

April 1996

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Gladyshev, Vadim N.; Boyington, Jeffrey C.; Khangulov, Sergei V.; Grahame, David ; Stadtman, Thressa C.; and Sun, Peter D., "Characterization of Crystalline Formate Dehydrogenase H from *Escherichia coli*" (1996). *Vadim Gladyshev Publications*. 45. <http://digitalcommons.unl.edu/biochemgladyshev/45>

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Characterization of Crystalline Formate Dehydrogenase H from *Escherichia coli*

STABILIZATION, EPR SPECTROSCOPY, AND PRELIMINARY CRYSTALLOGRAPHIC ANALYSIS*

(Received for publication, September 6, 1995, and in revised form, January 23, 1996)

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The selenocysteine-containing formate dehydrogenase H (FDH) is an 80-kDa component of the *Escherichia coli* formate-hydrogen lyase complex. The molybdenum-coordinated selenocysteine is essential for catalytic activity of the native enzyme. FDH in dilute solutions (30 µg/ml) was rapidly inactivated at basic pH or in the presence of formate under anaerobic conditions, but at higher enzyme concentrations (3 mg/ml) the enzyme was relatively stable. The formate-reduced enzyme was extremely sensitive to air inactivation under all conditions examined. Active formate-reduced FDH was crystallized under anaerobic conditions in the presence of ammonium sulfate and PEG 400. The crystals diffract to 2.6 Å resolution and belong to a space group of P4₁2₁2 or P4₃2₁2 with unit cell dimensions $a = b = 146.1$ Å and $c = 82.7$ Å. There is one monomer of FDH per crystallographic asymmetric unit. Similar diffraction quality crystals of oxidized FDH could be obtained by oxidation of crystals of formate-reduced enzyme with benzyl viologen. By EPR spectroscopy, a signal of a single reduced FeS cluster was found in a crystal of reduced FDH, but not in a crystal of oxidized enzyme, whereas Mo(V) signal was not detected in either form of crystalline FDH. This suggests that Mo(IV)- and the reduced FeS cluster-containing form of the enzyme was crystallized and this could be converted into Mo(VI)- and oxidized FeS cluster form upon oxidation. A procedure that combines anaerobic and cryocrystallography has been developed that is generally applicable to crystallographic studies of oxygen-sensitive enzymes. These data provide the first example of crystallization of a substrate-reduced form of a Se- and Mo-containing enzyme.

Molybdenum-containing enzymes are widely distributed in both eukaryotes and prokaryotes and have important functional roles in living organisms and in biogeochemical cycles (1, 2). All known Mo-containing enzymes except nitrogenase (3) are members of a large family of proteins in which molybdenum is coordinated to a molybdopterin (4, 5). In *Escherichia coli* formate dehydrogenase, one of two known selenium-dependent

molybdoenzymes (6, 7), molybdenum is also coordinated to selenium of a selenocysteine residue (7, 8).

Although several molybdopterin-dependent enzymes have been studied extensively by mechanistic and structural methods for decades (9, 10), there is still a lack of definitive information on reaction mechanisms and three-dimensional structures. X-ray structural analyses of some of these proteins have been hampered by microheterogeneity due to the partial loss of cofactors or their components, as well as by enzyme instability which usually resulted in failure in crystallization or in decreased diffraction of the crystals. The crystallization of two molybdopterin-containing enzymes, a native aldehyde oxidoreductase from *Desulfovibrio gigas* (11, 12) and a bovine xanthine oxidase, has been reported (13).

Important information on molybdenum-containing enzymes perhaps can be inferred from studies on tungsten-containing enzymes. The structure of the tungstoenzyme, aldehyde ferredoxin oxidoreductase, from *Pyrococcus furiosus* was solved recently at 2.3-Å resolution (14). The active center tungsten is not bound to amino acid residues of the polypeptide chain as suggested for certain bacterial molybdoenzymes (7); rather, tungsten is coordinated to two identical molecules of molybdopterin, each molybdopterin providing two thiolene sulfurs for tungsten coordination. By analogy, these studies supported the proposed structure of the molybdopterin molecule and the proposed type of metal-molybdopterin coordination. However, the archaeobacterial aldehyde ferredoxin oxidoreductase does not have amino acid sequence homology to known molybdenum-containing enzymes.

Substrates of molybdoenzymes react with molybdenum active centers resulting in the redox transformations of these centers and the formation of unstable enzymic intermediates. Some of these are paramagnetic species which have been the subject of extensive studies by EPR, ENDOR (electron nuclear double resonance), and EXAFS (extended x-ray absorption fine structure) spectroscopies (2, 9). However, the interpretation of the data obtained with these techniques depends largely upon our knowledge of three-dimensional structures, especially the structures of reduced forms of the enzymes.

