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Purification of the *Azotobacter vinelandii nifV*-Encoded Homocitrate Synthase

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The *nifV* gene product (NifV) from *Azotobacter vinelandii* was recombinantly expressed at high levels in *Escherichia coli* and purified. NifV is a homodimer that catalyzes the condensation of acetyl coenzyme A (acetyl-CoA) and α-ketoglutarate. Although α-ketoglutarate supports the highest level of activity, NifV will also catalyze the condensation of acetyl-CoA and certain other keto acids. *E. coli* cells in which a high level of *nifV* expression is induced excrete homocitrate into the growth medium.

A metallocluster called iron-molybdenum cofactor (FeMo cofactor) is believed to provide the substrate binding and reduction site for biological nitrogen fixation (16). FeMo cofactor is contained within the nitrogenase MoFe protein, and X-ray crystallographic analysis has revealed that it consists of a metal sulfur core [Fe_7S_9Mo] and one molecule of (*R*)-homocitrate (9) (Fig. 1). The core is constructed from [$MoFe_3S_3$] and [Fe_4S_3] subfragments that are connected by three inorganic sulfide bridges located between pairs of Fe atoms from opposing fragments. Homocitrate [(*R*)-2-hydroxy-1,2,4-butanetricarboxylic acid] is coordinated to the Mo atom through its 2-hydroxy and 2-carboxyl groups. Although it is not yet known how the substrate interacts with FeMo cofactor during turnover, the presence of six coordinately unsaturated Fe atoms, as well as the attachment of homocitrate to the Mo atom, has invited speculation about the nature of substrate binding. For example, in one model it has been proposed that the carboxylate group coordinated to the Mo atom might serve as a leaving group in a mechanism that activates Mo to provide a substrate coordination site (7).

Even prior to the emergence of the crystallographic model, biochemical and genetic evidence had established that (*R*)-homocitrate is the organic constituent of FeMo cofactor and that the *nifV* gene product (NifV) is required for homocitrate formation. This evidence can be summarized as follows. First, it was shown that a small organic molecule, later shown to be homocitrate (4), is required for the in vitro biosynthesis of FeMo cofactor (3). Second, the addition of homocitrate to cultures of *Klebsiella pneumoniae* or *Azotobacter vinelandii nifV* mutants cured both their diazotrophic growth and their biochemical phenotypes (5, 12). Third, [^{3}H]homocitrate could be incorporated into FeMo cofactor during its in vitro formation and an organic constituent extracted from purified MoFe protein was identified by nuclear magnetic resonance spectroscopic analysis as homocitrate (6). Fourth, it was shown that a *nifV* mutant from *K. pneumoniae* contains citrate rather than homocitrate (10) and that citrate-substituted FeMo cofactor is responsible for the altered catalytic features of MoFe protein produced by *nifV* mutants (2, 10). Fifth, there is substantial primary-sequence identity among *nifV* gene products and the *leuA* gene product from *Salmonella typhimurium* (20). The *leuA* gene product catalyzes the condensation of acetyl coenzyme A (acetyl-CoA) and α-ketoisovalerate to form α-isopropylmalate (14, 17). These sequence conservations have been interpreted to indicate similar reaction mechanisms where certain structural features of the acetyl-CoA and α-keto acid-binding regions within the respective enzymes are conserved (20). Finally, comparison of the deduced sequences of *nifV* gene products from various diazotrophs and a gene from *Saccharomyces cerevisiae* that encodes a putative homocitrate synthase (19) also reveals substantial sequence identity. Homocitrate is a precursor within the *S. cerevisiae* lysine biosynthetic pathway, and disruption of the *nifV*-like gene results in both a decrease in crude extract homocitrate synthase activity and a decrease in the lysine pool (13). In the present work we describe the heterologous production, purification, and characterization of NifV from *A. vinelandii.*

