Properties of the Selenium- and Molybdenum-Containing Nicotinic Acid Hydroxylase from *Clostridium barkeri*

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ABSTRACT: $NADP^+$-coupled nicotinic acid hydroxylase (NAH) has been purified to near-homogeneity from *Clostridium barkeri* by an improved purification scheme that allowed the isolation of milligram amounts of enzyme of higher specific activity then previously reported. NAH is most stable at alkaline pH in the presence of glycerol. The protein which consists of four dissimilar subunits occurs in forms of different molecular masses. There are 5−7 Fe, 1 FAD, and 1 Mo per 160 kDa protein protomer. Mo in the enzyme is bound to a dinucleotide form of molybdopterin and is coordinated with selenium. Mo(V), flavin radical, and two Fe_2S_2 clusters could be observed with EPR spectroscopy. The Se cofactor which is essential for nicotinic acid hydroxylase activity could be released from NAH as a reactive low molecular weight compound by a number of denaturing procedures. Parallel losses of Se and catalytic activity were observed during purification and storage of the enzyme. Addition of sodium selenide or selenophosphate did not restore the catalytic activity of the enzyme. Instead, NAH is reversibly inactivated by these compounds and also by sulfide. Cyanide, a common inhibitor of Mo-containing hydroxylases, does not affect NAH catalytic activity. The "as isolated" enzyme exhibits a Mo(V) EPR signal (2.067 signal) that was detected at early stages of purification. NAH exhibits a high substrate specificity toward electron donor substrates. The ability of a nicotinate analog to reduce NAH (disappearance of 2.067 signal) correlates with the rate of oxidation of the analog in the standard assay mixture. The properties of NAH differentiate the enzyme from known Mo-containing hydroxylases.

Selenium, an essential trace element for many forms of life, has a key role as a redox center in several important enzymes in eukaryotes and prokaryotes (Stadtman, 1994, 1995). In most of these enzymes, selenium is present in the form of selenocysteine residues (SeCys) that are inserted cotranslationally as directed by the UGA codon (Zinoni et al., 1986; Leinfelder et al., 1988). On this basis, selenocysteine was recently named the 21st amino acid (Böck et al., 1991). A number of SeCys-containing enzymes and proteins from prokaryotes [formate dehydrogenases (Jones et al., 1979; Stadtman et al., 1991), hydrogenases (Yamazaki, 1982; Eidsness et al., 1991), and glycine reductase (Cone et al., 1976; Arkowitz & Abeles, 1991)] and eukaryotes [the glutathione peroxidase family (Ursini et al., 1985; Flohé, 1989; Schuckelt et al., 1991), 5′-deiodinase (Berry et al., 1991), selenoprotein P (Read et al., 1990), and selenoprotein W (Vendeland et al., 1993)] have been characterized in detail. For some of these enzymes, their roles in metabolic pathways and/or their importance for human health have been determined (Heider & Böck, 1993). In addition to these selenoproteins that contain relatively stable covalently attached selenium, there are a few bacterial enzymes, namely, nicotinic acid hydroxylase and xanthine dehydrogenase, that lack selenocysteine and instead contain selenium in a relatively unstable form (Dilworth, 1982, 1983; Gladyshev et al., 1994a; Imhoff & Andreesen, 1979; Wagner & Andreesen, 1979).

Nicotinic acid hydroxylase (NAH)[1] was originally purified by Holcenberg and Stadtman (1969), and later by Dilworth (1982). We recently initiated studies on NAH in order to determine the structure of an unusual selenium-containing cofactor of the enzyme. Release of this cofactor from NAH by treatment with urea or SDS or by heating has been reported (Dilworth, 1982), but the selenium-containing moiety has not been characterized in detail. Dialkylselenides were identified when the enzyme was inactivated by treatment with various alkylating agents (Dilworth, 1982). Three types of catalytic activities are exhibited by NAH: nicotinic acid hydroxylase (conversion of nicotinic acid to 6-hydroxynicotinic acid with the concomitant reduction of $NADP^+$ to NADPH), NADPH oxidase, and diaphorase (NADPH oxidation with concomitant reduction of various artificial dyes as electron acceptors) (Holcenberg & Stadtman, 1969).

NAH is a multisubunit protein that contains a number of redox centers: in addition to selenium, there are FeS clusters, FAD, and a molybdenum cofactor consisting of a Mo atom bound to a molybdopterin. Surprisingly, NAH as isolated contains a Mo(V) rather than a Mo(VI) species. Whereas the latter has been shown to be present in most Mo-containing enzymes in the "as isolated" state, Mo(V) species have been described as unstable and intermediate forms during catalysis (Bray, 1988). By use of Mo(V) EPR spectroscopy of NAH containing either natural-isotope abundance Se or ^{77}Se, it was shown that Se is directly coordinated to Mo (Gladyshev et al., 1994a). Direct Se−Mo coordination was later confirmed or suggested for other

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[1] Abbreviations: NAH, nicotinic acid hydroxylase; DTT, dithiothreitol; EPR, electron paramagnetic resonance; PAGE, polyacrylamide gel electrophoresis.

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known Se- and Mo-containing enzymes (Gladyshev et al., 1994b).

The Se−Mo center of NAH has been shown to be required for enzymic oxidation of nicotinate, thus providing an example of the essential role of Se in catalysis by Se-containing molybdo-enzymes. When cultured in selenium-deficient media, *Clostridium barkeri* expressed an inactive isozyme of NAH that contained FeS clusters, FAD, and Mo, but no Se. Since expression of the protein was independent of selenium, it is clear that cotranslational incorporation does not occur; rather, a bound cofactor form of selenium appears to be indicated (Gladyshev et al., 1994a).

The present study was initiated to gain further information concerning the composition and catalytic properties of NAH and the properties of the labile selenium cofactor. An improved purification scheme that allows isolation of milligram amounts of pure enzyme has been developed for this purpose.

MATERIALS AND METHODS

Materials

Sodium [^{75}Se]selenite (1000 Ci/mmol) was purchased from the Research Reactor Facility, University of Missouri, Columbia, MO. The following materials were from the indicated sources: phenyl-Sepharose CL-4B, AcA-34, and FPLC G-25 desalting column from Pharmacia, TSK-Gel G3000SW HPLC gel filtration column (7.5 × 600 mm) from TosoHaas; Bio-Gel DEAE-5-PW HPLC ion exchange column (21.5 × 150 mm) and gel filtration protein standards from Bio-Rad; C_{18} reverse-phase HPLC column from Vydac; precast polyacrylamide gels and molecular weight standards from Novex (San Diego, CA); DNase I from Boehringer Mannheim; 6-hydroxynicotinic acid, 2-hydroxynicotinic acid, 3,5-pyridinedicarboxylic acid, 3-pyridinesulfonic acid, 2,3-pyrazinedicarboxylic acid, pyrazine-2-carboxylic acid, nicotinamide *N*-oxide, nicotinic acid *N*-oxide, 6-methylnicotinic acid, and 6-chloronicotinic acid from Aldrich; isonicotinic acid and thionicotinamide from Janssen Chimica; cobinamide dicyanide, picolinic acid, and trigonelline (1-methyl-pyridinium-3-carboxylate) from Sigma. Sodium selenide was prepared from sodium selenite and dithiothreitol (DTT) as described (Veres et al., 1992). Selenophosphate was a gift of Dr. Zsuzsa Veres. All other reagents were of the highest grade available.

Methods

Cell Culture. *C. barkeri* was cultured anaerobically in a nicotinic acid−mineral salts−yeast extract medium supplemented with 1 mM sodium selenite as described previously (Pastan et al., 1964; Dilworth, 1982). For ^{75}Se-labeled cells, *C. barkeri* was grown in 2 L cultures containing l μM [^{75}Se]-selenite (0.5 mCi). To obtain ^{77}Se-enriched NAH, *C. barkeri* was grown in the presence of 1 μM [^{77}Se]selenite as described (Gladyshev et al., 1994a).