Selenium- and molybdenum-containing formate dehydrogenase H (FDH)¹ and hydrogenase 3 are components of the formate-hydrogen lyase complex, which *in vivo* decomposes formic acid to carbon dioxide and hydrogen under anaerobic conditions (15). FDH was purified recently (16-18) and was shown to contain a number of redox centers: a molybdopterin cofactor

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¹ The abbreviations used are: FDH, formate dehydrogenase; PAGE, polyacrylamide gel electrophoresis; MES, 2-(*N*-morpholino)ethanesulfonic acid; HPLC, high performance liquid chromatography.

consisting of molybdenum coordinated to molybdopterin guanine dinucleotide (16), a selenocysteine (SeCys) residue (8) encoded by UGA (19), and a FeS cluster (16). The catalytic activity of mutant enzyme in which SeCys-140 was substituted with Cys was only 0.3% that of the wild type enzyme (18), and a Ser-140 mutant was inactive (20). EPR studies of formate-reduced Mo(V)-containing FDH have demonstrated the direct coordination of selenium to molybdenum (7). FDH is extremely oxygen-sensitive, and isolation of the active enzyme can be achieved only under strictly anaerobic conditions. In the presence of substrate, formate, the enzyme is inactivated in a time-dependent manner (16).

In spite of its oxygen sensitivity, FDH may be a useful model for studies on molybdenum- and selenium-containing metalloenzymes. Since the enzyme is a monomer of 80 kDa and lacks flavin, it has certain advantages over more complex multicomponent systems. Due to the presence of selenium in the molybdenum coordination sphere, additional methods could be applied to investigate enzyme structure and reaction mechanism.

Herein we present an improved purification procedure, methods of stabilization, and crystallization of substrate-reduced enzyme, together with a procedure for oxidation of the enzyme in crystals and preliminary crystallographic analysis of formate dehydrogenase.

EXPERIMENTAL PROCEDURES

Purification—Formate dehydrogenase H was isolated from *E. coli* strain FM911 containing plasmid pFM20 (16). Cells were grown anaerobically in the presence of 1 μ M sodium selenite as described previously (16, 20). All purification steps were performed in a nitrogen atmosphere containing 1–5% hydrogen in the NIH Anaerobic Laboratory operating at <1 ppm of oxygen (21). All solutions were prepared anaerobically and sparged additionally for 15 min with oxygen-free argon prior to use. All plastic vessels and equipment were kept for 3–4 days under strictly anaerobic conditions prior to use.

The procedure for isolation of FDH from 300 g of cells was essentially the same as described previously (16), except that cells were ruptured by sonication and the isolation procedure was scaled up 15 times to obtain larger amounts of protein. The entire purification procedure was completed in 3–4 days. FDH obtained after the phenyl-Sepharose and hydroxyapatite chromatographic steps was more than 95% pure according to analytical gel filtration and SDS-PAGE analyses. 50 mg of enzyme was obtained with a specific activity of 950 μ mol/min/mg of protein. To further purify the enzyme, an additional ion-exchange chromatographic step was designed, in which FDH in 25 mM MES/NaOH, pH 6.0, 3 mM sodium azide, was applied to a DEAE-HPLC column equilibrated with 25 mM sodium phosphate, pH 6.3, 3 mM sodium azide (buffer A). The enzyme was eluted with a linear gradient of 0 to 200 mM sodium sulfate in buffer A. The active enzyme fractions were pooled and made to 25 mM MES/NaOH, pH 6.0, 5 mM sodium azide, 1 mM sodium phosphate, 3 mM sodium sulfate by diafiltration on an Amicon ultrafiltration unit fitted with a YM-10 membrane. FDH was concentrated to 11 mg/ml, passed through a 0.22- μ m filter and frozen by dripping into liquid nitrogen, then stored in liquid nitrogen. No loss of activity was detected in thawed samples. For experiments with FDH, droplets of the frozen enzyme were brought into an anaerobic chamber or the NIH Anaerobic Laboratory and thawed in anaerobic microcentrifuge tubes.

Studies on Stability—The stability of the DEAE-purified FDH was studied at high and low protein concentrations in pH 6.0 and pH 7.5 buffers in the NIH Anaerobic Laboratory. Solutions containing 300 μ g or 3 μ g of FDH in 100 μ l of 50 mM MES/NaOH, pH 6.0, 3 mM sodium azide or 50 mM HEPES/NaOH, pH 7.5, 3 mM sodium azide were incubated in 1.5-ml microcentrifuge tubes (gas phase 99% N₂, 1% H₂) at room temperature for the indicated periods of time, and, thereafter, activity of the enzyme was measured in the standard assay. To test the effect of formate on stability of FDH under these conditions, 10 mM sodium formate was included in the incubation solutions. The stability of FDH under crystallization conditions in the presence of 2 M ammonium sulfate and 2% PEG 400 was also tested in the presence and in the absence of sodium formate.

