JOURNAL OF BACTERIOLOGY, June 1993, p. 3414–3421
0021-9193/93/113414-08$02.00/0

Vol. 175, No. 11

In *Azotobacter vinelandii* Hydrogenase, Substitution of Serine for the Cysteine Residues at Positions 62, 65, 289, and 292 in the Small (HoxK) Subunit Affects H_2 Oxidation

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Received 8 February 1993/Accepted 29 March 1993

The essential role of the small (HoxK) subunit of hydrogenase of *Azotobacter vinelandii* in H_2 oxidation was established. This was achieved by modification of the two Cys-X_2-Cys amino acid motifs at the N and C termini of the HoxK subunit (Cys-62, -65, -289, and -292). The Cys codons were individually mutated to Ser codons. Modifications in these two motifs resulted in loss of hydrogenase activity. At the N terminus, the mutations of the codons for the motif Cys-62–Thr–Cys-64–Cys-65 decreased the activity of hydrogenase to levels no higher than 30% of those of the parental strain. H_2 oxidation with the alternate electron acceptors methylene blue and benzyl viologen was decreased. H_2 evolution and exchange activities were also affected. Cys-64 possibly substitutes for either Cys-62 or Cys-65, allowing for partial activity. Mutation of the codons for Cys-289 and Cys-292 to Ser codons resulted in no hydrogenase activity. The results are consistent with alterations of the ligands of FeS clusters in the HoxK subunit of hydrogenase.

Dihydrogen (H_2) is intrinsically produced during the reduction of N_2 to NH_3 in biological nitrogen fixation. Nitrogen-fixing microorganisms with an active mechanism to recycle the evolved H_2 have more-efficient N_2 fixation systems (1, 10). Hydrogenases from a number of N_2-fixing microorganisms (e.g., *Bradyrhizobium japonicum* [3], *Rhodobacter capsulatus* [35], *Azotobacter vinelandii* [34], and *Thiocapsa roseopersicina* [12]) as well as a number of nonfixing microorganisms (e.g., *Alcaligenes eutrophus* [33], *Desulfovibrio baculatus* [14], and *Desulfovibrio gigas* [13]) have two subunits with molecular masses of approximately 30 (HoxK) and 60 (HoxG) kDa (28, 29). These hydrogenases possess very similar catalytic properties and can use alternate electron acceptors for H_2 oxidation. *A. vinelandii* is an excellent model system to study these homologous hydrogenases, since the genetics, biochemistry, and physiology of *A. vinelandii* are well characterized (38). The hydrogenase expressed by *A. vinelandii* is a membrane-bound enzyme which catalyzes the oxidation of H_2. It is an NiFe dimer (34) with a likely redox inventory of one Ni, two Fe_4S_4 clusters, and one Fe_3S_4 cluster and, as in the case of other metalloenzymes, possibly uses the metal ions to bind its substrate, to maintain structure, and to effect catalysis and for allosteric control and regulation.

Recently, the cloning and characterization of the genes coding for the small and large subunits of the hydrogenases of *A. vinelandii* (24), *B. japonicum* (32), *Escherichia coli* (26), *D. baculatus* (25), *D. gigas* (20), and *R. capsulatus* (18) have been made possible in different laboratories because of the similarity of their nucleotide sequences. From the deduced primary structures of these hydrogenases, the most notable similarities are among the Cys and His residues. In particular, two Cys-X_2-Cys arrangements, each located at the N and C termini of the small (HoxK) and large (HoxG) subunits, are consistently observed. In other iron sulfur proteins with roles as electron carriers (i.e., ferredoxins and rubredoxin) or roles for catalyses (i.e., aconitase and [de]hydratases [8, 17]), the individual amino acid motif Cys-X_2-Cys has been identified to provide two ligands to FeS clusters (4, 5). It is likely, although not proven, that the conserved Cys residues in the hydrogenases play a role in the binding of cofactor metal ions of these enzymes in a form analogous to those of other FeS cluster-containing proteins.

To understand the extent of the involvement of the motif Cys-X_2-Cys in the oxidation of H_2, it is necessary to assign specific roles to the HoxK and HoxG subunits of hydrogenase. The HoxG subunit of hydrogenase apparently binds the Ni atom and is believed to contain the site of H_2 activation and oxidation. All of the HoxG subunits of NiFe hydrogenases possess paired cysteine residues (Cys-X_2-Cys) at the N- and C-terminal ends. The assignment of Ni to the HoxG subunit is based primarily on the fact that selenocysteine replaces the first Cys of the Cys-X_2-Cys motif proximal to the C-terminal end in *D. baculatus* (28, 39). The selenium in this selenocysteine has been shown to interact with Ni by ^{77}Se electron paramagnetic resonance (14) and extended X-ray absorption fine structure (9). The role of HoxG as the site of H_2 activation is based on the fact that acetylene is a specific, slow, tight-binding inhibitor of hydrogenase (16). Radiolabeled acetylene binds specifically to the HoxG subunit but does not bind to the HoxK subunit (37). Because this inhibition and the binding of acetylene are competitive versus H_2 binding, it was concluded that the site for H_2 activation was located in the HoxG subunit. If the Ni atom (the likely site for H_2 activation) is to be held by Cys-X_2-Cys motifs in HoxG, it would include four Cys residues of a total of five conserved Cys residues in this subunit. The likelihood of locating three other metal cofactors (the FeS clusters) in HoxG would thus be small.