Purification. All steps of the standard isolation procedure were performed at 4 °C using buffer solutions deoxygenated by sparging with argon. Chromatography column matrices were deoxygenated by extensive washing with anaerobic buffers prior to use. Frozen *C. barkeri* cells were thawed in an equal volume of a solution containing 100 mM potassium phosphate, pH 7.8, 1 mM EDTA, and 2 mM DTT and disrupted by sonication. DNA was digested by addition of a few milligrams of DNase I. Insoluble material was removed by centrifugation. Saturated ammonium sulfate solution (4.1 M ammonium sulfate, 0.1 M potassium phosphate, pH 7.8) was added to 46% of saturation, and the precipitate was removed by centrifugation. NAH in the supernatant solution was precipitated by the further addition of ammonium sulfate to 64% of saturation and collected by centrifugation. Precipitated proteins were dissolved in a minimal volume of buffer A (100 mM potassium phosphate, 1 M ammonium sulfate, 1 mM EDTA, and 2 mM DTT, pH 7.8) and applied to a phenyl-Sepharose CL-4B column equilibrated with buffer A. After washing with 2 column volumes of buffer A, NAH was eluted by application of a linear gradient from buffer A to buffer B (20 mM potassium phosphate, pH 7.8, 1 mM EDTA, and 2 mM DTT). At the end of the gradient, the column was washed further with buffer B. NAH emerged from the column after a red-colored protein. Fractions containing NAH were collected and concentrated to about 50 mg of protein/mL on an Amicon stirred-cell ultrafiltration unit fitted with a YM-30 membrane. The concentrated protein solution was applied to an AcA-34 gel filtration column (2.5 × 100 cm) equilibrated with buffer B, and the enzyme was eluted with the same buffer. Fractions containing NAH were pooled and concentrated as above to about 20 mg of protein/mL. The enzyme then was applied to a DEAE HPLC column and eluted with a linear gradient increasing from 100 mM to 350 mM NaCl in buffer B. Active fractions were concentrated on Centricon-30 microconcentrators to about 20 mg of protein/mL and stored at −80 °C.

^{75}Se-labeled NAH was purified from ^{75}Se-labeled cells using the above procedure. ^{75}Se in fractions was measured using a Beckman 5500 gamma counter. A similar procedure was used for isolation of NAH under strictly anaerobic conditions except that all steps were performed in the NIH Anaerobic Laboratory (Poston et al., 1971) operating in an N_2 atmosphere containing 1−5% H_2 and less then 1 ppm of oxygen. Solutions were made anaerobic by sparging with oxygen-free argon or nitrogen. Columns were made anaerobic by application of 5−10 mL of 10 mM sodium dithionite followed by extensive washing of the columns with anaerobic buffer. Except for omission of the gel filtration step, the anaerobic purification procedure was essentially the same as described above. Purified, anaerobic NAH was frozen in small portions in liquid nitrogen and stored at −80 °C. For studies under strictly anaerobic conditions, these preparations were thawed, and experiments were carried out in the NIH Anaerobic Laboratory.

Enzyme Assay. The nicotinic acid hydroxylase standard assay mixture (1 mL) contained 670 μL of 0.1 M potassium phosphate, pH 7.0, 100 μL of 500 mM potassium nicotinate, 100 μL of 10 mM $NADP^+$, 100 μL of 0.5 M DTT, and 20 μL of 1 mM cobinamide. The mixture of nicotinate, $NADP^+$, and phosphate buffer was sparged with argon for 5 min prior to addition of anaerobic solutions of DTT and cobinamide. The DTT/cobinamide system was previously used for the removal of traces of oxygen from the assay mixtures (Holcenberg & Stadtman, 1969). The reaction was initiated by the addition of 10 μL of NAH, and NADPH production was monitored by the increase in absorbance at 340 nm. One unit of activity is defined as the formation of 1 μmol of NADPH per minute at room temperature. When

NAH activity measurements were carried out in the NIH Anaerobic Laboratory, the oxygen-scrubbing DTT/cobinamide system was not necessary, and these compounds were omitted from the assay mixture.

The NADPH oxidase assay mixture (1 mL) contained 50 μL of 3 mM NADPH and 940 μL of aerobic 0.1 M potassium phosphate buffer, pH 7.0. The reaction was initiated by the addition of 10−20 μL of NAH and monitored as a decrease in absorbance at 340 nm. The diaphorase assay mixture (1 mL) contained 900 μL of 0.1 M potassium phosphate buffer, pH 7.0, 50 μL of 3 mM NADPH, and a sufficient amount of methylene blue to give an absorbance of 2.0 at 600 nm. The reaction was initiated by the addition of 1−10 μL of NAH and monitored by the decrease in absorbance at 600 nm.

Purity and Molecular Weight Determinations. Native and SDS−PAGE were performed according to standard procedures using 8%, 10%, 12%, 16%, or gradient 4−20% acrylamide gels. Gels were stained with Coomassie Blue R-250. The molecular masses of subunits were estimated previously (Gladyshev et al., 1994a) with different PAGE standards and percentage of gels used.

Molecular weight determination by gel filtration chromatography was carried out with a TSK G3000SW column in 50 mM Tris-HCl, pH 7.8, 100 mM NaCl. The column was calibrated with gel filtration protein standards (thyroglobulin, γ-globulin, ovalbumin, myoglobin), vitamin B_{12}, and additionally with proteins (ferritin, catalase, and aldolase) similar to NAH in molecular weight or elution time. The molecular weight of NAH was determined from a plot of log(molecular weight) versus elution time.

N-Terminal Amino Acid Sequencing. Amino acid sequence analysis of protein subunits was performed according to two protocols. In the first protocol, NAH subunits were separated by SDS−PAGE. After staining and destaining, the protein bands were cut from the gel and eluted using 0.1% SDS in 100 mM Tris-HCl, pH 8.2. The protein−SDS−Tris mixture was used for sequencing on a Hewlett Packard Protein Sequencer G100A. In the second protocol, NAH was alkylated with iodoacetic acid (Stadtman, 1984), and the subunits were separated on a C_{18} reverse-phase column using a gradient of acetonitrile from 2% to 50% in 0.05% TFA. Fractions containing the NAH subunits were collected and dried on a SpeedVac SC110 (Savant). The peptides were dissolved in 6 M guanidine hydrochloride, 2 mM DTT and subjected to automatic sequencing as described above.

Metal Analysis. Selenium, molybdenum, and iron present in NAH were analyzed by atomic absorption spectroscopy at Galbraith Labs, Knoxville, TN. The selenium content of NAH was previously determined by ^{75}Se specific radioactivity measurements (Dilworth, 1982; Gladyshev et al., 1994a).

Molybdopterin Cofactor Analysis. Molybdopterin was detected by measuring the characteristic fluorescence of the cofactor after oxidation (Krüger et al., 1987). The molecular weight of the molybdopterin cofactor was estimated after separation from the enzyme by treatment with guanidine hydrochloride. The enzyme was denatured for 30 min at room temperature with 4 M guanidine hydrochloride, pH 7.0, in the presence of 2 mM DTT and 1 mM EDTA under argon. The released cofactors were separated from protein by chromatography on a FPLC G-25 column equilibrated and eluted at a 0.3 mL/min flow rate with 4 M guanidine hydrochloride, pH 2.0, 2 mM DTT, and 1 mM EDTA. Fractions were collected and neutralized with ammonium hydroxide, and the fluorescence (excitation at 400 nm, emission at 465 nm) was measured after air oxidation of the samples. The G-25 column was calibrated with bovine serum albumin, FAD, riboflavin, folic acid, xanthine, and 5,6-dimethylbenzimidazole.

Release of Cofactors from NAH. To study the release of Se and cofactors from NAH, ^{75}Se-labeled enzyme was denatured with 1−6 M guanidine hydrochloride for 0−60 min at 4−22 °C in the presence or absence of 2 mM sodium dithionite. In different experiments, NAH was denatured also by heating at 95 °C for 3−30 min in the presence or in the absence of 2 mM sodium dithionite followed by centrifugation in order to precipitate the denatured polypeptides. For the reduction of NAH with sodium dithionite, enzyme samples in 1.5 mL microcentrifuge tubes were flushed with argon prior to and after addition of sodium dithionite.

Cofactors were separated on a G-25 column equilibrated with the solution used for denaturation, and fractions were collected. FAD was detected by its characteristic fluorescence (excitation at 480 nm, emission at 540 nm) or by absorption spectra in solutions that did not contain reducing agents. Molybdopterin was detected by its fluorescence. Protein was detected by the absorption at 280 nm. Selenium was determined by ^{75}Se radioactivity measurements in a Beckman 5500 gamma counter.

Effect of pH, Salts, and Other Compounds on the Stability of NAH. For the determination of the effect of pH on the stability of NAH, the enzyme was incubated in buffer solutions consisting of 50 mM MES, 50 mM potassium phosphate, and 50 mM Tris adjusted to pH values varying from 4.8 to 9.0 with HCl or KOH. Samples containing 20 μL of NAH and 180 μL of buffer at the indicated pH were incubated in closed 1.5 mL tubes (gas phase air) at room temperature in the dark for the indicated time periods. Activities of samples were then measured by the standard assay. For the determination of the effect of salts and other compounds on the stability of NAH, enzyme in 50 mM Tris-HCl, pH 8.2, was incubated under the above conditions in the presence of the compound at the indicated concentration. Activities of the samples were determined after the indicated time periods in the standard assay.

