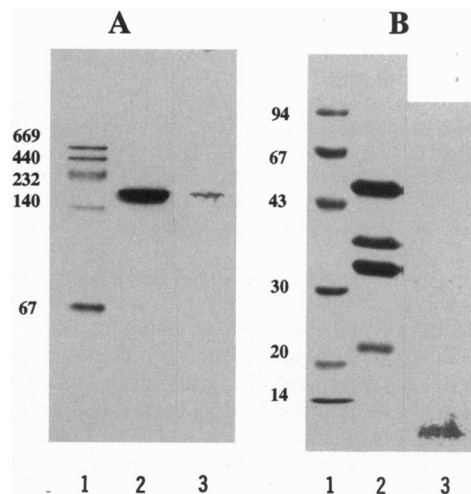

FIG. 1. PAGE analysis of active ^{75}Se-labeled nicotinic acid hydroxylase. (*A*) PAGE (12% gel) of native enzyme (lanes 2 and 3) and standards (lane 1) under nondenaturing conditions. (*B*) SDS/PAGE (12% gel) of enzyme (lanes 2 and 3) and standards (lane 1) after heating in 2% (wt/vol) SDS/2.5% (vol/vol) 2-mercaptoethanol buffer. Coomassie blue staining (lanes 1 and 2) and PhosphorImage detection of ^{75}Se (lane 3) of each gel is shown. The native protein band in an unstained gel slice also was detected by nicotinate-dependent reduction of a tetrazolium dye as described by Dilworth (6). The molecular weights (× 10^{-3}) of standards in lane 1 of each gel are shown.
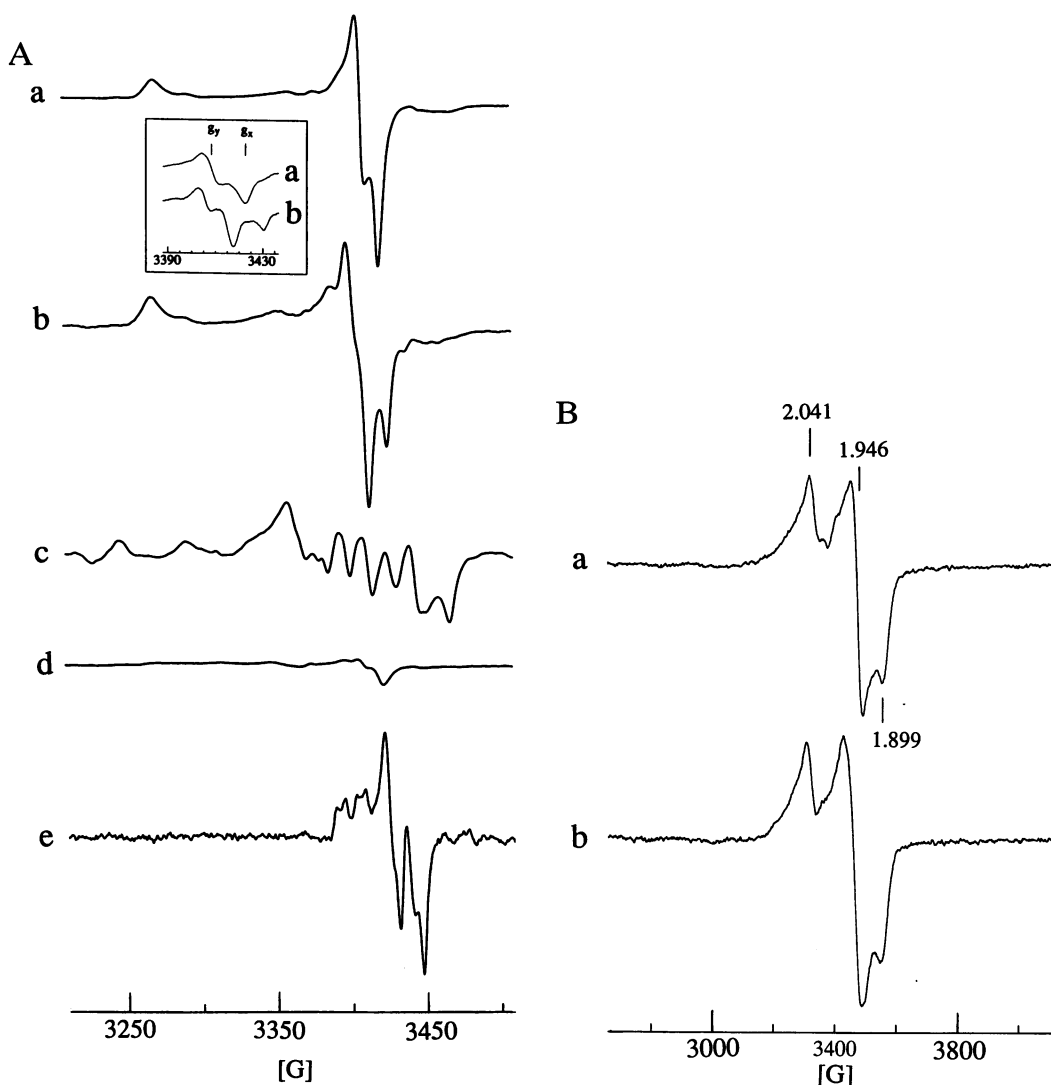
FIG. 2. EPR spectra of Mo(V) and [2Fe–2S] clusters of Se-containing and Se-deficient nicotinic acid hydroxylase. (*A*) Mo(V) spectra recorded at 130 K and 7.8-mW microwave power. Spectra: a, signal of native "as isolated" nicotinic acid hydroxylase; b, signal of nicotinic acid hydroxylase-enriched with ^{77}Se [(*Inset*) High-field components of spectra a and b in expanded scale]; c, signal of nicotinic acid hydroxylase-enriched with ^{95}Mo; d, signal of Se-deficient nicotinic acid hydroxylase purified from Se-deficient cells; e, signal of Se-deficient nicotinic acid hydroxylase incubated with 20 mM nicotinate for 1 min before freezing (the difference spectrum obtained by subtraction of spectrum d is shown). All samples contained about 0.5 mg of nicotinic acid hydroxylase in 100–200 μl of 50 mM potassium phosphate (pH 7.0). (*B*) Spectra of [2Fe–2S] clusters recorded at 43 K and 3.8-mW microwave power. Spectra a and b are dithionite-induced spectra of Se-containing and Se-deficient nicotinic acid hydroxylase, respectively. Nicotinic acid hydroxylase samples (0.5 mg) in 150 μl of buffer in 4-mm EPR tubes were flushed with argon for 10 min prior to addition of a freshly prepared solution of sodium dithionite (sodium hydrosulfite), at 10 mM final concentration. Samples were frozen after 5 min.