To test the effect of oxygen on stability of FDH, 50- μ l samples containing 150 μ g, 15 μ g, or 1.5 μ g of FDH in 25 mM MES/NaOH, pH 6.0, and 3 mM sodium azide were incubated for 2 h in closed 1.5-ml

microcentrifuge tubes (gas phase air) at room temperature. The samples then were brought into the NIH Anaerobic Laboratory, flushed with argon, transferred into deoxygenated microcentrifuge tubes, and incubated overnight (gas phase 99% N₂, 1% H₂) at 4 °C. Catalytic activities were measured in the standard assays and compared to the activity of a control sample (150 μ g of FDH/50 μ l), which was not exposed to air and had been kept in the NIH Anaerobic Laboratory. These experiments were performed both in the presence and in the absence of 20 mM sodium formate.

Crystallization—Due to the extreme oxygen sensitivity of the enzyme, crystallization experiments were carried out in an anaerobic glove chamber (B-type, Coy Laboratory Products, Inc., Grass Lake, MI) under a nitrogen atmosphere in the presence of 1–4% H₂. Oxygen concentration inside the chamber was controlled to less than 1 ppm. All buffers and solutions used in crystallization experiments were sparged with oxygen-free nitrogen gas to remove traces of dissolved oxygen prior to use. Protein samples of FDH were transported into the chamber in liquid nitrogen and thawed prior to use. To grow crystals, 5–10 aliquots of 11 mg/ml FDH was mixed with an equal amount of precipitation reagent and equilibrated in a hanging drop against the well precipitation solution using a Linbro plate (22). The well solution contained 1.5–1.7 M ammonium sulfate, 1% PEG 400, 20 mM sodium formate, and 100 mM HEPES/NaOH at pH 7.5. Crystals grew at room temperature for a period of 1 to 2 weeks.

Cryofreezing of FDH Crystals—FDH crystals were first equilibrated with the well solution before cryofreezing. A pre-equilibrated FDH crystal was then transferred together with about 5–10 μ l of the original well solution to a reservoir of 0.1 ml of cryoprotectant solution. The cryoprotectant solution contained 1.4 M lithium sulfate, 1% PEG 400, 20 mM sodium formate, 100 mM HEPES/NaOH at pH 7.5, and 25% glycerol. Immediately after the first transfer, the crystal was picked up on a cryoloop that was mounted on a stainless steel cap (Charles Supper Catalog No. 485SS), in a drop-and-pick fashion and was frozen in liquid nitrogen.

X-ray Crystallographic Data Collection and Processing—Frozen crystals were quickly transferred from storage liquid nitrogen to a cold –180 °C nitrogen stream centered at the goniometer head in the x-ray beam path. Data were collected using an R-AXIS imaging plate system with a focusing mirror (Molecular Structure Corp.) mounted on a Rigaku RU-200 rotating anode x-ray generator. Crystals were oscillated at 2-degree intervals with a detector distance of 175 mm. The oscillation images were processed using either R-AXIS software package (23) or HKL version 1.0 (DENZO and SCALEPACK) (24). To prevent ice formation on the frozen crystals during data collection, a complete enclosure was built surrounding the crystal to provide a local near zero humidity atmospheric environment.

EPR Spectroscopy—Preparation of EPR samples of FDH was performed as described previously (7). To prepare formate-reduced enzyme samples, 11 mg/ml FDH in 100 mM HEPES/NaOH, pH 7.5, was incubated in the presence of 10 mM sodium formate at room temperature for 1 min followed by freezing of the samples in liquid nitrogen. For EPR study of crystallized FDH, crystals were frozen in liquid nitrogen in a manner similar to freezing of the crystals for x-ray data collection. Frozen crystals were then transferred into EPR tubes. EPR spectra were recorded with a Bruker X-band ESP300 spectrometer (7). Spectra accumulation was performed at 130 K and at 35 K, 5 G modulation amplitude, and 9.46 GHz microwave frequency.

Activity Assessment of FDH Crystals—To determine whether crystallized FDH was active, crystals of 0.2–0.4 mm maximal dimensions were incubated for 5 min in a solution similar to the well solution except that formate was omitted. Crystals were then dissolved in 300 μ l of 25 mM MES/NaOH, pH 6.0, 3 mM sodium azide. Formate dehydrogenase and hydrogenase specific activities of the enzyme were measured in the standard assays and compared to those of the enzyme that was used for crystallization.