Specific roles for HoxK have not yet been proposed. In hydrogenases from several microorganisms, the HoxK subunits have leader peptides which are removed in the mature enzyme. The function of the leader peptides appears to be to transport the enzyme through the cell membrane (27). Less is known about the catalytic role of HoxK in the oxidation of H_2. Among the NiFe hydrogenases, nine Cys residues in HoxK are located in similar arrangements. Of these Cys

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TABLE 1. Plasmids and bacterial strains

Plasmid or strain	Relevant characteristic(s)	Reference or source
Plasmids		
pTZ19R	Amp^r LacZ	22
pALMZ'1	pTZ19R with the structural genes cloned into *Xba*I and *Bam*HI sites	24
pDB303	*A. vinelandii* Rif^r genomic DNA fragment in pUC8; Amp^r	D. R. Dean (Virginia Polytechnic Institute and State University)
$pAVhox^-$	pALMZ'1 with the deletion of the *Sph*I DNA fragment at the *hoxKG* loci	31
pAVH1	pSelect with the structural hydrogenase genes	This work
pAVc series[a]	pAVH1 with the specific Cys codons mutated to Ser	This work
Strains		
E. coli JM109	*lac-proAB*	40
E. coli BMH 71-18 mut S	*lac-proAB* (*mutS*::Tn*10*)	Promega
A. vinelandii DJ	High-transformation strain	D. R. Dean (Virginia Polytechnic Institute and State University)
A. vinelandii hoxKG mutant	Strain with nonfunctional *hoxKG* genes derived from *A. vinelandii* DJ	31
A. vinelandii $Cys^a \rightarrow Ser$ (series)	*A. vinelandii* with the specific Cys residues changed to Ser in HoxK	This work

[a] Cys residues 62, 64, 65, 289, and 292 were targeted.

residues, four are in two Cys-X_2-Cys motifs; one is located proximal to the N terminus (Cys-62,65), and one is located proximal to the C terminus (Cys-289,292). These Cys residues likely serve as ligands to one or more FeS clusters. The regions in the hydrogenases which have striking similarity are expected to possess key properties for catalysis and so offer points for site-directed mutagenesis studies. To investigate the involvement of Cys-X_2-Cys motifs of the HoxK subunit in the oxidation of H_2, we isolated mutants in which individual Cys residues were changed to Ser. In this paper, we show that the specific substitutions of the Cys for Ser (a conservative substitution) in the Cys-X_2-Cys motifs in HoxK have a dramatic effect on hydrogenase activity. The extent of the mutations on hydrogenase activity was such that, in some mutants, no hydrogenase activity was observed with a variety of electron acceptors. To our knowledge, this is the first evidence that clearly demonstrates that the HoxK subunit of hydrogenase plays an essential role in H_2 oxidation. The HoxK Cys-X_2-Cys motifs possibly serve as ligands to the FeS clusters.

MATERIALS AND METHODS

Plasmids, directed mutagenesis, bacterial strains, and DNA handling. Plasmids, bacterial strains, and their relevant characteristics are listed in Table 1. Starter DNA was pALMZ'1, kindly provided by R. L. Robson (University of Georgia). The 8.9-kb insert of this plasmid was subcloned into pSelect (Promega, Madison, Wis.) to produce pAVH1. Plasmid $pAVhox^-$, derived from pALMZ'1, was produced by deletion of the 1.4-kb *Sph*I DNA fragment at the *hoxKG* loci. Synthetic oligonucleotides homologous to 18 to 24 bases, except at the Cys codon (changed to Ser), were used to induce the point-specific mutations in pAVc62s, pAVc64-65s, pAVc65s, pAVc289s, and pAVc292s. Site-directed mutagenesis was carried out in pAVH1 by using a commercial kit according to the directions of the manufacturer (Promega). DNA sequencing was performed in double-stranded DNA with a Sequenase kit (U.S. Biochemical, Cleveland, Ohio). Plasmid DNA from *E. coli*, grown in antibiotic–Luria-Bertani medium, and genomic *A. vinelandii* DNA, grown in either basal (2) or rifampin-basal medium, were isolated as previously described (30). *E. coli* strains were the mismatch repair minus strain BMH-18 mut S and strain JM109. *A. vinelandii* DJ (a strain with a high rate of transformation) and pDB303 (a plasmid used as selection marker during transformation) were kindly supplied by D. R. Dean (Virginia Polytechnic Institute and State University). The negative control for H_2 oxidation, an *A. vinelandii hoxKG* mutant, was derived from *A. vinelandii* DJ after transformation with $pAVhox^-$. DNA was handled by established protocols as previously described (30).