The high-level expression of *nifV* in *Escherichia coli* was accomplished by constructing a *nifV* gene cartridge in vitro and cloning this cartridge into the pT_7-7 plasmid (18) such that *nifV* gene expression is controlled by the T7 phage transcriptional and translational regulatory elements, which in turn are regulated by the *lac* control elements. The same methods were used as were previously described for similar constructs for the high-level recombinant production of the *nifU* (1) and *nifS* (21) gene products. The *nifV*-containing plasmid is designated pDB555. Oligonucleotide primers used to direct PCR formation of the *nifV* cartridge during construction of pDB555 had the following sequences: 5′CATGCATATGGGTAGCGTGATC3′ and 5′CTACGGATCCATTGCGTAAGCA3′. The amino terminus-coding region for NifV is underlined in the first primer sequence. The functional integrity of the cloned *nifV* gene contained within pDB555 was demonstrated by transforming the *A. vinelandii nifV* deletion strain DJ107 (8) to prototrophy via reciprocal recombination with the chromosome, with isolated pDB555 DNA as the donor. For expression of *nifV* in *E. coli*, pDB555 was transformed into the host strain BL21(DE3), and the transformed cells were maintained on Luria-Bertani medium supplemented with 100 μg of ampicillin per ml. Cells were grown in 500-ml batches in Luria-Bertani medium at 30°C, and NifV production was induced at approximately 160 Klett units (red filter) by the addition of lactose to a 1% (wt/vol) final concentration. After the addition of lactose, cells were cultured for another 2 h and then harvested by centrifugation and frozen at −20°C until used.

For purification of NifV, approximately 10 g of cells was thawed in a buffer containing 25 mM Tris-HCl (pH 7.4), 10% (vol/vol) glycerol, 10 mM α-ketoglutarate, and 1 mM dithiothreitol. Glycerol, α-ketoglutarate, and dithiothreitol were included in the purification buffer because they were all found to

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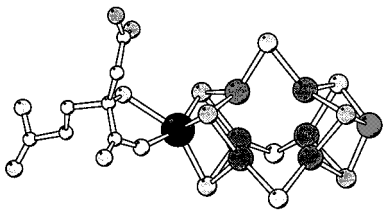

FIG. 1. FeMo cofactor model. The homocitrate [(*R*)-2-hydroxy-1,2,4-butanetricarboxylic acid] molecule is shown on the left and is attached to the Mo atom (darkest sphere) through its 2-hydroxy and 2-carboxy groups. A description of the metal-sulfur core is in the text.

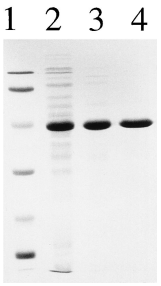

FIG. 2. Analysis of the stages of NifV purification by denaturing polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue. Lane 1, M_r standards (phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme); lane 2, crude extract fraction; lane 3, 35% ammonium sulfate pellet fraction; lane 4, pooled Sephacryl S300 HR peak fraction.

stabilize the enzyme during the purification steps. All manipulations were performed in an Ar atmosphere. Cells were disrupted by sonication, and 1% *p*-methyl sulfonylfluoride was added to yield a final concentration of 1 mM and leupeptin was added to 0.5 mg/liter. Disrupted cells were spun at 35,000 rpm in a Beckman TY35 rotor for 20 min. Solid streptomycin sulfate was added to the ice-chilled supernatant (1% [wt/vol]), and then ammonium sulfate was added to 20% saturation. The sample was then spun at 35,000 rpm in a TY35 rotor, and the subsequent supernatant was brought to 35% ammonium sulfate saturation, and spun an additional time at 35,000 rpm. At this stage NifV was located in the pellet, which was then dissolved in 5 ml of the same buffer as described above, except that the buffer also contained 100 mM NaCl. The resuspended sample was then loaded onto a Sephacryl S300 HR 5- by 50-cm column equilibrated in the same buffer, and NifV was eluted as the second major peak as monitored by optical density at 280 nm. Purification steps were monitored by activity assays (Table 1) and by gel electrophoresis (Fig. 2). The native M_r of NifV was estimated by gel filtration chromatography with a 1- by 30-cm Superose 12 fast protein liquid chromatography column (data not shown). Cytochrome *c*, carbonic anhydrase, bovine serum albumin, yeast alcohol dehydrogenase, and β-amylase were used as standards for estimation of the M_r of NifV. The estimated M_r of native NifV was approximately 89,000, indicating that it is a dimer, because the approximate M_r of NifV determined by denaturing gel electrophoresis was 44,000 (Fig. 2). Assays included 10 mM α-ketoglutarate, 0.2 mM acetyl-CoA, 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 10 mM HEPES buffer, and 5 mM $MgCl_2$, pH 8.0, and were initiated by the addition of 5 to 10 μl of the enzyme fraction to the reaction cocktail. During the course of the optimization of the NifV assay, it was discovered that NifV is oxygen labile, with an irreversible loss of approximately 50% of its activity within 2 h upon exposure to air. The additions of MoO_4^{2-}, Fe^{2+}, and Mg^{2+} were tested for their stimulation of NifV activity or protection of NifV from oxygen inactivation. No effects were observed. NifV activity was measured by the previously described DTNB assay (15). In addition to α-ketoglutarate and acetyl-CoA, pyruvate, α-ketoadipate, α-ketopimelate, and propionyl-CoA were also tested as substrates by the DTNB assay. Kinetic parameters (Table 2) were established by varying the concentrations of the various substrates in the assay mixture. Results of these experiments showed that propionyl-CoA did not support appreciable enzymatic activity but that both oxaloacetate and α-ketoadipate could serve as substrates. The identities of the products when oxaloacetate or α-ketoadipate was used as the keto acid substrate were not investigated further.