analysis (7). Since the isolated purified enzyme has a tendency to exist as higher molecular weight species that are approximate multiples of 160,000 (data not shown), the selenium content of Dilworth's preparations on a molar basis probably were even lower. A content of 1 gram-atom of selenium per mol of native active nicotinic acid hydroxylase is indicated on the basis of the present work.

By analogy with other molybdenum-containing hydroxylases [e.g., xanthine oxidase (10, 11)], the molybdenum center of nicotinic acid hydroxylase is expected to have a role in the reaction step involving hydroxylation of the heterocyclic ring with oxygen derived from water. At 130 K, the "as isolated" catalytically active nicotinic acid hydroxylase reveals a highly axial EPR signal (Fig. 2*A*, spectrum a), which we have named "resting" signal.[†] To avoid saturation effects upon signal detection at 130 K, it was necessary to use a low microwave power of 7.8 mW. The temperature dependence

of this unsaturated EPR signal amplitude is in accord with the Curie Law (proportional to $1/T$, where T is temperature) and thus is consistent with an $S = 1/2$ paramagnetic species. At higher gain, the signal exhibits a multiline hyperfine structure (data not shown) similar to that observed for Mo(V) EPR signals of xanthine oxidase and attributed to the natural abundance of ^{95}Mo($I = 5/2$; 15.7%) and ^{97}Mo($I = 5/2$; 9.45%) isotope species (where I is a nuclear spin). Based on these considerations, the paramagnetic species responsible for the observed "resting" signal of nicotinic acid hydroxylase are assumed to be Mo(V) ions. Although the line shape of the "resting" signal (Fig. 2*A*, spectrum a) is similar to that of the "very rapid" signal of xanthine oxidase observed upon addition of substrate (12), the g values 2.067, 1.982, and 1.974, determined at 130 K for nicotinic acid hydroxylase, differ from those recorded for xanthine oxidase (2.025, 1.955, and 1.949). To determine whether the unusually high g_z value of 2.067 is indicative of an interaction of selenium with molybdenum in the nicotinic acid hydroxylase, enzyme en-

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[†]Consistent with terminology introduced by Bray (10).

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riched with ^{77}Se was studied. As shown in Fig. 2A, spectrum b, this substitution of ^{77}Se ($I = 1/2$) for natural Se isotope abundance resulted in splitting of the "resting" signal providing evidence of direct coordination of Se with Mo (see also later). Fig. 2A *Inset* showing expanded spectra of the high-field components of spectra a and b illustrates this splitting more clearly. The line shape of the "resting" signal changes dramatically after ^{95}Mo substitution (Fig. 2A, spectrum c) due to hyperfine splitting from $I(^{95}$Mo$) = 5/2$ nucleus. Accurate evaluation of the hyperfine constants from the EPR spectrum of ^{95}Mo enzyme requires computer simulation. Nevertheless, direct comparison of the "very rapid" signal in the spectrum of ^{95}Mo-enriched xanthine oxidase (13) with the signal presented in Fig. 2A, spectrum c, shows that in both cases the hyperfine splittings from ^{95}Mo are close. These observations provide additional evidence that selenium is coordinated with molybdenum in the native catalytically active enzyme form of nicotinic acid hydroxylase.

In contrast to the EPR spectrum of the active selenium-containing enzyme, the "resting" signal is not detected in the selenium-deficient enzyme (Fig. 2A, spectrum d), which is inactive as a hydroxylase but retains NADPH oxidase activity. Upon incubation of this enzyme with substrate (20 mM nicotinate) for 1 min, a signal (Fig. 2A, spectrum e) similar to xanthine oxidase "rapid type I" Mo(V) signal (10) is observed. This demonstrates that molybdenum is present in the selenium-deficient enzyme and that it can interact with nicotinate to produce a Mo(V) EPR signal, although this signal is different from the "resting" signal observed in the presence of selenium (Fig. 2A, spectrum a). The "resting" signal displayed by the "as isolated" selenium-containing enzyme (Fig. 2A, spectrum a) is not altered by a short-time (1 min) incubation of enzyme with 20 mM nicotinate in the absence of NADP$^+$ or by incubation of enzyme with an oxidizing agent (10 mM ferricyanide) for 5 min. However, fast (less than 5–10 sec) nicotinate-induced disappearance of the "resting" signal was observed under strictly anaerobic conditions if, in addition, NADP$^+$ was added. Thus, to dissipate the "resting" signal observed with catalytically competent selenium-containing nicotinic acid hydroxylase, NADP$^+$, the ultimate electron acceptor, appears to be required. These are precisely the conditions required for enzyme turnover and catalysis of the overall hydroxylase reaction.