Oxidation of Formate-reduced Crystals—Prior to oxidation, formate-reduced crystals were equilibrated in a formate-free solution. A 0.4-mm crystal in a 10- μ l drop was quickly washed 4 times with 50 μ l of the well solution lacking sodium formate. The crystal in a 1–2- μ l solution was transferred into 750 μ l of fresh formate-free well solution and incubated for 15 min, then transferred into another 750 μ l of solution and again incubated for 15 min. The formate-free crystals obtained in this way were oxidized with 0.1–10 mM benzyl viologen in 750 μ l of formate-free well solution for 30–60 min. For experiments with inhibitors, benzyl viologen-oxidized crystals were incubated in 750 μ l of formate-free well solution containing either 10 mM sodium azide, 50 mM sodium hypophosphate, or 50 mM sodium nitrite for 30 min. Crystals were frozen as described above except that the cryoprotectant solution did not

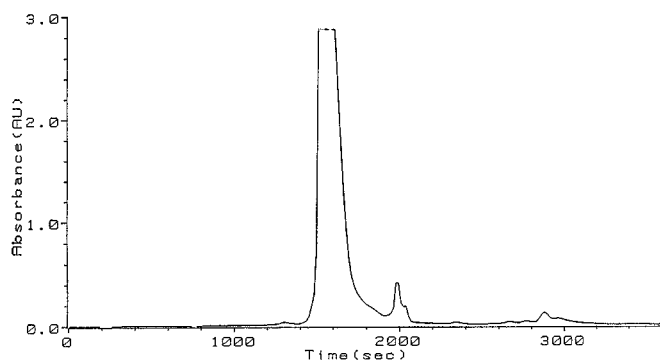


FIG. 1. **Purification of FDH on a DEAE-HPLC column.** FDH purified by phenyl-Sepharose and hydroxyapatite chromatography was further purified on a DEAE-HPLC column as described under "Experimental Procedures." Elution was followed by absorption measurements at 280 nm. The main peak eluted at 1570 s represents FDH. Minor peaks represent impurities.

contain sodium formate.

Other Methods—Benzyl viologen-dependent formate dehydrogenase assays were performed as described (16). To measure hydrogenase activity, a 1-ml mixture containing 2 mM benzyl viologen and 50 mM Tris/HCl, pH 7.5, was bubbled with hydrogen gas for 5 min. Reaction was initiated by the addition of 10 μ l of enzyme and monitored by the increase in absorption at 578 nm due to the reduction of benzyl viologen. Electronic absorption spectra of FDH samples were recorded anaerobically with a Hewlett Packard model 8452A spectrophotometer. Native and SDS-PAGE analyses were carried out aerobically on 12% gels (Novex, San Diego). Analytical gel filtration was performed aerobically using a TSK G3000 HPLC column equilibrated with 100 mM Tris/HCl, pH 7.5, 100 mM NaCl.

RESULTS AND DISCUSSION

Purity of FDH—The preliminary experiments on crystallization of FDH were performed using enzyme of more than 95% purity isolated according to a previously published procedure (16). In addition to FDH activity, this enzyme exhibited detectable hydrogenase activity. With this enzyme preparation, formation of enzyme nucleations was observed in the presence of ammonium sulfate and PEG 400, but no crystals suitable for data collection were obtained.

An additional ion-exchange chromatographic purification step separated some impurities from the main FDH peak (Fig. 1). The purity of the resulting FDH preparation was confirmed with native and SDS-PAGE, analytical gel filtration and mass spectrometry (MALDI) analyses (not shown). A molecular mass of about 80 kDa estimated with these techniques matched the molecular mass predicted from the FDH gene sequence (79.1 kDa), indicating the presence of a full-length polypeptide. The UV-visible absorption spectrum (Fig. 2) was characteristic of a protein containing iron-sulfur clusters with a 280 nm to 400 nm absorbance ratio of 9. Residual trace amounts of hydrogenase activity were not separated from formate dehydrogenase activity by the DEAE-chromatographic step and later by crystallization. The ratio of formate dehydrogenase specific activity to hydrogenase specific activity was 733 for the isolated enzyme and 933 for the enzyme obtained from dissolved crystals.

Stability of Enzyme—It was found previously that FDH is most stable at lower pH (maximum stability at pH 6.0) and most active at higher pH (maximum activity at pH 8.0). It was suggested that both pH profiles might reflect the ionization of a single functional group (16). Sodium formate, the physiological substrate for FDH, inactivated the enzyme in a time-dependent manner (16). These results have been reproduced in our hands. The enzyme was found to have higher stability at pH 6.0 than at pH 7.5, and at each of these pH values the presence of formate resulted in inactivation of FDH (Fig. 3A). It was observed, however, that stability of FDH was highly de-

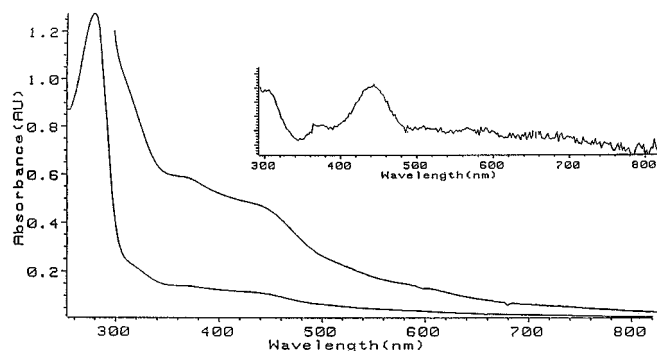


FIG. 2. **Absorption spectra of FDH.** The electronic absorption spectrum of "as isolated" FDH is shown (*lower spectrum*). The area where FeS clusters absorb is shown in the enlarged scale (*upper spectrum*). *Inset* shows the difference spectrum (oxidized enzyme minus formate reduced enzyme). The maximum in the difference spectrum is at 450 nm due to the reduction of FeS clusters. The enzyme was reduced with 10 mM formate for 5 min at room temperature.