Effect of Nicotinic Acid Analogs. Nicotinic acid analogs (10 mM) were assayed as substrates for NAH in the standard assay mixture in the absence of nicotinic acid. A base line activity of 0−0.050 A_{340} unit/min, presumably due to the oxidation of DTT, was observed in these assays. The K_m value for 2-pyrazinecarboxylic acid was determined from the Lineweaver−Burk plot. The effects of nicotinic acid analogs on nicotinate hydroxylation were tested in two separate experiments using equimolar concentrations (10 mM or 0.1 mM) of nicotinic acid and of the analog in the reaction mixtures. Analogs that were not soluble at 10 mM were tested only at 0.1 mM. To determine whether nicotinic acid analogs were able to reduce NAH, the enzyme was mixed in EPR tubes with 10 mM nicotinate or with 10 mM nicotinate analog and incubated for 1 h at 4 °C under argon. Samples were frozen in liquid nitrogen and measured with an EPR spectrometer, and the amplitude of the 2.067 signal of the Mo(V) species was determined. In some cases, the 2.067 Mo(V) signal completely disappeared, and a new signal (signal A) was produced.

Table 1: Purification of Nicotinic Acid Hydroxylase (NAH) from *Clostridium barkeri*

purification step	vol, mL	protein, mg	sp act., μmol min^{-1} mg^{-1}	total act., units	yield, %	purif factor
ammonium sulfate fractionation	200	5300	0.16	745	100	1
phenyl-Sepharose	100	1060	0.81	700	94	5.1
AcA-34	30	230	1.96	450	60	12
DEAE	18	21	18	380	51	112

EPR Spectroscopy. EPR spectra were recorded on a Bruker ESR-300 spectrometer operating on X-band and equipped with a 5352B microwave frequency counter. Low-temperature measurements of Mo(V) species and FeS clusters were performed with an Oxford Instruments ESR910 cryostat. For the detection of spectra of Mo(V) species at 130 K, a Bruker variable-temperature unit ER4111 VT was used. The EPR spectra of Mo(V) species at 130 K and of iron−sulfur clusters at 35 K were detected at microwave powers of 7.8 mW and 3.2 mW, respectively. A microwave frequency of 9.45 GHz, a modulation frequency of 100 kHz, and modulation amplitude of 5 G were used.

RESULTS

Enzyme Purification. The results of a typical purification are shown in Table 1. The enzyme is relatively abundant in *C. barkeri* and can be purified in large amounts using standard isolation procedures consisting of ammonium sulfate fractionation and hydrophobic interaction, gel filtration, and ion exchange chromatography. After the phenyl-Sepharose step, NAH was detected as the major Se-containing protein by ^{75}Se-radioactivity measurements. After gel filtration chromatography, NAH was the major yellow−brown-colored protein in the preparations. These observations made control of the isolation procedure easier and faster. NAH was relatively stable prior to the phenyl-Sepharose step, but gradual loss of activity was observed during further purification. Since an efficient stabilizer of the enzyme was not found, it was necessary to carry out the isolation procedure rapidly at low temperature, keeping the enzyme as concentrated as possible in order to obtain NAH with high specific activity. Under these conditions, a typical enzyme preparation exhibited a specific activity of 15−25 μmol min^{-1} (mg of protein)$^{-1}$. The highest specific activity reached so far was 35 μmol min^{-1} mg^{-1}, and our studies suggest that the maximal specific activity would be close to 40 μmol min^{-1} mg^{-1} under the assay conditions employed. For a purification procedure starting with 1−5 g of cells, a 1 day procedure was developed; 3−4 days were required for the purification of NAH starting with 100−300 g of cells. The yellow−brown-colored NAH obtained was soluble to a high concentration (>1 mM) of the enzyme.

The oxygen sensitivity of NAH and of Se-containing proteins in general has been documented previously, and therefore the purification steps were performed in deoxygenated solutions. NAH was also purified under strictly anaerobic conditions in the NIH Anaerobic Laboratory. This procedure, however, did not result in increased enzyme specific activity which suggests that oxygen sensitivity might not be the major destabilizing factor for the enzyme. Comparison of NAH purified anaerobically and NAH purified in the presence of air revealed no significant differences in catalytic activities, absorption spectra, or subunit and cofactor compositions of enzymes.

Purified NAH exhibited three types of enzymic activities: nicotinic acid hydroxylase, NADPH oxidase, and diaphorase which appeared in a ratio of about 1:0.5:6, respectively. To test the hypothesis that the NADPH oxidizing activity that separated from NAH on the gel filtration purification step might represent an inactive NAH isozyme, the fractions containing this activity were further purified on a DEAE cellulose column. Proteins eluted from the column that exhibited NADPH oxidase activity differed from NAH both in subunit and in cofactor composition (data not shown). It was calculated that NAH represented essentially all of the nicotinic acid hydroxylase activity in *C. barkeri* extracts, about 30% of the NADPH oxidase activity, and about 50% of the diaphorase activity.

Subunit Composition. Analysis by native and by SDS−PAGE and by analytical gel filtration (Figure 1) revealed that purified NAH was typically 90−99% pure. NAH was eluted as an approximately 400 kDa nonsymmetrical peak from the calibrated analytical gel filtration column (Figure 1A). Fractions eluted at the front, middle, and back positions of the protein peak were collected separately, and gradient native PAGE analysis was performed on these samples. Native PAGE (Figure 1B) showed a progression from the front to the back of the peak of 400−120 kDa bands with a major band at 160 kDa throughout. SDS−PAGE analysis performed with isolated NAH (Gladyshev et al., 1994a) or with separated 120, 160, and 400 kDa protein bands eluted from native PAGE gels (Figure 1C) revealed that each consisted of subunits of 33, 37, and 50 kDa present in about equal proportion. A 23 kDa protein band was also present in all protein species eluted from native PAGE gels, but it had significantly lower staining intensity.

The amino acid sequences of the amino terminal regions of the 23, 33, 37, and 50 kDa subunits of NAH (Table 2) showed no significant similarity to other Mo-containing proteins.

Spectral Properties. The electronic absorption spectrum of purified NAH (Figure 2) determined in the present study is identical to that described previously (Holcenberg & Stadtman, 1969). This spectrum is characteristic of FAD/FeS-containing molybdoenzymes such as xanthine oxidase (Hille, 1994). The ratio of 4.8 for the absorbancies at 280 nm to 450 nm obtained for NAH is also typical of xanthine oxidase and other Mo-containing hydroxylases. This ratio and the absorption spectrum itself are characteristic of a pure protein with a full set of FAD and FeS centers. Reduction of NAH with sodium dithionite or other strong reducing agents caused a decrease in absorbance in the 350−500 nm region due to reduction of FAD and FeS centers. The enzyme was reduced slowly when incubated anaerobically with nicotinate (Figure 2), but no reduction was observed when the enzyme was incubated for short times (5 min) with nicotinate under aerobic conditions. Oxidation of nicotinate-reduced NAH with air led to the oxidation of FAD and FeS