Assays of hydrogenase activity, D_2 exchange, and H_2 evolution. For hydrogenase activity, D_2 exchange, and H_2 evolution assays, whole cells and cell extracts were used. The cell extracts were prepared in a French pressure cell disrupter at 9,000 kg/cm^2. The cell material was assayed immediately or stored at −70°C until used. For all determinations, the cell material used was normalized to protein content. Protein content was determined by the Bradford method as described previously (6).

H_2 oxidation was detected by one of three methods. (i) The H_2 content in the gas phase was determined by gas chromatography with a Molecular Sieve 5A (80/100) column at 40°C and a thermal conductivity detector. Approximately 8.9 μmol of H_2 gas was injected into stoppered 10-ml test tubes containing approximately 2 ml of a culture grown to an A_{600} of 1.0, and the tubes were incubated at 30°C with agitation (150 rpm in a 5-cm orbital shaker). The amounts of H_2 oxidized were normalized with respect to the N_2 content in the injection. Analyses were performed with at least three replicates and in three independently isolated clones. (ii) H_2 in solution was determined amperometrically with a Clark style electrode (Yellow Springs Instruments, Yellow Springs, Ohio). The reaction chamber (1.6 ml) contained 0.05 M phosphate buffer (pH 7.0), 0.2 mM methylene blue as alternate electron acceptor, 0.15 mM NaF as respiratory inhibitor, and 4 mM EDTA. Sodium dithionite ($Na_2S_2O_4$) from a stock solution (2 mM) was used to activate hydrogenase when necessary. (iii) The reduction of 0.2 mM methylene blue or 100 mM benzyl viologen linked to H_2 oxidation was determined spectrophotometrically in a stoppered cuvette in 0.05 M MES (morpholineethanesulfonic acid) buffer (pH 6.0) at 30°C. The optical density at 690 nm of the reaction mixture containing methylene blue was adjusted to approximately 3 with $Na_2S_2O_4$ from a stock solution (2 mM).

When benzyl viologen was used, a small amount of $Na_2S_2O_4$ was added to the reaction mixture to ensure complete absence of O_2. The extinction coefficients used were 11.4 mM^{-1} at 690 nm for methylene blue and 8.3 mM^{-1} at 600 nm for benzyl viologen.

The exchange reaction mixture with D_2 was assayed in 0.5 ml of a 10×-concentrated cell extract in a quadrupole gas analyzer (Dycor; Ametek Thermox Instruments Division, Philadelphia, Pa.). The exchange reaction was carried out at room temperature in a 12-ml sealed vial under anaerobic conditions in a solution containing 0.05 M phosphate buffer (pH 7), 2 mM $Na_2S_2O_4$, and 8.9 μM D_2 with vigorous stirring. The rates of change in quantity for the masses of H_2, HD, and D_2 (2, 3, and 4, respectively) were monitored for at least 45 min. To normalize the quantities and ensure the absence of O_2 in the assay vial, the masses of N_2 and O_2 (28 and 32, respectively) were also monitored.

Evolution of H_2 was monitored for 5 min amperometrically in 0.1 ml of a 10X-concentrated cell extract with a Clark style electrode in 0.05 M succinate buffer (pH 5.0) with 0.1 M methyl viologen and 2 mM $Na_2S_2O_4$.

Production, screening, and corroboration of *A. vinelandii* mutants. In vitro site-directed mutagenesis was carried out as described above. The mutation was corroborated by dideoxy sequencing with double-stranded DNA as template. The overall integrity in pAVc62s, pAVc64-65s, pAVc65s, pAVc289s, and pAVc292s was checked by restriction mapping to ensure that the DNA was unaltered except at the point of mutation. The sequencing reactions were primed approximately 100 nucleotides from the point mutation to allow the determination of at least 50 bases flanking the base change. The mutated pAVc plasmids were used as a source of DNA for reciprocal recombination in wild-type *A. vinelandii* and *hoxKG* mutant (*hox*-negative) *A. vinelandii*. Mutations were transferred to the chromosomes by cotransformation as previously described except that the selection marker pDB303 was used (7). After 12 h of recovery in 3 ml of basal medium supplemented with NH_4Cl, the transformants were isolated on basal medium agar plates containing 20 μg of rifampin per ml. Primary screening of mutants was by gas chromatography. If accelerated growth was desired, the strains were grown at 37°C; otherwise, the strains were grown at 30°C. Mutants exhibiting deficient hydrogenase activity were isolated for further characterization. The introduced mutation was corroborated by dideoxy sequencing on an amplified DNA fragment produced by the polymerase chain reaction (PCR; Perkin-Elmer Cetus, Alameda, Calif.) with genomic DNA as template. The PCR was carried out for 30 cycles (95°C for 1 min, 55°C for 1 min, and 72°C for 2 min) with primers complementary to two sequences flanking the *hoxKG* genes. Sequencing was performed on the amplified DNA fragment with an AmpliTaq cycle sequencing kit (Perkin-Elmer Cetus) or by the snap-freeze method. For the former, the sequencing primer was end labeled with [γ-^{32}P]ATP (10 mCi/ml; 3,000 Ci/mmol) and sequenced according to the directions of the manufacturer at 68°C for the anneal-extend reaction. For the latter, the primer was annealed by heating the template and primer to 100°C for 1 min and quickly freezing it in liquid N_2. The reaction mixture was then allowed to warm to room temperature in a prechilled (−20°C) aluminum block. The sequencing reaction in this method was as described for double-stranded plasmid DNA. The primers used for sequencing were 100 bases apart from the point mutation, to allow determination of the sequence of at least 50 nucleotides flanking each mutation. Independently isolated mutant clones of *A. vinelandii* exhibited identical phenotypes.