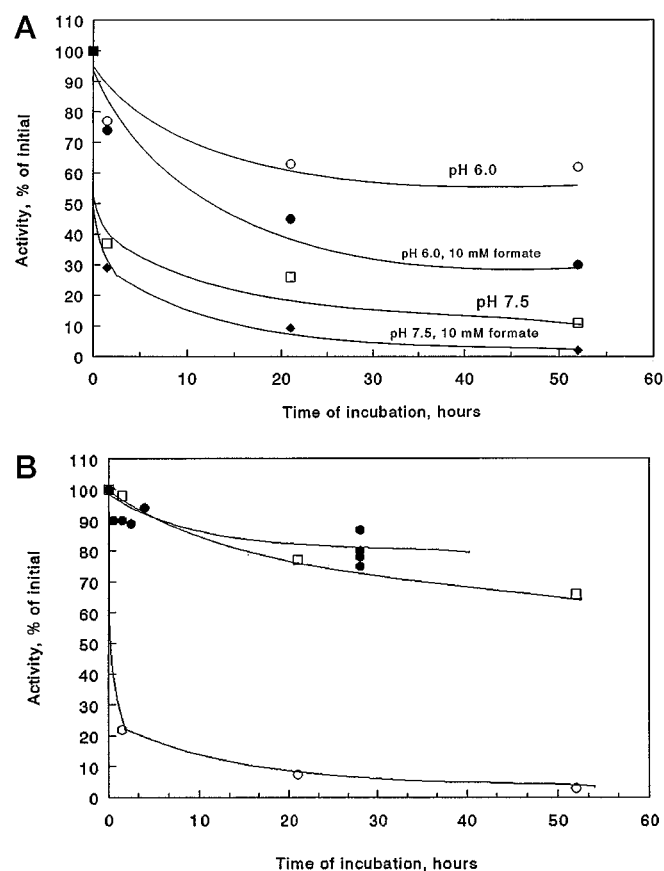


FIG. 3. **Stability of FDH: effect of pH, sodium formate, and enzyme concentration.** FDH samples were incubated in the presence of various compounds at room temperature as described under "Experimental Procedures." At the indicated times, aliquots of the solutions were taken for activity measurements. Activities remaining are expressed as percent of initial activity. *A*, samples contained 3 μ g of FDH per 100 μ l. Buffers were 50 mM MES/NaOH, pH 6.0, or 50 mM HEPES/NaOH, pH 7.5. *B*, 3 μ g of FDH/100 μ l in 50 mM HEPES/NaOH, pH 7.5, 10 mM sodium formate (○); 300 μ g of FDH/100 μ l in 50 mM HEPES/NaOH, pH 7.5, 10 mM sodium formate (●).

pendent on the concentration of the enzyme. At 3 mg/ml, FDH was very stable both at pH 7.5 and in the presence of 10 mM sodium formate (Fig. 3B). Thus, an increase in enzyme concentration led to stabilization of FDH in a state where it exhibited maximal activity. This stabilization effect was presumably due to an increase in the ratio of highly reactive reduced FDH to

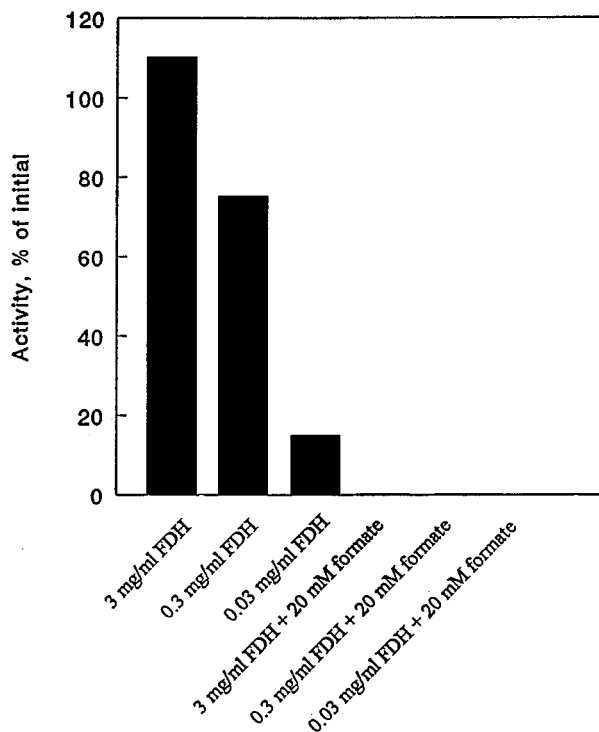


FIG. 4. **Oxygen sensitivity of FDH.** FDH samples of varying enzyme concentration in the presence or in the absence of sodium formate were prepared and exposed to air as described under "Experimental Procedures." Activities of FDH samples are expressed as percent of activity of a FDH control sample which was not exposed to air.

traces of oxygen in the solution.