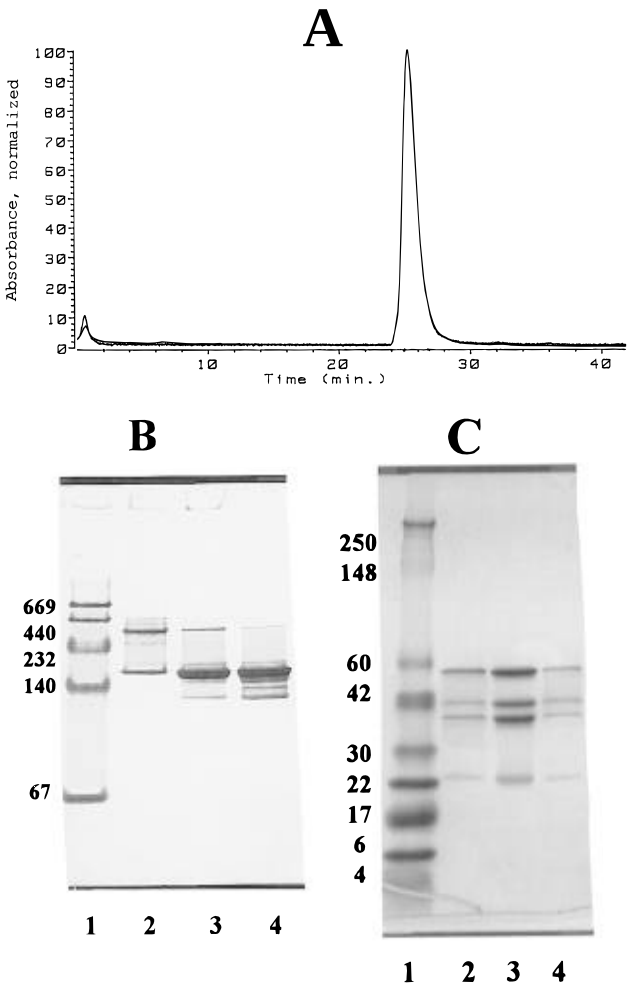

FIGURE 1: Gel electrophoresis and gel filtration analysis of NAH preparations. (A) Gel filtration of an NAH sample on an HPLC TSK-Gel G3000SW column. Elution was monitored by the absorption at 280 nm and at 450 nm, and both absorption profiles were normalized. (B) Native PAGE of NAH. Lane 1, molecular weight standards (molecular masses in kilodaltons are indicated); lane 2, fraction from the front of the protein peak from the gel filtration column; lane 3, fraction from the middle of the protein peak from the gel filtration column; lane 4, fraction from the back of the protein peak from the gel filtration column. A 4−20% gradient gel was used. (C) SDS−PAGE of NAH. Lane 1, molecular weight standards (molecular masses in kilodaltons are indicated); lane 2, 400 kDa NAH species; lane 3, 160 kDa NAH species; lane 4, 120 kDa NAH species. A 4−20% gradient gel was used. To prepare SDS samples, a preparative native 4−20% gradient gel was used to separate 120, 160, and 400 kDa enzyme species. The gel was quickly stained and destained. Bands of interest were cut and incubated overnight in microcentrifuge tubes (gas phase air) in minimal volumes of SDS sample buffer. Eluted polypeptides were heated and used for SDS−PAGE.

Table 2: N-Terminal Sequences of NAH Subunits

subunit, kDa	N-terminal sequence
50[a,b]	$G^{1}KDYQVLGKN^{10}KVKVDSLEKVMGTAK^{25}$
37[b]	$M^{1}KKRGKGVGA^{10}$
33[a,b]	$M^{1}KDFEFFAPK^{10}TLEEAKGLLHQYKDV^{25}$
23[b]	$M^{1}NKXTXNLNL^{10}$

[a] Sequence determined after isolation of the carboxymethylated subunit on a C_{18} column. [b] Sequence determined after elution of the subunit from a gel after SDS−PAGE analysis. The residues designated as X in positions 4 and 6 of 23 kDa subunit are I or F.

centers, and the spectrum of the reoxidized enzyme was indistinguishable from the spectrum of the isolated oxidized enzyme species. The enzyme after reduction with substrate,

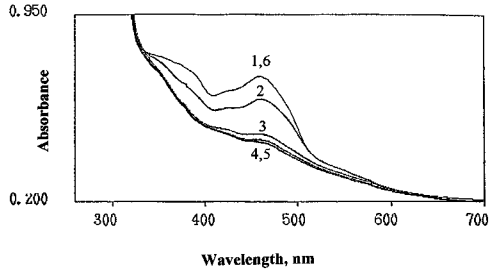

FIGURE 2: Nicotinate-induced reduction of NAH. NAH was made anaerobic in a sealed cuvette, and an anaerobic solution of nicotinate (final concentration 20 mM) was added. Absorption spectra of NAH were recorded at 0 min (1), 1 min (2), 3 min (3), 10 min (4), and 20 min (5) after the addition of nicotinate. The reduced NAH was then oxidized by introduction of air. The spectrum of reoxidized NAH (6) was indistinguishable from the spectrum of NAH before reduction.

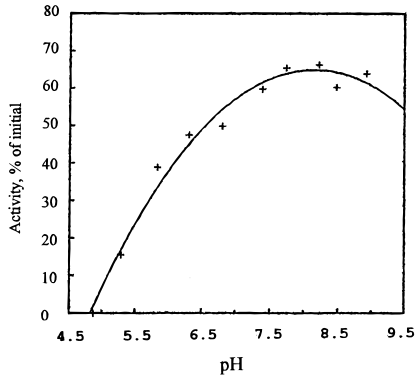

FIGURE 3: Stability of NAH as a function of pH. The tubes containing NAH and buffers of the indicated pH values were incubated (gas phase air) at room temperature as described under Materials and Methods. Activity remaining at 14 h of incubation is expressed as the percentage of initial activity.

however, was much more sensitive to oxygen inactivation than the form of the enzyme originally isolated. Exposure of the substrate-reduced NAH to air resulted in a complete loss of enzymic activity.

Stability Studies. Studies on the stability of NAH during storage at room temperature in solutions of varying pH are summarized in Figure 3. NAH was particularly sensitive to storage at acidic pH. Under the conditions described in the legend to Figure 3, maximal stability during incubation for 14 h was observed at pH 8.0. Similar pH−stability profiles with maxima at pH 8.0 were observed after prolonged incubations, and under these conditions, about 25% of the initial activity remained after 9 days storage at pH 8.0. The pH−stability profile (Figure 3) is similar to the pH−activity profiles reported earlier (Holcenberg & Stadtman, 1969).

The effects of various salts, substrates, substrate analogs, "antioxidant enzymes", and other compounds on the stability of NAH during storage are shown in Table 3. Most of these substances including nicotinate and its analogs, $NADP^{+}$, antioxidant proteins (catalase, glutathione peroxidase, and superoxide dismutase), ferricyanide, and DTT inactivated NAH under the storage conditions used. Only 20% glycerol which is commonly used for stabilization of proteins or 400 mM KCl proved to be effective as a stabilizing agent. High concentrations of KCl have been reported to stabilize another Mo-containing enzyme, formylmethanofuran dehydrogenase (Schmitz et al., 1992).

Sensitivity to potassium cyanide is a common property of Mo-containing hydroxylases, and in most cases Mo-contain-

Table 3: Stability of NAH in the Presence of Different Compounds[a]

compound	activity, % of initial			
	3 h	1 day	4 days	7 days
50 mM Tris-HCl, pH 8.2 (control)	100	60	39	38
50 mM Tris-HCl, pH 8.2 (control)	100	56	39	37
20% glycerol	100	70	50	51
400 mM KCl	95	71	42	40
400 mM $(NH_4)_2SO_4$	92	53	33	27
0.1 mg/mL SOD	82	67	35	22
1 mg/mL catalase	73	37	21	10
2 mM 6-hydroxynicotinate	95	54	29	20
0.1 mg/mL GPx + 1 mM GSH	83	26	3	0
50 mM DTT	100	17	1	0
1 mM $NADP^+$	58	20	8	0
50 mM nicotinate	61	13	1	0
2 mM pyridinesulfonic acid	80	29	3	0
2 mM KCN	71	16	1	0
1 mM potassium ferriyanide	52	8	2	0

[a] NAH was incubated in the presence of the indicated compounds in 50 mM Tris-HCl, pH 8.2, at room temperature as described under Materials and Methods. Aliquots were taken for activity measurements after the indicated incubation times. Abbreviations: SOD, superoxide dismutase; GPx, glutathione peroxidase; GSH, reduced glutathione.

ing enzymes are completely inactivated by treatment for l h with l mM KCN (Massey & Edmondson, 1970; Malthouse et al., 1981). It is believed that cyanide reacts with the external sulfo ligand of molybdenum, resulting in the replacement of sulfur with oxygen. Inactive desulfoenzyme formed in this way can be partially converted to the active enzyme by addition of sulfide or dithionite. Surprisingly, even 100 mM KCN did not significantly inhibit NAH catalytic activity upon incubation for 1 h at room temperature. However, after prolonged treatment with KCN (Table 3), NAH is inactivated, but this process may or may not be directly related to interaction of cyanide with the molybdenum center of the active enzyme.

Substrate Specificity. During previous studies it was noted that NAH exhibits high substrate specificity (Holcenberg & Stadtman, 1969). To examine this point further, a number of nicotinate analogs were tested as substrate and for their effects on NAH activity (Table 4). Only 2-pyrazinecarboxylic acid was equally as effective as nicotinate as substrate for NAH. However, the K_m value (0.83 mM) was about 7 times higher than the K_m value for nicotinate. 2-Pyrazinecarboxylic acid differs from nicotinate in that it has nitrogen in the 4-position of the heterocyclic ring instead of carbon. Several other nicotinate analogs were oxidized by NAH, but the rates were slow. Most of the compounds tested, even those structurally related to nicotinate, were ineffective as substrates. This high substrate specificity distinguishes this enzyme from xanthine oxidase and xanthine dehydrogenase which utilize a number of purine analogs of xanthine (Bradshow & Barker, 1960; Smith et al., 1967). Analysis of the data presented in Table 4 suggests that an unsubstituted nitrogen and a carboxyl group at position 3 are absolutely required for substrate hydroxylation. Unsubstituted carbon-5 is also important for oxidation at carbon-6 of the substrate. None of the nicotinate analogs inhibited NAH effectively except the product of nicotinate oxidation, 6-hydroxynicotinate.