At low temperatures (<60 K), dithionite-reduced nicotinic acid hydroxylase preparations exhibit an EPR signal typical of FeS centers (Fig. 2B, spectra a and b). The signal reveals temperature dependence without an increase in line width up to 60 K, in accordance with the Curie Law. At 43 K, the signal can easily be saturated at microwave powers higher than 3.8 mW. These spectral properties are characteristic of [2Fe–2S] centers rather than [4Fe–4S] centers (14) because the EPR signals of the latter are much smaller in amplitude or may not be detectable at $T > 35$ K (15). Based on these considerations plus the observed average g value of 1.96, the signals shown in Fig. 2B can be attributed to [2Fe–2S] centers in the enzyme (16). From the fact that the EPR spectra recorded at 43 K (Fig. 2B, spectra a and b) of dithionite-reduced [2Fe–2S] clusters in selenium-containing (either normal isotope abundance or ^{77}Se-enriched) and selenium-deficient forms of nicotinic acid hydroxylase are identical, it can be concluded that selenium is not present in the active enzyme in the form of an iron–selenium cluster. Indeed, it has been shown by Orme-Johnson *et al.* (17) that substitution of Se for S in a [2Fe–2S] cluster results in dramatic transformation of line shape of [2Fe–2S] EPR signals.

Other evidence that the catalytic activity of nicotinic acid hydroxylase is associated with the "resting" Mo(V) EPR signal observed in the enzyme "as isolated" is presented in Figs. 3 and 4. In Fig. 3 it can be seen that there is an
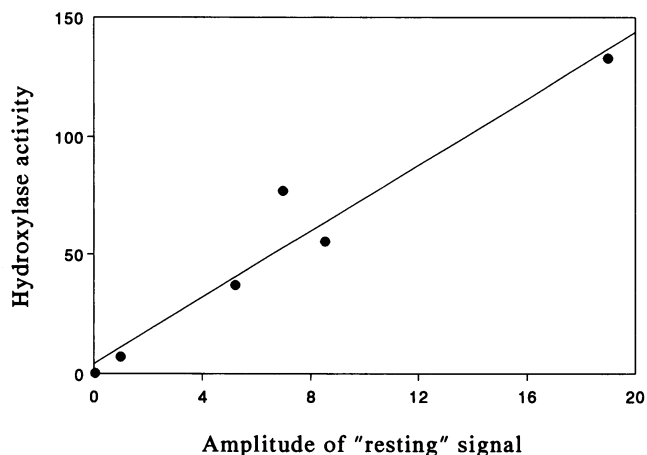

FIG. 3. Correlation between Mo(V) EPR "resting" signals and hydroxylase activities of different nicotinic acid hydroxylase preparations. Nicotinic acid hydroxylase activity, expressed as μmol of NADPH produced per min per ml of enzyme solution, is plotted vs. amplitude of Mo(V) EPR "resting" signal (relative units) measured in 100- to 160-μl samples of each enzyme preparation and normalized to 1-ml sample volumes. The highly purified enzyme samples ($\approx$5 mg of protein per ml) were isolated from various batches of *C. barkeri* cells grown in media containing low or adequate levels of selenium using identical purification procedures. Each point represents a different enzyme preparation. Values of signal amplitudes were normalized for microwave power and splitting of molybdenum signal due to ^{77}Se enrichment in the case of ^{77}Se-enriched enzyme samples.

approximately linear relationship between amplitude of the Mo(V) "resting" signal and nicotinate hydroxylase activity of various enzyme preparations. The preparation with barely detectable catalytic activity was isolated from cells grown in selenium-deficient medium and thus consists mostly of the "apoenzyme" form of the hydroxylase. Incubation of an active preparation of the enzyme at an elevated temperature, which is known to increase the rate of loss of selenium from the protein, resulted in roughly parallel decreases in catalytic activity and the amplitude of the "resting" Mo(V) EPR signal (Fig. 4).
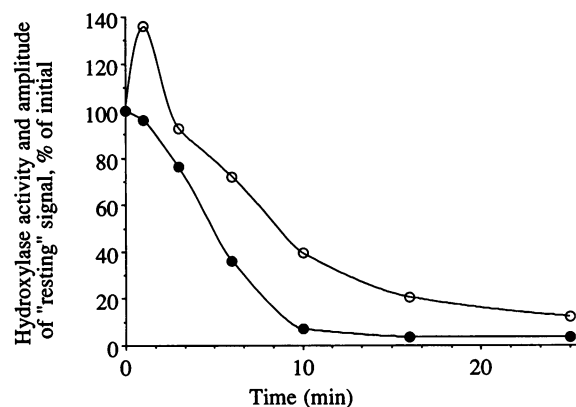