To test the stability of reduced enzyme at high pH in the presence of oxygen, FDH samples were exposed to air for 2 h (Fig. 4). After this treatment, no activity was detected in the reduced FDH samples at any concentration of the enzyme studied. Interestingly, as isolated FDH was much less sensitive to oxygen. In one experiment, 94% of initial activity remained in the enzyme sample after a 1-h exposure of FDH (6 mg/ml protein) to air. Concentrated FDH (3 mg/ml) was more stable than enzyme diluted 100-fold (0.03 mg/ml), although even diluted enzyme had significant activity (Fig. 4).

The previously observed greater sensitivity of an isolated FDH toward oxygen exposure (16) could possibly be explained by the presence of oxygen-sensitive reduced FDH species in native FDH preparations. This is consistent with our observations that a low reduced Mo(V) EPR signal could be detected in certain isolated FDH preparations.

Crystallization and Cryoprotection of FDH Crystals—Diffraction quality FDH crystals were obtained at room temperature by the hanging drop method using ammonium sulfate and PEG 400 as precipitants (Fig. 5). These crystals grew only under strictly anaerobic conditions. Attempts to carry out crystallizations aerobically failed to yield any crystals. The most competent state of the enzyme for crystallization proved to be freshly prepared protein samples from liquid nitrogen storage. Protein remained competent for crystallization for about 2 weeks. This strong time dependence of crystallization indicates a partial inactivation of the enzyme in 2 weeks even under anaerobic conditions, presumably due to oxidative damage to the enzyme by traces of oxygen. The diffraction quality of already grown crystals was markedly reduced by oxygen and, as a result, only freshly grown crystals were used in data collections.

Crystals were first frozen in liquid nitrogen before removal from the anaerobic chamber for data collection. Once frozen,

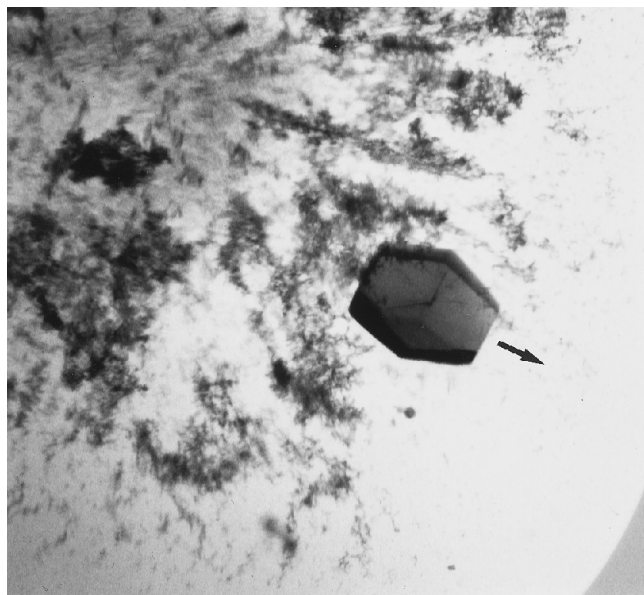


FIG. 5. **Crystal of formate dehydrogenase H.** The crystal grew in approximately 1 week and measured $0.3 \times 0.3 \times 0.4$ mm in size. The direction of a 4-fold symmetry axis is shown with an arrow.

these crystals appear to be insensitive to the level of oxygen and thus can be handled outside the anaerobic chamber. A number of cryoprotectant solutions, including ethylene glycol, PEG 400, sucrose, and glycerol at different concentrations, were tested to obtain satisfactory freezing of the crystals. The most effective reagents for cryofreezing of FDH crystals contain 25%–30% glycerol or sucrose with 1.7 M ammonium sulfate, 1% PEG 400, and 0.1 M HEPES/NaOH at pH 7.5. Glycerol solution gave less mosaicity of diffraction than the sucrose solution. The requirement of a high concentration of glycerol is thought to be necessary to suppress a high spontaneous crystal-forming tendency of ammonium sulfate during cryofreezing, and the elevated concentration of ammonium sulfate might be needed to stabilize crystals in 25–30% glycerol. In order to reduce the crystallization tendency of ammonium sulfate and reduce the amount of glycerol used, we subsequently replaced ammonium sulfate in the cryoprotectant with lithium sulfate and achieved the same or better diffraction of crystals using 1.4 M lithium sulfate and 20–25% glycerol (25). Both a step transfer procedure, in which a crystal was transferred stepwise to 5, 10, 15, 20, and 25% glycerol solutions, and a quick exchange procedure, in which a crystal was directly transferred into the final cryopreserving solution, were tested for applying the cryopreserving solution. Only the quick exchange procedure gave satisfactory cryofreezing results.