Cofactors. Experiments were carried out to determine which type of naturally occurring molybdopterin was bound to the enzyme. A simple molybdopterin has been shown to

Table 4: Study on Nicotinic Acid Analogs: NAH Has a High Substrate Specificity[a]

nicotinic acid analog	hydroxylation of analog (NADPH formation)	inhibition of hydroxylation	changes in 2.067 EPR signal
nicotinic acid	+++		+++
2-pyrazinecarboxylic acid	+++	ND	++
2,3-pyrazinedicarboxylic acid	++	−	ND
2-hydroxynicotinic acid	ND	−	++
3,5-pyridinedicarboxylic acid	+	−	+
trigonelline	+	−	+
picolinic acid	−	ND	−
isonicotinic acid	−	−	ND
nicotinic acid *N*-oxide	−	−	−
nicotinamide *N*-oxide	−	−	ND
nicotinamide	−	ND	ND
3-pyridinesulfonic acid	−	+	ND
6-methylnicotinic acid	+	+	−
6-chloronicotinic acid	⁻	+	⁻
6-hydroxynicotinic acid	−	+++	−

[a] To determine the ability of NAH to oxidize nicotinic acid analogs, the various compounds were added to the standard assay mixture in place of nicotinate. Activities exhibited by NAH with those compounds were compared with activity of NAH when nicotinate was used. Data are shown in the "hydroxylation of analog" column (+++, 100% activity; ++, 10−20% activity; +, 5−10% activity; −, less than 5% activity). The effects of nicotinic acid analogs on nicotinate hydroxylation were determined as described under Materials and Methods. Data are shown in the "inhibition of hydroxylation" column (+++, 10−20% activity remained; +, more then 60% activity remained; −, 100% activity remained). To determine the ability of nicotinic acid analogs to reduce NAH, enzyme samples were mixed with the test compounds (final concentration 25 mM) in EPR cuvettes and incubated anaerobically for 1 h at 4 °C. EPR spectra of samples were measured at 130 K. Data are shown in the "changes in 2.067 EPR signal" column (+++, 2.067 signal disappeared, signal A was detected; ++, amplitude of 2.067 signal decreased significantly or signal disappeared; +, amplitude of 2.067 signal slightly decreased; −, no change in 2.067 signal). ND - not determined.

be present in all eukaryotic systems and in some prokaryotic organisms. Dinucleotide forms of molybdopterin which additionally contain the 5′-phosphate derivative of either guanosine, cytidine, adenosine, or hypoxanthosine have been found in prokaryotes (Rajagopalan & Johnson, 1992; Rajagopalan, 1993). The dinucleotide forms of molybdopterin can be distinguished from the simple molybdopterin on the basis of molecular weight (Krüger et al., 1987). By use of gel filtration chromatography on a calibrated G-25 column, the molybdopterin released from NAH migrated as an 850 Da component (Figure 4), indicating that NAH contains a dinucleotide form of molybdopterin.

The molecular weights of other cofactors released from NAH were also estimated by gel filtration (Figure 5). FAD eluted from the column at the same position as the molybdopterin. There was essentially complete release of FAD, molybdopterin, and FeS clusters from the protein as judged by the lack of 350−500 nm absorbance in the void volume fractions that contained the polypeptides. Most of the Se released from the protein appeared in fractions of the ^{75}Se radioactivity profile corresponding to molecular weights below 500. Sodium selenite, molecular mass of 173 Da, when chromatographed as a reference compound was eluted in fractions 15 and 16. The nonsymmetrical elution pattern of selenium in fractions 11−19 suggests that the form of the selenium eliminated from the enzyme might be a series of polyselenides (Se) ranging from Se_2 (molecular mass of

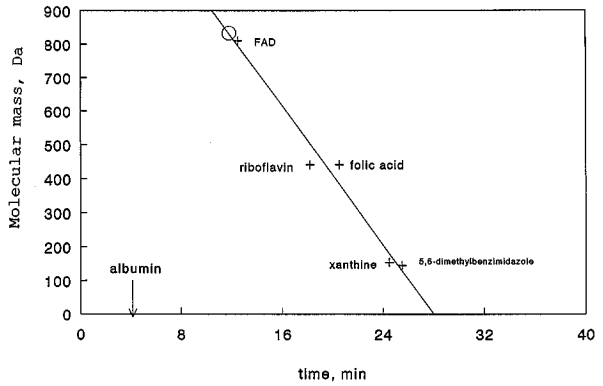

FIGURE 4: Determination of the molecular weight of molybdopterin released from NAH. G-25 column was equilibrated with 4 M guanidine hydrochloride, 2 mM DTT, and 1 mM EDTA, pH 2.0, and was calibrated as described under Materials and Methods. NAH was reduced with sodium dithionite and denatured with 4 M guanidine hydrochloride for 30 min. The released molybdopterin (○) eluted from the column was monitored by fluorescence (exitation at 400 nm, emission at 465 nm).

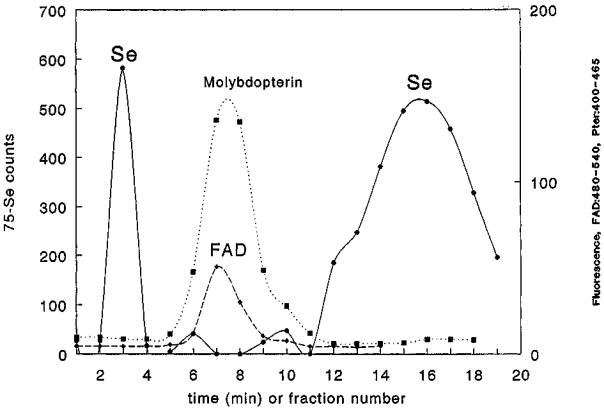

FIGURE 5: Separation of cofactors released from NAH. ^{75}Se-labeled NAH was incubated in a solution containing 4 M guanidine hydrochloride, 2 mM DTT, and 1 mM EDTA for 40 min at room temperature and applied to a G-25 column equilibrated with the same solution. Fractions were collected at 0.3 mL/min flow rate. ^{75}Se (●), FAD (◆), and molybdopterin (■) were determined as described under Materials and Methods. Se eluting at 3 min is bound to the denatured polypeptides. Se eluting at 15−16 min is released from the protein.

158 Da) and upward analogous to inorganic polysulfides (sulfane sulfur) which consist of sulfur bonded to sulfur.

The unstable Se moiety that was released from NAH by treatment with guanidine hydrochloride (Figure 5) also can be released by a number of other strong denaturation procedures including methods such as heating at 95 °C, incubation with detergents such as SDS, or incubation with high concentrations of other chaotrophic agents, e.g., urea. Various mild techniques including freezing, digestion with proteases, or incubation with DMSO also cause loss of selenium. Strong methods released most of the Se, while 50% or less of the Se moiety was lost under the mild conditions. The released Se resembled colloidal elemental selenium in that it showed a high tendency to bind to denatured protein, column matrices, microconcentrators, and plastic membranes.

The amounts of Mo, Fe, and Se in different preparations of NAH were determined by atomic absorption spectroscopy. The values of 0.82, 0.96, and 1.2 atoms of Mo per 160 kDa enzyme molecule suggest the presence of 1 mol of Mo. The

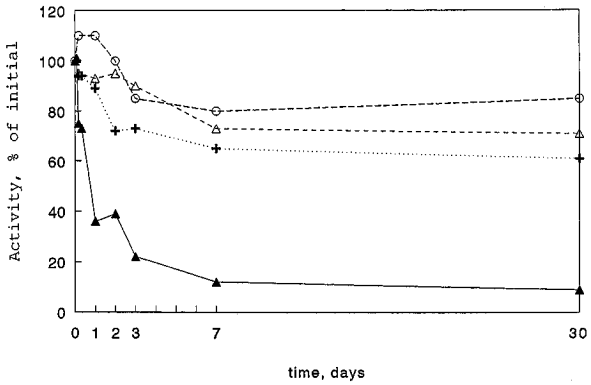

FIGURE 6: Inactivation of NAH and release of Se upon storage of the enzyme. ^{75}Se-labeled nicotinic acid hydroxylase in 20 mM potassium phosphate buffer, pH 7.0, was incubated (gas phase air) at 4 °C. At the indicated times, nicotinic acid hydroxylase (▲), NADPH oxidase (△), and diaphorase (○) activities of NAH were determined as described under Materials and Methods. At the same time, enzyme was equilibrated with fresh 20 mM potassium phosphate buffer, pH 7.0, on a Centricon-30 microconcentrator to remove released Se, and the radioactivity remaining with NAH was determined (+). ^{75}Se radioactivity and catalytic activities of NAH were normalized at each measurement to the protein concentration and are expressed as percentages of initial values.

value of 7 atoms of Fe per 160 kDa enzyme molecule obtained in this study may be compared with the previously obtained value of 5.87 atoms of Fe (recalculated for 160 kDa) (Holcenberg & Stadtman, 1969). The values of 0.47 and 0.55 atom of Se per 160 kDa enzyme species obtained in the present study are substoichiometric. This is likely to be due to inaccuracies of the determination of Se in proteins of molecular weight greater than about 50 000 by atomic absorption. Based on these studies, it is concluded that NAH contains 1 FAD, 5−7 atoms of Fe, and 1 Mo atom coordinated to the dinucleotide form of molybdopterin and up to 1 Se atom.