	62 65	289 292
Av	---LECTCCSE---	---HGCIGCSE---
Bj	---LECTCCSE---	---HGCIGCSE---
Db	---QGCTGCSV---	---AVCIGCVE---
Dg	---AECTGCSE---	---HPCIACSE---
Ec	---LECTCCTE---	---HGCLGCAE---
Rc	---LECTCCSE---	---HGCIGCSE---

FIG. 1. Cys residues in which the mutations were produced; comparison of the amino acid sequences among different microorganisms and their relative locations. The Cys residues were changed to Ser. Av, *A. vinelandii*; Bj, *B. japonicum*; Rc, *R. capsulatus*; Ec, *E. coli*; Dg, *D. gigas*; Db, *D. baculatus*. Numbers indicate the Cys residue targeted at the Cys-X_2-Cys motifs and are derived from the gene sequence.

The gene product was determined by Western blotting (immunoblotting) as described elsewhere (30) with rabbit antiserum raised against the hydrogenase of *A. vinelandii* and a commercial alkaline phosphatase-conjugated antiserum as secondary antibody according to the directions of the manufacturer (Promega). The amount of protein precipitated on the immunoblots was measured as transmittance through a photographic negative as determined with a scanning densitometer (Hoefer Scientific Instruments, San Francisco, Calif.).

RESULTS

Mutated DNA and mutant strains. The targeted Cys residues in HoxK were residues 62, 65, 289, and 292 (the numbers were derived from the gene sequence). In each case, the Cys residues were changed to Ser. One mutant was constructed with a double substitution in Cys residues 64 and 65 (Fig. 1). The DNA used to induce the mutations was confirmed to be unaltered, except at the point mutation, by restriction endonuclease mapping and by sequencing at least 50 nucleotides flanking the mutation. Mutant strains were produced with an efficiency of 3 to 5% of that of the rifampin-resistant colonies. Colonies were individually picked and assayed by gas chromatography for altered hydrogenase activity as described above. This method proved to be a fast and reliable way to screen large numbers of possible mutants. Isolated mutant strains were considered stable after reisolation from a single colony three to four times with expression of the observed phenotype. Although there are reports that mutants of *A. vinelandii* are often unstable because of segregation or instability of the mutated DNA (38), mutants in the gene *hoxK* of hydrogenase were frequently stable after one generation. The incorporation of the mutation into the *A. vinelandii* genome was corroborated by determining the nucleotide sequence of the regions next to the targeted Cys in a PCR-amplified DNA fragment containing the genes of hydrogenase. In addition, the predicted sizes of the amplified mutated DNA fragment by PCR were in all instances the same as that of the PCR product of the wild-type strain, confirming that the genes for hydrogenase were unaltered except at the site of the mutation. We concluded that the induced mutations were the cause for the altered hydrogenase phenotypes that we observed. The presence of the protein of hydrogenase was detected with

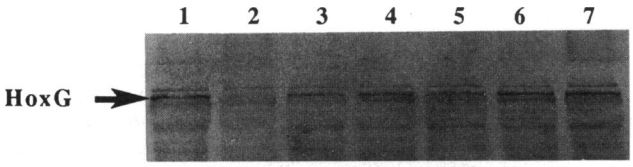

FIG. 2. The protein HoxG in the parental strain and the hydrogenase mutant strains of *A. vinelandii*. The samples were prepared and probed with an antibody for the whole hydrogenase in an immunoblot as described in Materials and Methods. Lanes: 1, the parental strain; 2, the HoxKG mutant (negative control); 3, mutant Cys-62→Ser; 4, mutant Cys-65→Ser; 5, mutant Cys-64,65→Ser; 6, mutant Cys-289→Ser; 7, mutant Cys-292→Ser. The arrow points to the protein highlighted (HoxG [molecular mass, 66.8 kDa]).