Preliminary Crystallographic Analysis of FDH Crystals—A typical FDH crystal measured $0.3 \times 0.3 \times 0.3$ mm and diffracted to 2.6-Å resolution (Fig. 6) on a Rigaku RU200 rotating anode x-ray generator equipped with an R-AXIS imaging plate system. The crystals belonged to a space group $P4_12_12$ or $P4_32_12$ with cell dimensions $a = b = 146.1$ Å and $c = 82.7$ Å. The calculated Matthews' volume (26) was 2.8 assuming one monomer in each crystallographic asymmetric unit. A native data set was collected to 3.0-Å resolution that consisted of 17,511 unique reflections with a merging *R*-factor of 6.6% (Table I). The completeness of the data was 96%.

Properties of Crystals—An important issue of these studies was to determine whether the FDH crystals obtained represented active, formate-reduced enzyme. A 1-month-old crystal of FDH was dissolved in 25 mM MES/NaOH, pH 6.0, and FDH specific activity was determined in the standard assay and

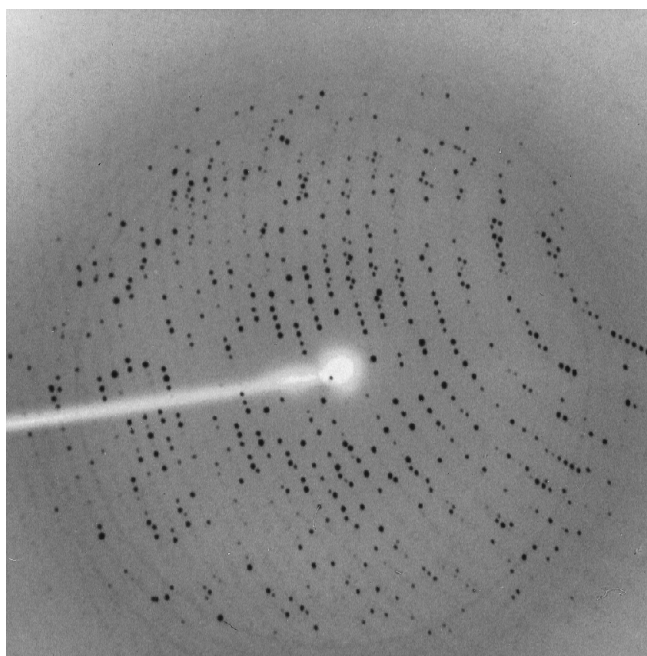


FIG. 6. A 1-degree oscillation image of a native FDH crystal. The image was taken with an R-AXIS detector at a crystal to detector distance of 175 mm and a 5-degree 2θ swing angle. The exposure time was 30 min.

TABLE I
Data collection statistics of the native FDH crystals

Cell dimensions	
a, b (Å)	146.1
c (Å)	82.7
Resolution (Å)	3.0
Observed reflections	31,476
Unique reflections	17,511
Completeness (%)	96
R_{merge}^a	0.066

^a $R_{\text{merge}} = \sum_h |I_h - \langle I_h \rangle| / \sum_h I_h$ is the intensity of reflection h . \sum_h is the sum over all reflections.

compared to the specific activity of the enzyme before crystallization. The formate dehydrogenase specific activity of the enzyme from the dissolved crystal was 41% of the original. In another experiment, 70% of the formate dehydrogenase specific activity and 56% of the hydrogenase specific activity were recovered from six 2-week-old crystals combined and redissolved in one solution. Assuming partial inactivation of the enzyme during crystallization, storage, and dissolving the crystals, activity of FDH present in a freshly formed crystal might be as high as 100%. The electronic absorption spectrum of the redissolved crystal was typical of formate-reduced FDH indicating that the FeS center of the enzyme was in the reduced state.

The redox states of cofactors in FDH crystals were studied with EPR spectroscopy. The signal of a single reduced FeS cluster was observed at 35 K with components corresponding to those of the FeS cluster of FDH in solution (Fig. 7). The FeS signal in the crystal was dependent on the orientation of the sample in the magnetic field of the EPR spectrometer. The complexity of the FeS cluster signal of crystallized FDH suggests that FeS clusters of several orientations are present in the crystal. None of the EPR signals presented in Fig. 7, spectra *b* and *c*, were observed at 130 K, providing further evidence that these signals are derived from FeS clusters. The Mo(V) signal which is observed in solutions of FDH both at 35 and 130 K (Fig. 7, spectrum *a*) was not detected in crystals at these temperatures; therefore, FDH crystals do not contain Mo(V)