In the development of the assay for NifV activity, it was shown that formation of the TNB anion is specifically dependent upon the presence of a keto acid. This result is a good indication, but does not prove, that homocitrate is the product of the NifV-catalyzed reaction when α-ketoglutarate is the keto acid substrate. That homocitrate is the organic acid formed by the NifV-catalyzed condensation of acetyl-CoA and α-ketoglutarate was confirmed in two different ways. In the first approach homocitrate was identified as the product, by gas chromatographic-mass spectrometric analysis (GC-MS), when acetyl-CoA and α-ketoglutarate were incubated in the presence of NifV. In this analysis a sample obtained from a 1-ml assay, performed as described above, was passed through a Centricon 30 filter to remove the protein and then passed over a Dowex 50W-8X H^+ column (25 by 55 mm). The eluted material was evaporated to dryness with a stream of nitrogen gas and then dissolved in 0.5 ml of 3 M HCl either in *n*-butanol (in which case it was heated at 100°C for 3 h) or in methanol (in which case it was kept at room temperature overnight). After evaporation of the butanol or methanol with a stream of dry nitrogen gas, the sample was dissolved in 1 ml of methylene chloride, washed twice with 0.5 ml of water, and dried with Na_2SO_4, and the solvent was evaporated. The butylated sample was dissolved in 20 μl of methylene chloride and analyzed by GC-MS at 70 eV by using a VG-70-70EHF mass spectrometer fitted with an HP-1 column (0.32 mm by 30 m) and programmed from 100 to 280°C at 10°C/min. Both the NifV-generated product and a known sample of homocitrate purchased from Sigma produced two separate GC peaks under

TABLE 1. Purification of NifV

Purification step	Vol (ml)	Protein concn (mg/ml)	Total amt of protein (mg)	Total activity (nmol/min)	Sp act (nmol/min/mg)	Yield (%)
Crude extract	38.0	17.8	676.4	77,610	72	100.0
35% AS[a] precipitate	5.5	21.5	118.3	37,600	318	48.5
S300 HR column fraction	15.0	3.1	46.5	30,540	657	39.3

[a] AS, ammonium sulfate saturation.

TABLE 2. NifV substrate specificity

Substrate[a]	V_{max} (nmol/min/mg)	K_m (apparent) (mM)
Acetyl-CoA	730	0.06
α-Ketoglutarate	750	2.24
Oxaloacetate	350	2.83
α-Ketoadipate	380	1.24

[a] Kinetic parameters for acetyl-CoA were determined with 10 mM α-ketoglutarate as the other substrate. Kinetic parameters for the other keto acids were determined with 0.2 mM acetyl-CoA as the other substrate. Neither propionyl-CoA, pyruvate, nor α-ketopimelate was found to be an effective substrate.

these conditions. The ratio between these peaks, their mass spectra, and their retention times were the same for the known and unknown samples. Based on their mass spectra, the earliest peak was the dibutyl ester of homocitrate lactone (M^+ = *m/z* 300) and the later-eluted peak was the tributyl ester of homocitrate (M^+ = *m/z* 374). The mass spectra of the dibutyl esters of the homocitrate lactones are shown in Fig. 3. The *R* and *S* isomers of the dimethyl ester of homocitrate lactone can be resolved on a type G-TA Chiraldex column (0.25 mm by 40 m; Advanced Separation Technologies Inc., Whippany, N.J.) programmed from 95 to 180°C at 3°C/min. Under these conditions the *R* isomer, the isomer of homocitrate present in nitrogenase, had a retention time of 31.15 min and the *S* isomer had a retention time of 31.29 min. The corresponding butyl esters of homocitrate cannot be resolved by this method. Detection of the eluted isomers was accomplished by MS to ensure compound identity. Because of the similar retention times for the two isomers, the isomer identity was established by coinjecting the unknown isomer with a racemic mixture of the two isomers and noting which of the two peaks increased. In this way it was shown that NifV catalyzes the in vitro formation of (*R*)-homocitrate.