rabbit antiserum raised against the whole enzyme of *A. vinelandii*. Unfortunately, the antibody presented cross-reactivity against three other proteins of *A. vinelandii* with molecular weights similar to that of the HoxK subunit. The precise assignment for a peptide of 31 kDa to the HoxK subunit was therefore not possible. Nevertheless, an unmistakable peptide identifying the HoxG subunit (66.8 kDa) of hydrogenase was detected (Fig. 2). Since hydrogenases (including that of *A. vinelandii*) are expressed from a single operon (15, 19, 23), it was assumed that HoxK and HoxG were present in stoichiometric amounts in the protein samples separated by sodium dodecyl sulfate-gel electrophoresis prior to immunoblotting. The HoxG subunit of hydrogenase was detected by scanning densitometry of the immunoblots at similar intensities in the mutants and the parental strain. The proteins cross-reacting with the antibody were present in all of the mutant and control strains and served as a reference to judge the concentrations of HoxG. The *A. vinelandii* KG mutant strain, which lacks functional *hoxKG* genes, did not show a peptide for HoxG. This corroborated the specificity of the antibody for the HoxG subunit of hydrogenase. Since equal amounts of protein from extracts showed a similar amount of the HoxG subunit of hydrogenase, protein concentration of extracts was used to normalize the results of enzyme activity tests.

Phenotypes of the mutants. The phenotypes observed for the isolated mutants are summarized in Table 2. The oxidation of H_2 in the wild-type strain and the mutants was tested with the terminal electron acceptors O_2, methylene blue, and benzyl viologen. The evolution of H_2 from hydrogenase was tested with the electron donor methyl viologen. The exchange activity in the absence of an electron acceptor was assayed with D_2. Since rifampin-resistant *A. vinelandii* grew at rates different from those for non-rifampin-resistant strains, the control strains used in this work were also made rifampin resistant. Overall, the rifampin-resistant strains altered in hydrogenase activity did not show any additional differences (e.g., in growth rates or appearance) compared with the rifampin-resistant control strains. Hydrogenase activity varied from batch to batch and depended on the age of the cultures. For comparison, all assays were performed with a control culture and are reported as percentages of the activity observed in the parental strain. Table 2 contains a representative set of cultures, and specific activities are given.

H_2 oxidation with O_2 as terminal electron acceptor. O_2 is the terminal electron acceptor in vivo for hydrogenase. We tested the hydrogenase activity in the presence of O_2 in intact mutant cells using the gas-chromatographic and amperometric assays. The gas-chromatographic assay consisted of a 12-h incubation in a sealed vial with finite concentrations of H_2 (8.9 μmol) and O_2. In the wild-type strain H_2 was oxidized to completion, usually before O_2 was depleted (i.e., less than 6 h of incubation). In contrast, the mutants showed residual H_2 after the complete depletion of O_2 (approximately 12 h). The negative-control strain KG mutant was used to account for the possible losses of H_2 due to leakage (less than 5% of the initial concentration). Mutant Cys-62→Ser did not oxidize more than 20% of the H_2 concentration consumed by the wild-type control strain. Mutant Cys-65→Ser consumed 45% of the H_2 added before the O_2 was depleted. No H_2 was consumed by the double mutant Cys-64,65→Ser. Mutants Cys-289→Ser and Cys-292→Ser presented phenotypes similar to that of the negative-control KG mutant.

Using the amperometric method, we determined H_2 oxidation rates over a 5-min period in whole cells and cell extracts. In this assay, activity of less than 1% was observed in whole cells of mutant Cys-62→Ser. Whole cells of mutant Cys-65→Ser were in the range of 1 to 2% (Fig. 3). In cell extracts, the parental strain showed 85% loss of activity, indicating that coupling with O_2 was disrupted in cell extracts. Less than 1% of the activity of the wild type was observed for mutant Cys-62→Ser in cell extracts. Mutant Cys-65→Ser cell extracts showed 2 to 3% of the activity observed in the wild type. With O_2 as the electron acceptor, no H_2 oxidation was observed in cell extracts from *A. vinelandii* mutants Cys-289→Ser and Cys-292→Ser or mutant Cys-64,65→Ser.

TABLE 2. Hydrogenase activities of the parental strain and the hydrogenase mutant strains of *A. vinelandii*[a]

Strain	Activity in whole cells (%)		Activity in cell extracts (%)				
	O_2 Amp	Methylene blue	O_2 Amp	Methylene blue	Benzyl viologen	H_2 evolution	D_2 exchange
DJ	100 (485)	100 (485)	100 (75)	100 (193)	100 (86)	100 (25)	100 (170)
HoxKG$^-$	0	0	0	0	0	0	ND[b]
Cys-62→Ser	<1	2	<1	8	21	0	ND
Cys-65→Ser	<2	2 and 35[c]	<3	18	16	58	10
Cys-64,65→Ser	0	0	0	0	0	0	ND
Cys-289→Ser	0	0	0	0	0	0	ND
Cys-292→Ser	0	0	0	0	0	0	ND

[a] Data corresponding to a representative set of cultures. Activities are given in percentages for comparison with the parental strain. The numbers in parentheses indicate the maximal specific activity for the parental strain in whole cells and extracts in nanomoles of $H_2 \cdot min^{-1} \cdot mg^{-1}$ of protein. Amp, amperometric assay.
[b] ND, not detected by this method.
[c] Before and after activation with $Na_2S_2O_4$.