Relative Stabilities of Hydroxylase, Oxidase, and Diaphorase Activities. The time course of loss of the three catalytic activities exhibited by NAH during storage of an enzyme preparation at 4 °C is shown in Figure 6. The marked loss of hydroxylase activity after l day and the virtually complete loss after 1 week of storage are in marked contrast to the stabilities of the NADPH oxidase and diaphorase activities. Similar relative stabilities of these catalytic activities to heat denaturation have been observed (Holcenberg & Stadtman, 1969; Gladyshev et al., 1994a). The rate of selenium release from the enzyme could not be measured accurately since in the absence of detergents or chaotropic reagents much of the released selenium remains bound to protein. Hence, the loss of the active cofactor form of selenium was possibly much greater than shown in Figure 6. It is known that selenium in the enzyme, which is coordinated directly to molybdenum, is essential only for the hydroxylase activity of NAH and not for the oxidase and diaphorase activities (Gladyshev et al., 1994a).

Attempts To Reconstitute Hydroxylase Activity with Selenium Compounds. Enzyme that had undergone partial loss of nicotinate hydroxylase activity during storage was incubated under aerobic or anaerobic conditions with selenite, selenide, and selenophosphate. Surprisingly, treatment of the enzyme with selenide (1 mM, 10 min) or with selenophosphate (7 mM, 30 min) under anaerobic conditions led to complete inactivation of hydroxylase activity in a time-

dependent process. Selenite neither activated nor inhibited when incubated with the enzyme under aerobic conditions (10 mM, 1 h).

Incubation of a *C. barkeri* crude extract with 0.l mM selenite and 2 mM DTT for l h under anaerobic conditions led to the complete loss of NAH hydroxylase activity. Since selenite is reduced to selenide under these conditions, the latter rather than selenite presumably caused the inhibition. The inactivated enzyme was purified anaerobically from the treated crude extract using phenyl-Sepharose and DEAE-cellulose chromatographic column steps. In a parallel experiment, active NAH was purified anaerobically from nontreated crude extract. Both of the isolated enzymes had the same specific activity, indicating that inactivation with selenide was reversible.

Reversible inactivation of NAH was not specific for selenide since sulfide also inactivated the purified enzyme in a time-dependent manner (1 mM, 10 min). Sulfide-inactivated NAH regained full activity after diafiltration with 100 mM potassium phosphate, pH 7.0, 2 mM DTT on a Centricon-30 microconcentrator.

EPR Spectroscopy. Cofactors of NAH were additionally characterized by EPR spectroscopy. Figure 7 shows the EPR signals obtained for various cofactors of NAH. The isolated enzyme exhibits a "resting" Mo(V) signal with $g_z = 2.067$, $g_y = 1.982$, and $g_x = 1.974$ (2.067 signal, Figure 7A, spectrum a) (Gladyshev et al., 1994a). It was shown that this signal was stable toward oxidation with ferricyanide and towards short-time (5 s) reduction with nicotinate. It was suggested previously that this signal represents Mo(V) centers of catalytically active enzyme. The "as isolated" ^{77}Se-containing NAH exhibited a 2.067 signal in which the g_x and g_y components are transformed due to the hyperfine interaction of Mo(V) centers with ^{77}Se (nuclear spin $I = {}^1/_2$), indicating the direct coordination of Mo with Se (Figure 7A, spectrum b) (Gladyshev et al., 1994a).

Prolonged anaerobic treatment of NAH with nicotinate led to the complete disappearance of the 2.067 signal. This reaction of NAH with substrate which also caused changes in the electronic absorption spectra (Figure 2), eventually led to the formation of a FAD radical EPR signal and to the appearance of new EPR signals (Figure 7A, spectra c, c′, d). Two new signals obtained at slightly different conditions upon long time incubations with substrate developed slowly in a time-dependent manner. By analogy with xanthine oxidase and other Mo-containing enzymes (Bray, 1988; Hille, 1994), these signals presumably belong to the Mo(V) centers of NAH. In this paper, these signals are called signal A and signal B.

Signal A was much smaller in amplitude than the 2.067 signal and was developed upon incubation of NAH with nicotinate for 1−3 h under argon flow at 4 °C. Signal A was similar for ^{77}Se-enriched NAH (Figure 7A, spectrum c′) and for NAH with natural Se isotope abundance (Figure 7A, spectrum c).

Signal B had about the same amplitude as the 2.067 signal and was obtained when NAH was incubated with nicotinate for 1−3 h under strictly anaerobic conditions at room temperature (Figure 7A, spectrum d). This signal could also be obtained upon reduction of NAH with sodium dithionite (not shown). The signal B of ^{77}Se-containing NAH was similar to the signal B of NAH-containing natural-abundance selenium isotopes (not shown). Because the line shape of signal B slightly varies from preparation to preparation, at present we are unable to make definite conclusions whether Mo−Se coordination is preserved upon prolonged reduction

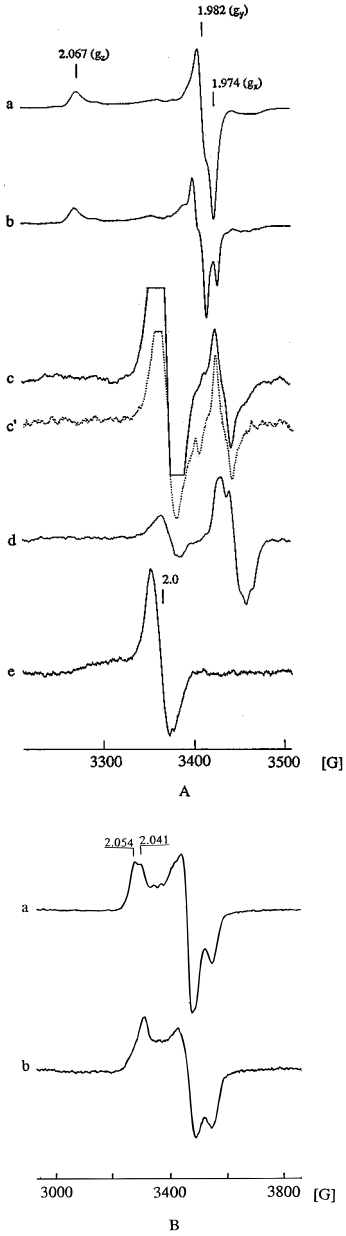

FIGURE 7: EPR signals of various cofactors of NAH. (A) Signals detected at 130 K. Spectrum a: As-isolated NAH exhibits a 2.067 Mo(V) signal. Spectrum b: As-isolated ^{77}Se-enriched NAH; g_x, and g_y components of the signal are transformed due to the hyperfine interaction of Mo(V) species on ^{77}Se (Gladyshev et al., 1994a). Spectrum c: Signal A is exhibited by NAH after 2 h reduction of enzyme with 20 mM nicotinate under argon flow at 4 °C. Spectrum c′: Signal A of ^{77}Se-containing NAH obtained after incubation of enzyme with 20 mM nicotinate for 2 h under argon flow at 4 °C. Spectrum d: Signal B is exhibited by NAH after 2 h reduction of enzyme with 20 mM nicotinate under strictly anaerobic conditions at room temperature. Spectrum e: Signal of flavin radical is obtained after 1 min reduction with 2 mM NADPH at room temperature. FAD radical signal is present also in spectra c, c′, and d. (B) Signals of iron−sulfur clusters detected at 35 K. Spectrum a: Signals of Fe_2S_2(I) and Fe_2S_2(II) are obtained after 5 min reduction of NAH with 1 mM sodium dithionite, 0.05 mM methyl viologen, and 100 mM Tris-HCl, pH 8.5. g_z components of Fe_2S_2(I) and Fe_2S_2(II) signals are indicated. Spectrum b: Signal of Fe_2S_2(I) is obtained after 2 h reduction of NAH with 20 mM nicotinate under strictly anaerobic conditions at room temperature. The spectrum contains a small amount of the Fe_2S_2(II) signal.

of NAH with nicotinate. The presence of these signals at significantly higher magnetic field compared to the 2.067 signal and the similarity between signals A and between signals B of ^{77}Se-containing and natural-abundance selenium-containing enzymes suggest a significant change in Mo active center structure, and perhaps the absence of Se in the first coordination sphere of Mo after prolonged reduction of NAH with substrate.