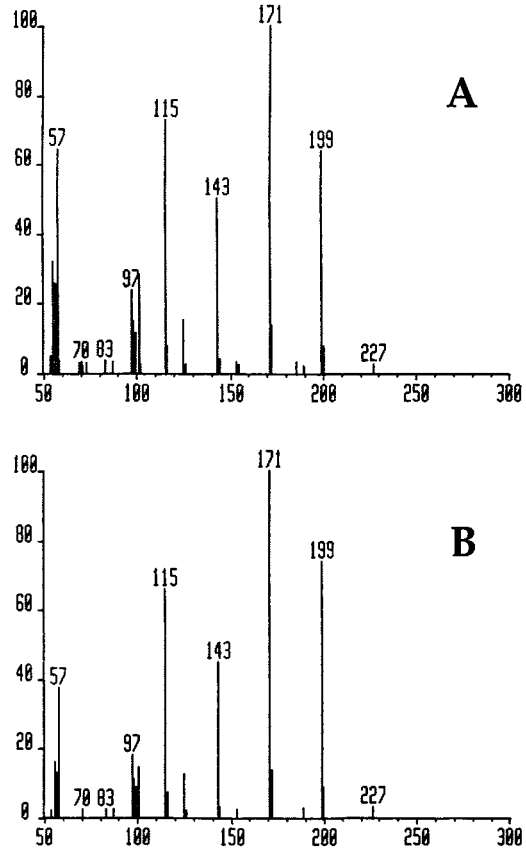

FIG. 3. GC-MS fragmentation patterns of dibutyl ester derivatives of homocitrate lactone produced by NifV activity (A) and authentic homocitrate lactone purchased from Sigma (B).

In the second approach it was shown that cells containing pDB555 excrete homocitrate into the growth medium when NifV expression is induced. For this analysis, *nifV* expression was induced for 2 h in cells that harbored pDB555, and then the cells were centrifuged. Ten milliliters of the clarified medium was then passed over a 5- by 30-mm Dowex 50W-8X H^+ column, and the eluant was evaporated. The resulting sample was dissolved in 1 ml of water and adjusted to pH 12 by the addition of 1 M NaOH. After the sample was heated at 100°C for 3 min, the solution was loaded onto a Dowex 1X8-200 OH^- column (5 by 30 mm) and washed with 5 ml of water, and the carboxylic acids were eluted with 3 ml of 1 M HCl. After evaporation of the HCl from the eluant with a stream of nitrogen gas, the sample was dissolved in 1 ml of methylene chloride. This sample was then washed with a saturated solution of $NaHCO_3$, dried with Na_2SO_4, evaporated to dryness, dissolved in 100 μl of methylene chloride, and analyzed by GC-MS as described above. Quantitation was accomplished by comparing peak areas from single ion plots for the unknown sample with those for a similarly processed sample of authentic homocitrate. The results (data not shown) indicated that upon induction of pDB555-encoded *nifV* expression, homocitrate is excreted into the growth medium, accumulates to at least 0.2 mM, and represents the major organic acid constituent in the growth medium.

In this work the *nifV* gene product was expressed in *E. coli* and purified. It was shown that NifV is a homodimer that catalyzes the condensation of acetyl-CoA and α-ketoglutarate to form homocitrate. NifV is the only gene product required for homocitrate formation. The availability of a strain that produces NifV at a high level and the facile purification of active NifV could serve several useful purposes. (i) The high level of homocitrate excretion into the growth medium by cells which harbor the *nifV* gene provides an inexpensive source for homocitrate. (ii) The availability of active NifV makes possible the design of experiments where specifically radiolabeled high-specific-activity homocitrate can be formed by using radiolabeled precursors in the biosynthetic assay. It might also be possible to use substituted forms or analogs of the homocitrate precursors to form homocitrate analogs. The availability of homocitrate analogs, as well as of radioactively labeled homocitrate, would be useful in expanding the approach described by Madden et al. (11, 12) to monitor the fate of homocitrate during FeMo cofactor formation and to determine the role of homocitrate in nitrogenase catalysis. (iii) Although it is clear that NifV catalyzes homocitrate formation, it is not yet known how Mo becomes coordinated to homocitrate, nor is it known how homocitrate and Mo become incorporated into FeMo cofactor. The availability of purified NifV now makes possible in vitro experiments designed to determine if NifV participates, either individually or in concert with other *nif*-specific gene products, in other aspects of FeMo cofactor biosynthesis.

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