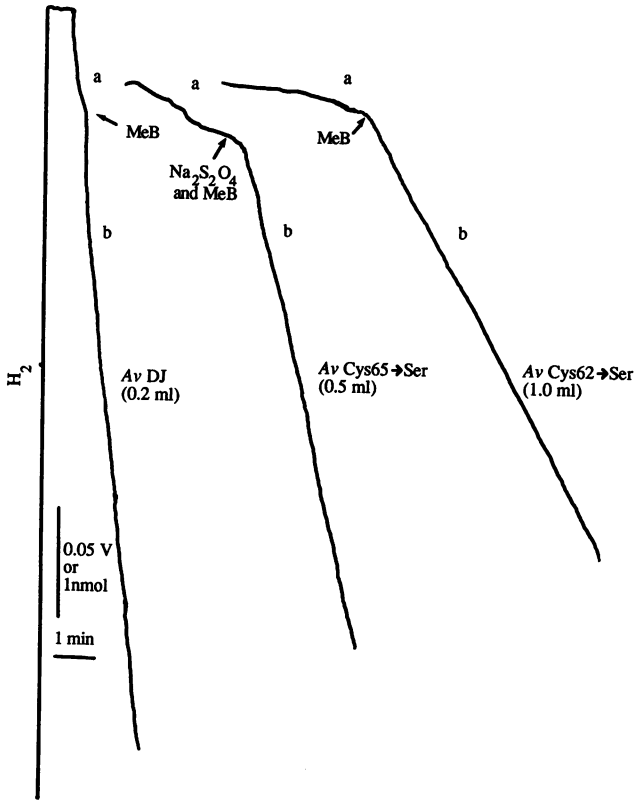

FIG. 3. Amperometric recordings of H_2 uptake of the parental strain and the hydrogenase mutant strains Cys-62→Ser and Cys-65→Ser of *A. vinelandii*. The traces represent H_2 oxidation by intact cells. H_2 oxidation was measured with (a) O_2 and (b) methylene blue (MeB) as the electron acceptor. The arrows point to the addition of $Na_2S_2O_4$ and methylene blue as indicated. The cells of *A. vinelandii* DJ (0.2 ml), *A. vinelandii* Cys-62→Ser (0.5 ml), and *A. vinelandii* Cys-65→Ser (1.0 ml) were from 3-day-old cultures.

H_2 oxidation with methylene blue as the electron acceptor. Methylene blue is an alternate electron acceptor of electrons from hydrogenase. The activity of hydrogenase with methylene blue could be tested by the spectrophotometric or amperometric assay. Both methods gave comparable results. We tested the hydrogenase activities of the mutants in whole cells and cell extracts. The activity in intact cells of *A. vinelandii* Cys-62→Ser with methylene blue was 2% of the wild-type rate, which is higher than the rate with O_2 as terminal electron acceptor by the amperometric method (Fig. 3). The activity in the cell extract of Cys-62→Ser was 8% of the wild-type rate. The addition of $Na_2S_2O_4$ resulted in the enhancement of activity in cell extracts of mutant Cys-62→Ser but not in intact cells. The hydrogenase activity of whole cells of the mutant Cys-65→Ser was 2% of that of the control but was enhanced by the addition of $Na_2S_2O_4$ to up to 35% of the activity of the control strain (Fig. 3). When cell extracts of this mutant were used, 18% of the control activity was observed only after the addition of $Na_2S_2O_4$. The full 35% activity observed in whole cells of mutant Cys-65→Ser could not be recovered; possibly some of the hydrogenase was irreversibly inhibited by O_2 during cell breakage. No activity in Cys-65→Ser was observed with methylene blue alone. It is known that O_2 inhibits *A. vinelandii* hydrogenase and that the enzyme can be reactivated by the addition of a reductant such as $Na_2S_2O_4$ (36). The low level of activity observed in cell extracts of Cys-62→Ser and the slight increase of activity after activation differed from those of Cys-65→Ser, in which no residual activity was observed after cell breakage and not all activity could be recovered after activation. The *A. vinelandii* mutants Cys-64,65→Ser, Cys-289→Ser, and Cys-292→Ser did not show activity with methylene blue, nor were they activated with $Na_2S_2O_4$ in whole cells or cell extracts.

H_2 oxidation with benzyl viologen as the electron acceptor. Benzyl viologen can be used as an alternate electron acceptor by hydrogenase in cell extracts of *A. vinelandii* but not in whole cells. In addition, benzyl viologen is a one-electron acceptor with low midpoint potential (−340 mV), while methylene blue is a two-electron acceptor with a more positive potential (+11 mV). The possibility existed that mutants that transferred electrons poorly or not at all to methylene blue or the natural electron acceptor could still transfer electrons to benzyl viologen. H_2 oxidation activity was observed in extracts of the mutants Cys-62→Ser and Cys-65→Ser and at similar rates. Mutant Cys-62→Ser showed 21% the activity observed in the wild type, while mutant Cys-65→Ser presented 16% of the wild-type activity. This is contrary to the observations with O_2 and methylene blue, in which Cys-65→Ser was generally more active than Cys-62→Ser. Mutants Cys-64,65→Ser, Cys-289→Ser, and Cys-292→Ser did not show activity with benzyl viologen.