FIG. 4. Thermal inactivation of nicotinic acid hydroxylase is correlated with decreases in amplitude of Mo(V) EPR signal. A 1-mg sample of enzyme (18 units) was incubated aerobically in 1 ml of 100 mM potassium phosphate (pH 7) at 62°C. At the indicated times, nicotinic acid hydroxylase activity (O) was measured and other aliquots of the sample were frozen in EPR tubes in liquid nitrogen. The Mo(V) "resting" signal was measured in each sample and the intensities of the signal were calculated (●). It was shown earlier (7) that in contrast to the heat sensitivity of the hydroxylase activity, NADPH oxidase and diaphorase activities of nicotinic acid hydroxylase decline only slightly during incubation at 57°C or 70°C for 30 min. EPR conditions are as described in the Fig. 2A except a modulation amplitude of 10 G was used.

Two other purified molybdenum-containing enzymes from bacteria, a carbon monoxide oxidase from *Pseudomonas carboxydovorans* (18–20) and xanthine dehydrogenase from *Veillonella alcalescens* (21), have been reported to exhibit Mo(V) signals in the absence of added substrate. Carbon monoxide oxidase activity with methylene blue as electron acceptor was stimulated severalfold by treatment of enzyme with selenite under aerobic conditions (19, 20), but the same extent of activation was brought about by anaerobic treatment with sulfide and dithionite. In this case, the effect of the bound selenium in the enzyme is not specific. Whether it may have a role comparable to that of the essential selenium moiety in nicotinic acid hydroxylase is unknown.

The *g* values of the "resting" Mo(V) signal observed in the present study are unusually high for molybdenum-containing hydroxylases. In studies recently initiated (in collaboration with M. Axley), we detected a similar signal in another enzyme, the anaerobic formate dehydrogenase component of *Escherichia coli* formate-hydrogen lyase. This 80-kDa enzyme contains molybdopterin and FeS centers and either a selenocysteine residue (wild type) or a cysteine residue (mutant) in the polypeptide (22–24). Although both of these oxygen-sensitive enzymes, as isolated, exhibit Mo(V) EPR signals, the *g* values of the selenium-containing enzyme are much higher than those of the sulfur enzyme. As shown earlier (24), the catalytic activity of the selenium-containing enzyme is at least 300 times that of the mutant sulfur enzyme. If selenium in this formate dehydrogenase proves to be coordinated with molybdenum, the other atom bonded to selenium is already known; i.e., it is carbon-3 of the selenocysteine residue (25) in the protein. In nicotinic acid hydroxylase, the selenium that is coordinated with molybdenum in the resting form of the enzyme is a labile dissociable species. The possibility that this selenium is present as an outer ligand to molybdenum (Se=Mo) instead of the usual sulfur ligand is not yet determined. That a mechanism for insertion of selenium as the outer molybdenum ligand may indeed exist *in vivo* is suggested by the discovery that a *Drosophila* ma-1 mutant specifically lacks the outer sulfur ligand to molybdenum in its molybdenum hydroxylase (26). The catalytically inactive hydroxylases in the mutant flies could be reactivated *in vitro* by treatment with dithionite and sulfide, a procedure known to reconstitute desulfomolybdopterin in xanthine oxidase. Also consistent with the known chemical properties of the selenium in nicotinic acid hydroxylase, it could be weakly bonded to a heteroatom of a separate cofactor.

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