Unlike nicotinate, NADPH was very effective in the reduction of NAH. Short-time incubation (1 min) of the enzyme with this compound produced a radical signal centered at a *g* value of 2.003 and led to the complete disappearance of the 2.067 signal (Figure 7A, spectrum e). It is known that NADPH is able to reduce the FAD cofactor; thus, the radical signal obtained after reduction with NADPH can probably be attributed to the flavin radical. It is interesting that although NAD^+ cannot serve as an electron acceptor for nicotinate hydroxylation (Holcenberg & Stadtman, 1969), NADH was about equally effective in reduction of NAH (disappearance of the 2.067 signal and production of the flavin radical) as NADPH (not shown).

It was shown earlier that at temperatures less than 50 K reduced NAH exhibits an EPR signal [Fe_2S_2(I), $g_{x,y,z}$ values of 1.899, 1.946, and 2.041] characteristic of an Fe_2S_2 cluster (Gladyshev at al., 1994a). This signal was obtained after reduction of the enzyme with sodium dithionite. In addition to this signal, the signal of a second Fe_2S_2 cluster [Fe_2S_2-(II), $g_{x,y,z}$ values of 1.897, 1.945, and 2.054] was obtained in the present study upon reduction of NAH with sodium dithionite in the presence of methylviologen. The superposition of these two signals leads to a splitting of the g_z component into a poorly resolved two line pattern with maxima at 2.041 and 2.054 (Figure 7B, spectrum a).

Reduction of NAH with nicotinate also led to the reduction of FeS clusters. This phenomenon was observed both by electronic absorption spectral measurements (Figure 2) and by EPR spectroscopy (Figure 7B, spectrum b). Interestingly, reduction of NAH with nicotinate produced predominantly the Fe_2S_2(I) signal.

The presence of a significant amount of the Mo(V) species in the isolated enzyme is rather unusual for Mo-containing hydroxylases (Bray, 1988). Therefore, it was of interest to see whether the 2.067 Mo(V) species is present *in vivo* or was formed during purification of NAH. In a standard isolation procedure, the 2.067 signal was detected in *C. barkeri* crude extracts and could be monitored throughout the purification. However, if NAH was purified under strictly anaerobic conditions, the 2.067 signal was not observed during the early stages of purification (ammonium sulfate fractionation and phenyl-Sepharose steps). These preliminary data could be interpreted in terms of formation of the 2.067 Mo(V) species upon purification of NAH. This could be due to interaction of Mo(IV) species of enzyme with oxygen or due to superoxide-induced reduction of Mo(VI) species.

The 2.067 Mo(V) signal had a reproducible line shape except that the position of the g_z component varied between 2.07 and 2.062 depending on the type of buffer and the presence of salts. It was found that small anions caused changes in the g_z value and in the width of the g_z component. Examples of changes in the g_z component observed upon addition of azide, cyanide, and glycerol are presented in Figure 8. Short-time incubations (5 s) of NAH with nicotinate (or with analogs of nicotinate) also caused small shifts in the g_z value of 2.067 without affecting g_y and g_x components of the signal. From these data, it could be suggested that the 2.067 Mo(V) centers possess an anion binding site. The same site might account for binding and oxidation of nicotinate. It has been reported previously that changes in g_z values of the Rapid signal of xanthine oxidase could be induced upon addition of anions such as nitrate (Gutteridge et al., 1978).

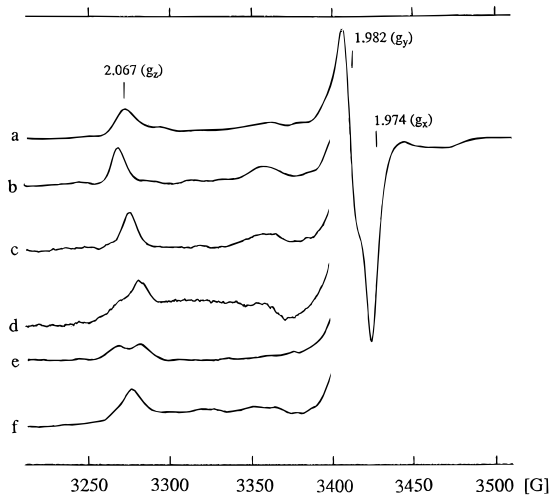

FIGURE 8: The anion binding site of 2.067 Mo(V) species of NAH. Spectrum a: 2.067 signal of as-isolated NAH in 100 mM NaCl, 20 mM potassium phosphate, pH 7.8. Spectrum b: as in spectrum a but in the presence of 30 mM sodium azide. Spectrum c: as in spectrum a but in the presence of 50% glycerol. Spectra d and e: as in spectrum a but in the presence of 50 mM potassium cyanide. Spectrum f: as in spectrum a but in the presence of 20 mM potassium nicotinate. g_x and g_y components of spectra b, c, d, e, and f are identical to those of spectrum a and are not shown. Most of the spectra contain detectable amounts of the original g_z component (as in spectrum a). Spectra b, c, and e were obtained after 1 min, spectrum d was obtained after 30 min and spectrum f was obtained after 5 s incubation with the indicated compound at room temperature in air. All spectra were detected at 130 K.

Various nicotinate analogs were used to study the reduction of NAH. EPR spectra obtained with the treated enzyme were compared to the spectrum of NAH obtained after reduction with nicotinate (Figure 9). A correlation was observed between the ability of the analog to reduce NAH (affect 2.067 signal) and the rate of oxidation of this analog in the reaction assay mixture (Table 4). Nicotinate, 2-pyrazinecarboxylic acid, and 2-hydroxynicotinic acid initiated signal A and caused disappearance of the 2.067 signal. Other nicotinate analogs, which were slowly oxidized by NAH in the reaction assay mixture, were only able to decrease the 2.067 signal. Compounds which were not oxidized by NAH in the reaction mixture were not able to reduce enzyme and did not have any effect on the amplitute of the 2.067 signal.

DISCUSSION

Nicotinic acid hydroxylase from *Clostridium barkeri* has been purified to near-homogeneity using standard chromatographic procedures. Under certain conditions, such as at high enzyme concentrations in deoxygenated solutions at 4 °C, NAH was shown to be reasonably stable during purification. Other bacterial Mo-containing enzymes such as xanthine dehydrogenases are more unstable, especially those that