D_2 exchange activity. The isotope exchange activity in which D_2 is converted to HD and H_2 was tested in cell extracts of the wild-type and mutant strains with a quadrupole gas analyzer. In the control strain, mass 4 (D_2) tended to decrease steadily as mass 2 (H_2) increased throughout the 45-min period of analysis. Mass 3 (HD) initially increased during the first 10 min and then stabilized to a steady amount. Presumably the diffusion of HD from the solution was sufficiently slow so that the HD could rebind to the enzyme and be converted to H_2. Of the mutants, we were able to detect exchange activity only in mutant Cys-65→Ser. The activity was less than 10% of the exchange activity of the control strain. No isotope exchange activity was observed in cell extracts of the mutants Cys-62→Ser, Cys-64,65→Ser, Cys-289→Ser, and Cys-292→Ser.

H_2 evolution. *A. vinelandii* hydrogenase catalyzes a reversible reaction; thus, in the presence of an appropriate electron donor, it is able to evolve H_2 at a low rate. To test whether the flow of electrons was impaired in this reversible reaction, H_2 evolution was assayed with methyl viologen as electron donor. H_2 evolution was tested in 10×-concentrated cell extracts. We were able to detect H_2 evolution in the control strain and in the mutant Cys-65→Ser. This mutant showed approximately 58% of the hydrogen evolution observed in the wild type. Mutants Cys-62→Ser, Cys-64,65→Ser, Cys-289→Ser, and Cys-292→Ser did not catalyze the evolution of H_2 in amounts large enough to be detected by the amperometric method.

DISCUSSION

It is hypothesized that HoxG, the large subunit of *A. vinelandii* hydrogenase, contains the H_2-activating site. This hypothesis is based on comparisons with other hydrogenases (23) and on the binding of C_2H_2, a competitive inhibitor of H_2 binding, to HoxG (37). On the basis of sequence similarities with other NiFe hydrogenases and the known binding of Ni to the large subunit of *D. baculatus* hydrogenase, a putative role for HoxG is also to bind Ni (28, 39). A role for HoxZ is to transfer electrons between the electron transport chain of

A. vinelandii and hydrogenase. This is based on the observation that deletion mutants in HoxZ no longer transfer electrons from H_2 to O_2 and are not capable of activating hydrogenase but can transfer electrons from H_2 to an artificial electron carrier when the hydrogenase is activated with a reductant (31). What, then, is the role of HoxK, the small subunit of *A. vinelandii* hydrogenase? Site-directed mutagenesis of amino acids in HoxK provides one approach to elucidating the role of HoxK in hydrogenase catalysis. We targeted four conserved Cys residues in HoxK. In each case, conversion of the residue from Cys to Ser, a conservative change, resulted in the partial or complete loss of hydrogenase activity. We conclude from these observations that HoxK has an essential role in hydrogenase catalysis in *A. vinelandii*. This observation is in contrast to a report with *E. coli* HYD2 (11) in which a form of the enzyme with only the large subunit was isolated. The preparation of hydrogenase retained high levels of activity with artificial electron acceptors (11). In the *E. coli* work, the enzyme was purified, while in the present work, the mutant enzyme remained on the membranes. Although it could be argued that purification facilitated access to the active site of the large subunit in *E. coli* hydrogenase, this is unlikely to be the reason for the decreased activity in *A. vinelandii*. First, the artificial electron acceptor methylene blue accepts electrons readily from hydrogenase in wild-type membranes and in membranes of the HoxZ deletion mutant, indicating that access to the electron-donating site is present even when the enzyme is bound to the membranes (31). Second, the isotope exchange activity is also decreased in the HoxK mutants. This reaction does not require an electron mediator, and therefore the question of electron mediator accessibility does not apply.