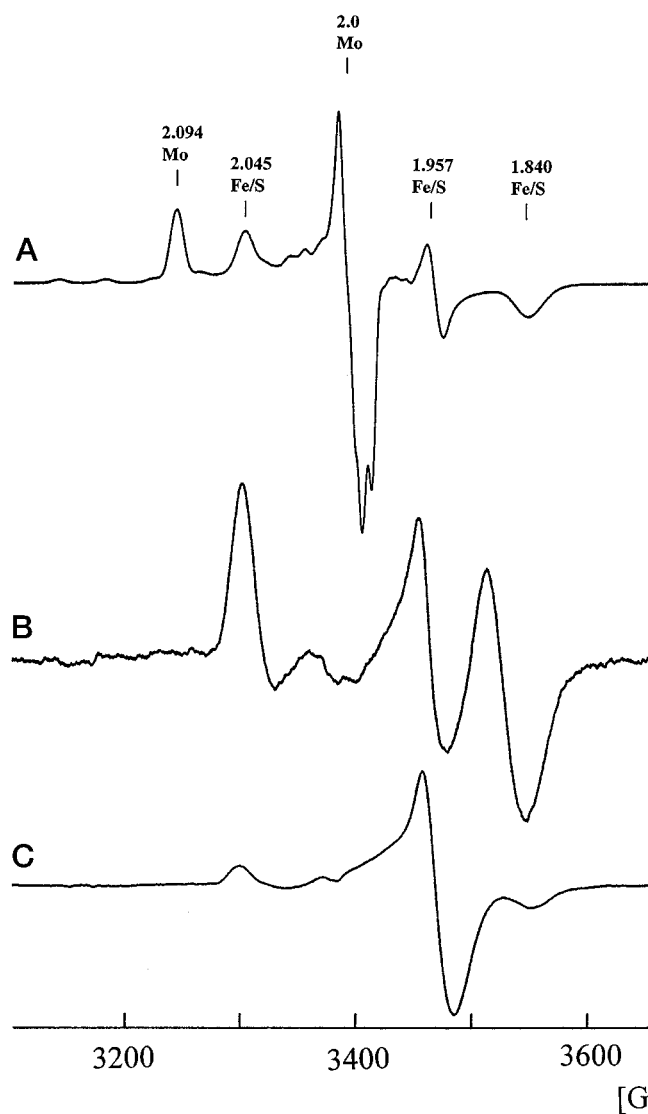


FIG. 7. EPR spectra of FDH in solution and FDH crystals. *a*, EPR signal of the frozen solution of FDH. Enzyme was treated with 10 mM formate for 1 min. The spectrum is a superposition of Fe_2S_2 EPR signal with g -factors of 1.840, 1.957, and 2.04, and Mo(V) EPR signal with g -factors of 2.094 and 2.0. The first signal can be detected at temperatures below 50 K, whereas the second signal can be observed at 130 K and below. *b*, EPR spectrum of a single frozen FDH crystal obtained as described under "Experimental Procedures." *c*, same as *b* but the sample was turned by 45 degrees in the magnetic field of the EPR spectrometer. Before plotting, spectrum *c* was multiplied by 0.25. Spectra *a*, *b*, and *c* were recorded at 35 K at microwave powers of 0.81, 3.8, and 3.8 milliwatts, respectively.

species. These data suggest that the Mo(IV)- and reduced FeS cluster-containing form of the enzyme was crystallized.

Although sodium formate was essential for FDH crystallization, the crystal remained intact when equilibrated in a similar solution lacking sodium formate. When a formate-free crystal was oxidized by 0.1–10 mM benzyl viologen, the crystal developed a dark blue color due to the reduced benzyl viologen. It was observed that reduced benzyl viologen binds tightly to FDH crystals and could not be easily oxidized with aerobic solutions. EPR spectroscopy was used to follow oxidation of crystals. A signal of reduced FeS cluster was observable after equilibration of the crystals in formate-free solutions. However, neither a Mo(V) signal nor a reduced FeS cluster signal was detected after a 30-min incubation of formate-free crystal in 10 mM benzyl viologen.

Ammonium bicarbonate (100 mM) was also tested as an

oxidant for formate-free reduced crystals. Although the reduction of FDH by formate in solutions is reversible (17), added bicarbonate did not reproducibly cause oxidation of crystals, presumably due to the low concentration of carbon dioxide in equilibrium with bicarbonate at pH 7.5. Oxidation of crystals from the Mo(IV)-reduced FeS cluster form to the Mo(VI)-oxidized FeS cluster form of the enzyme or incubations of oxidized crystals with inhibitors (azide, nitrite, and hypophosphate) did not change the diffraction quality of crystals. Since crystals of oxidized FDH could be obtained from formate-reduced crystals, a potential to solve the structures of both reduced and oxidized FDH is provided.

The studies described here for development of an improved purification and stabilization protocol have resulted in the first crystallization of a substrate-reduced molybdenum- and selenium-containing enzyme in its active form.

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