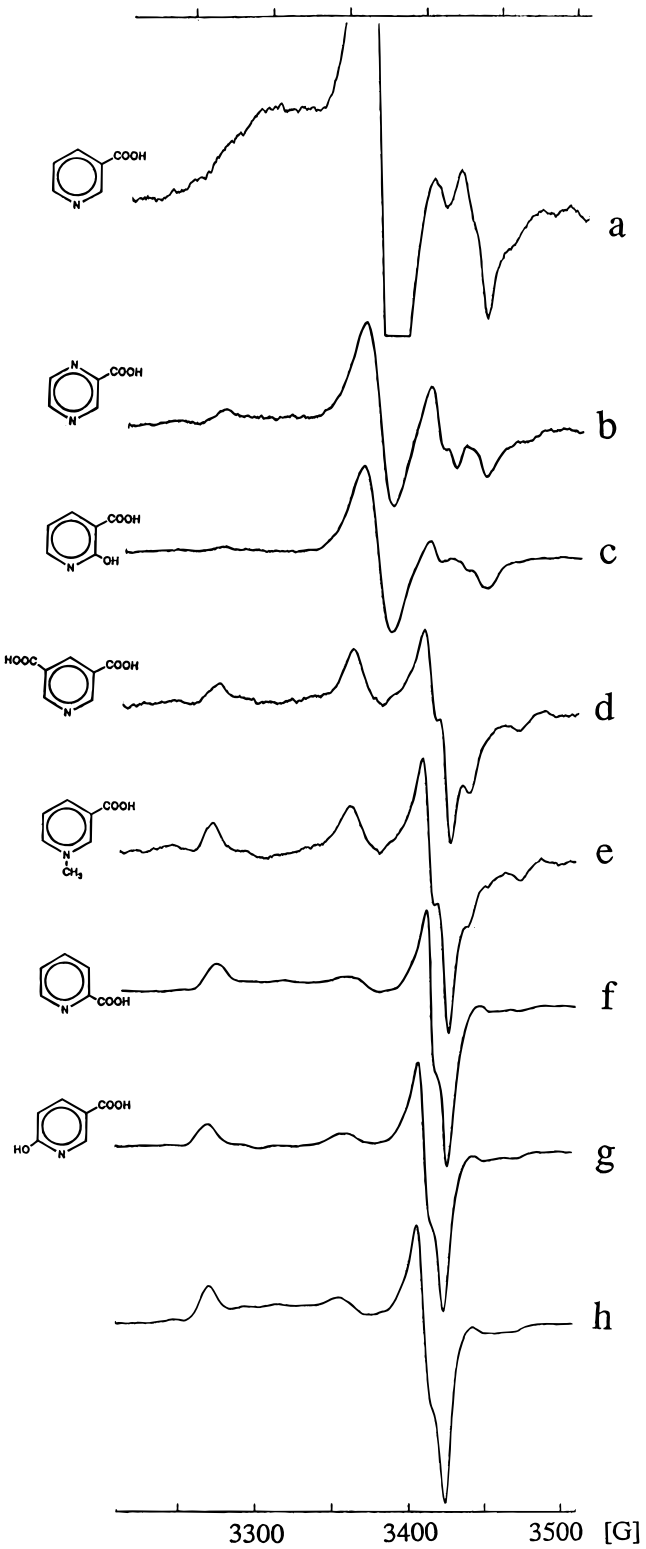

FIGURE 9: Effect of nicotinate and nicotinate analogs on the 2.067 signal of NAH. Spectra were obtained upon 1 h incubation of NAH in the presence of 25 mM potassium nicotinate or its analogs at 4 °C under argon flow. Spectrum a: NAH in the presence of nicotinate. Spectrum b: NAH in the presence of 2-pyrazinecarboxylic acid. Spectrum c: NAH in the presence of 2-hydroxynicotinic acid. Spectrum d: NAH in the presence of 3,5-pyridinedicarboxylic acid. Spectrum e: NAH in the presence of trigonelline. Spectrum f: NAH in the presence of picolinic acid. Spectrum g: NAH in the presence of 6-hydroxynicotinic acid. Spectrum h: as-isolated NAH without any additions. Spectra were obtained at 130 K.

contain a Se moiety (Wagner et al., 1984; V. N. Gladyshev and T. C. Stadtman, unpublished results). Detailed investigation of NAH may provide important information relevant to other bacterial Mo−Se-containing hydroxylases.

Analytical gel filtration and PAGE analyses of NAH revealed that purified enzyme is present in forms of different molecular masses. Distribution of enzyme species over the protein peak from a gel filtration column and association or aggregation of enzyme species under conditions of gel filtration were observed. Major 160 kDa and minor 400 and 120 kDa enzyme forms separated by gel filtration and native PAGE consisted of the same four dissimilar subunits. Subunits of 33, 37, and 50 kDa were present in about equal proportion, and a 23 kDa subunit had significantly lower staining intensity. At present, we cannot exclude the possibility that the 23 kDa subunit is a non-N-terminal degradation product of one of the higher molecular mass subunits. The occurrence of the enzyme in differing molecular mass forms would be in agreement with observations on other Mo-containing bacterial hydroxylases, which may possess 1, 2, or 3 copies of each subunit per enzyme molecule (Kretzer et al., 1993).

The classical scheme for subunit organization of Mo-containing hydroxylases describes enzymes as consisting of three dissimilar subunits: a large (L) subunit of 70−100 kDa containing the molybdenum cofactor, a medium (M) subunit of 25−35 kDa, and a small (S) subunit of 15−25 kDa. Other molybdopterin-containing enzymes have been described as containing 1, 2, or 4 subunits (Wootton et al., 1991; Jollie & Lipscomb, 1991). NAH, however, does not resemble any of the proposed schemes of subunit organization since it contains subunits of similar molecular masses. Moreover, none of the NAH subunits exhibit homology in their N-terminal regions to the published sequences of Mo-containing enzymes (Wootton et. al., 1991; Lehmann et al., 1995). Thus, it is difficult to suggest which of the subunits are responsible for the coordination of Se with the Mo cofactor.

Metal and cofactor analyses of NAH reported previously (Holcenberg & Stadtman, 1969; Dilworth, 1982, 1983; Gladyshev et al., 1994a) and determined here indicate contents of 1 Se, 1 Mo, 1 FAD, and 5−7 Fe per 160 kDa enzyme species. Two Fe_2S_2 clusters may account for four iron atoms. Thus, NAH might contain an additional Fe or FeS center. The presence of a dinucleotide form of molybdopterin in the enzyme is consistent with the reports that most bacterial Mo-containing enzymes contain a larger form of molybdopterin rather than the simple molybdopterin which is characteristic of eukaryotic enzymes.

Se in NAH is coordinated with Mo (Gladyshev et al., 1994a) and is essential for hydroxylation of nicotinate but not for the other enzymic activities exhibited by NAH. The Se moiety present in NAH is unstable and can be released by a number of denaturing procedures, most effective of which are heating or incubation with chaotrophic agents (urea, guanidine hydrochloride) or detergents (SDS). The selenium species obtained upon denaturation of NAH could be a series of polyselenides.

Se is also partially lost during purification of NAH, and none of the available selenium compounds tested were able to restore nicotinic acid hydroxylase activity of NAH. The lability of the Se moiety and therefore the nicotinic acid hydroxylase activity make it extremely difficult to obtain homogeneous preparations of NAH suitable for structural studies. In preliminary experiments, however, we were able

to crystallize the "as isolated" NAH (V. N. Gladyshev, T. C. Stadtman, and P. Sun, unpublished data). Although crystals of NAH are not suitable at present for data collection, this indicates that under certain conditions the NAH preparation is essentially homogeneous and can be crystallized in spite of the presence of impurities of inactive enzyme.

NAH was found to be most stable at alkaline pH, and the pH dependence of stability generally coincides with the pH dependence of activity. Both pH dependencies might reflect the ionization of a similar group(s) on the protein molecule.

It appears that the 2.067 Mo(V) species observed in "as isolated" NAH are formed upon purification of NAH in the presence of traces of oxygen. The 2.067 EPR signal could be first detected in crude extracts of *C. barkeri* and monitored throughout the purification. NAH incubated with nicotinic acid was inactivated in the presence of traces of oxygen, but anaerobic nicotinate-reduced NAH was reasonably stable. This inactivation of the reduced enzyme by oxygen could be due to the production of reactive oxygen species in the vicinity of the Se−Mo center.

An interesting phenomenon was observed during attempts to activate partially inactive NAH with various added selenium compounds. Those compounds which had Se in a −2 state of oxidation (selenide, selenophosphate) reversibly inactivated NAH in a time-dependent manner. If ^{75}Se-labeled compounds were used, radioactivity was incorporated into the protein (not shown). In a similar manner, sulfide also reversibly inhibited NAH. These data suggest nonspecific binding of Se^{2-} and S^{2-} to the surface groups of NAH.

It was found that all NAH cofactors (molybdenum cofactor, FeS clusters, and FAD) under certain conditions are paramagnetic and can be studied by EPR spectroscopy. Previous EPR experiments provided evidence for the direct coordination of Se to Mo in "as isolated" NAH. In the present studies, we found that other Mo signals could be generated in NAH under certain conditions, indicating the complexity of the Mo−Se center of NAH. These signals designated as signal A and signal B were obtained after long-term reduction with nicotinate. No difference has been found between these signals generated by ^{77}Se-enriched NAH or by NAH with natural Se isotope abundance, suggesting substantial change in structure at the Mo center, perhaps breaking the Mo−Se bond.

Amounts of paramagnetic species of NAH have been recently determined by double-integration of EPR signals (S. V. Khangulov and V. N. Gladyshev, manuscript in preparation). The 2.067 signal (0.44 spin per 160 kDa enzyme molecule) represented a significant if not the entire amount of the Mo−Se species of NAH. Signal B also had a significant amplitude (0.2−0.4 spin per enzyme molecule). Signal A accounted for only 0.01−0.05 spin, and iron−sulfur clusters accounted for 2.4 spins per 160 kDa enzyme.

It was indicated that NAH possesses an anion binding site and that this site might be occupied by nicotinate and its analogs. It is not clear at present why nicotinate, the physiological substrate for NAH, is not effective as a reductant of NAH. One possible explanation for this phenomenon is that oxidation of nicotinate is a two-electron process and in order to accept two electrons from the substrate Mo should be in a +6 rather than +5 state of oxidation.

Another distinctive property of NAH is the lack of inactivation by cyanide. This suggests the absence of a terminal sulfo group coordinated to Mo which is rather unusual since in all respects NAH can be considered as a Mo-containing hydroxylase (Wootton et al., 1991). Most bacterial Mo-containing hydroxylases involved in hydroxylation of heterocyclic compounds exhibit close similarity in molecular weight, subunit organization, cofactor composition, EPR characteristics and other spectroscopic, kinetic, and physicochemical properties (Kretzer et al., 1993). The properties of NAH presented in this paper serve to differentiate the enzyme from previously described hydroxylases and point to a unique place of NAH in this class of enzymes.

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