Hydrogenase from *A. vinelandii* has a likely redox inventory of 1 Ni, 2 Fe_4S_4 centers, and 1 Fe_3S_4 center. The Ni may be ligated by four cysteine residues, and the three iron sulfur centers would be expected to require a total 11 cysteines as ligands, as has been suggested for other hydrogenases (28). There are five conserved cysteines in HoxG and nine in HoxK. Clearly, the conserved cysteine residues in HoxK must play a role in binding the iron sulfur clusters to the protein. The cysteines we targeted were each in Cys-X_2-Cys motifs, typical of iron sulfur-binding domains. Although we have no proof, it is reasonable to assume that the loss of cysteines in these positions resulted in disruptions to iron sulfur clusters. In the case of the Cys-X_2-Cys motif located proximal to the C terminus of HoxK, conversion of either cysteine to serine resulted in complete loss of all activities of hydrogenase, including H_2 oxidation (methylene blue, benzyl viologen, or O_2 as electron acceptors), H_2 evolution, and isotope exchange. In contrast, conversion of either cysteine in the Cys-X_2-Cys motif proximal to the N terminus of HoxK resulted in partial activities in several cases. This Cys-X_2-Cys motif actually contains three cysteines (Cys-62–Thr–Cys-64–Cys-65); the internal Cys is conserved in some, but not all, small subunits of NiFe hydrogenases for which sequences are available (24). It is tempting to speculate that Cys-64 can substitute for the missing Cys in the altered hydrogenases and provide the missing ligand to an iron sulfur center. There is precedent for a neighboring cysteine to substitute for a missing cysteine as a ligand to an iron sulfur cluster. In ferredoxin I from *A. vinelandii*, the substitution of an iron sulfur cysteine ligand for alanine results in another, more distant cysteine serving as a ligand to the iron sulfur cluster (21). The redox properties of the substituted ferredoxin are also altered. Similarly, it is possible that the iron sulfur cluster that uses Cys-65 as a ligand can also use Cys-64, but the resultant hydrogenase has decreased catalytic capabilities. Likewise, Cys-64 might substitute for the more distant Cys-62, giving rise in general to even lower rates of activities. Support for this idea comes from the double mutant in which both the Cys-64 and Cys-65 codons were converted to serine codons. In this mutant, all hydrogenase activities were nil, possibly because Cys-64 was no longer present to substitute for Cys-65. An alternative explanation for residual activity in the altered proteins is the interconversion of an Fe_4S_4 to an Fe_3S_4 cluster analogous to that occurring in aconitase (4). Such a conversion would be expected to change the redox properties of the cluster, which could result in altered catalytic properties.

A likely function of HoxK and any iron sulfur centers that it harbors is the transfer of electrons from HoxG and the H_2-activating site to HoxZ. If this is the case, then it is not surprising that disruptions in putative iron sulfur-binding residues would result in disruptions of hydrogenase activities that require these iron sulfur clusters for transfer of electrons to an electron acceptor. This function for HoxK would adequately explain the inability of the HoxK mutants to transfer electrons efficiently to O_2 or to artificial electron acceptors (if one assumes that the electron acceptors bind to HoxK). Likewise, the inability of the mutants to evolve H_2 efficiently can be explained by inefficient transfer of electrons from an electron donor site on HoxK through HoxK to the H_2-activating site on HoxG. However, it is not clear why disruptions in electron transfer should affect the isotope exchange reaction. It has long been argued that the isotope exchange reaction is one of the most basic reactions of hydrogenase, requiring only the H_2 activation site. According to this argument, if the H_2 activation site is wholly contained on HoxG, then changes in electron transfer through HoxK should not influence isotope exchange activity. Perhaps the H_2 activation site is not wholly contained in HoxG but is actually formed by elements from both HoxK and HoxG. Alternatively, the isotope exchange reaction might not be as simple or basic a reaction as was previously imagined. Perhaps the isotope exchange reaction actually requires the participation of iron sulfur centers in HoxK as sites of electron storage while deuterons (from D_2 or HD) exchange with protons (from H_2O). Whichever model turns out to be accurate, a role for HoxK in the isotope exchange seems evident.

The partial activities that we observed in the Cys-62→Ser and Cys-65→Ser mutants are of interest. In whole cells, Cys-65→Ser could be activated to 35% of the wild-type activity by treatment with dithionite. In contrast, Cys-62→Ser was not activated by dithionite but gave the same low level of activity before and after treatment with dithionite. In cell extracts, both mutants gave similar activities in the H_2 oxidation assay with benzyl viologen as the electron acceptor. With methylene blue as the electron acceptor, Cys-65→Ser was twice as active as Cys-62→Ser. The most surprising differences between these two mutants were in the H_2 evolution and isotope exchange reactions. The only mutant in which we detected any isotope exchange activity was Cys-65→Ser. The H_2 evolution activity of Cys-65→Ser was 58% of that of the wild type. In contrast, Cys-62→Ser was inactive in both H_2 evolution and isotope exchange. Clearly, the different partial activities of Cys-65→Ser and Cys-62→Ser were not influenced to the same extent by the mutations. Instead of resulting in decreases in all activities by the same proportion, the mutations affected each activity differently. This supports the idea that these cysteines and/or

the redox clusters that they ligate play an integral role in hydrogenase catalysis.

In summary, the results that we present here indicate that HoxK has an integral role in hydrogenase catalysis in *A. vinelandii*. That role would appear to be more than simply electron transfer from HoxG to HoxZ, since isotope exchange activity, in addition to H_2 oxidation and H_2 evolution activities, was influenced by mutations in HoxK. Because the cysteines that we targeted for mutagenesis are in Cys-X_2-Cys motifs, we propose that these cysteines serve as ligands to Fe in the iron sulfur clusters. The different proportions of residual activities in some mutants suggest that HoxK even has a role in modulating the ratio of H_2 evolution to H_2 oxidation. Further studies are required to determine how the roles of HoxK and HoxG are integrated to produce a functioning hydrogenase.

ACKNOWLEDGMENTS

This research was funded by U.S. Department of Energy Grant DE FG6-90ER20013 to D.J.A.

We thank Norman Hommes and Sterling Russell for their technical assistance.

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