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Characterization of the Menaquinone Reduction Site in the Diheme Cytochrome *b* Membrane Anchor of *Wolinella succinogenes* NiFe-hydrogenase*

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The majority of bacterial membrane-bound NiFe-hydrogenases and formate dehydrogenases have homologous membrane-integral cytochrome *b* subunits. The prototypic NiFe-hydrogenase of *Wolinella succinogenes* (HydABC complex) catalyzes H_2 oxidation by menaquinone during anaerobic respiration and contains a membrane-integral cytochrome *b* subunit (HydC) that carries the menaquinone reduction site. Using the crystal structure of the homologous FdnI subunit of *Escherichia coli* formate dehydrogenase-N as a model, the HydC protein was modified to examine residues thought to be involved in menaquinone binding. Variant HydABC complexes were produced in *W. succinogenes*, and several conserved HydC residues were identified that are essential for growth with H_2 as electron donor and for quinone reduction by H_2. Modification of HydC with a C-terminal Strep-tag II enabled one-step purification of the HydABC complex by Strep-Tactin affinity chromatography. The tagged HydC, separated from HydAB by isoelectric focusing, was shown to contain 1.9 mol of heme b/mol of HydC demonstrating that HydC ligates both heme b groups. The four histidine residues predicted as axial heme b ligands were individually replaced by alanine in Strep-tagged HydC. Replacement of either histidine ligand of the heme b group proximal to HydAB led to HydABC preparations that contained only one heme b group. This remaining heme b could be completely reduced by quinone supporting the view that the menaquinone reduction site is located near the distal heme b group. The results indicate that both heme b groups are involved in electron transport and that the architecture of the menaquinone reduction site near the cytoplasmic side of the membrane is similar to that proposed for *E. coli* FdnI.

Hydrogen gas (H_2) and formate are commonly used as low potential electron donors in microbial metabolism, for example in anaerobic respiration. H_2 is converted by hydrogenases into protons and electrons, while formate dehydrogenase catalyzes formate oxidation to yield CO_2, a proton, and two electrons. NiFe-hydrogenases are a widespread class of hydrogenases that contain nickel and iron at the active site of H_2 oxidation (1). Minimally, NiFe-hydrogenases consist of a large subunit harboring the NiFe site and a small subunit with three iron-sulfur clusters that form an electron-transferring conduit to the NiFe center. High resolution structures of several heterodimeric NiFe-hydrogenases are available (1, 2). In many microorganisms, heterodimeric NiFe-hydrogenases are bound to the membrane via a cytochrome *b* subunit. Formate dehydrogenases are molybdoenzymes that consist of a formate-oxidizing subunit containing bismolybdopterin guanine dinucleotide and an iron-sulfur subunit with four [4Fe-4S] centers. The heterodimer is bound to the membrane by a cytochrome *b* that is similar to those anchoring NiFe-hydrogenases (3). These hydrophobic subunits are here collectively referred to as the HydC/FdnI family (see below for nomenclature). The primary structures of the family members contain four conserved histidine residues that are thought to axially ligate two heme b groups (3–5). An alignment of the primary structures of the HydC/FdnI family is presented by Berks *et al.* (3).

The crystal structure of *Escherichia coli* formate dehydrogenase-N was determined recently (6). This heterotrimeric complex catalyzes formate oxidation by menaquinone and comprises the molybdoprotein FdnG, the iron-sulfur protein FdnH, and the membrane-bound FdnI. The latter subunit is a diheme cytochrome *b* typical of the HydC/FdnI family and contains four transmembrane helices. Another transmembrane helix is formed by a hydrophobic segment near the C terminus of FdnH. The crystal structure proved that the four conserved histidines of FdnI serve as axial ligands to the heme b iron atoms. The quinone reduction inhibitor HQNO[1] is bound to FdnI in the crystal structure of the *E. coli* FdnGHI complex. The HQNO binding site, which is likely to be identical with the menaquinone reduction site, is situated near the cytoplasmic surface of the membrane, whereas the FdnG and FdnH subunits were shown to be oriented to the periplasmic side (7). It is therefore likely that protons required for menaquinone reduction are taken up from the cytoplasm, whereas protons generated upon formate oxidation are released into the periplasm. This would make formate oxidation by menaquinone an electrogenic process generating a Δp across the membrane by a so-called redox loop mechanism (3, 8–10).

The ϵ-proteobacterium *Wolinella succinogenes* grows by anaerobic respiration using hydrogen or formate as electron donor and various organic and inorganic compounds as electron acceptor such as fumarate, nitrate, nitrite, or polysulfide (11–15). The NiFe-hydrogenase of *W. succinogenes* is a membrane-bound heterotrimeric complex oriented to the periplasmic space that consists of two hydrophilic (HydA and HydB) and one hydrophobic subunit (HydC). HydB is the large subunit

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[1] The abbreviations used are: HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; Δp, electrochemical proton potential; DMN, 2,3-dimethyl-1,4-naphthoquinone; $DMNH_2$, 2,3-dimethyl-1,4-naphthoquinol.

containing the NiFe site, HydA is the iron-sulfur subunit, and HydC is the membrane-bound cytochrome *b* (11, 12). The HydABC complex is anchored to the membrane by both HydC and the hydrophobic C terminus of HydA, which is predicted to traverse the membrane once, similar to the C-terminal helix of *E. coli* FdnH. Mutants of *W. succinogenes* forming only one of these anchors still contained HydB bound to the membrane and catalyzed H_2 oxidation by benzyl viologen (16). In the absence of both anchors, hydrogenase was found to be present exclusively in the periplasm. A *W. succinogenes* mutant that lacks the consecutive *hydABC* genes did not grow by anaerobic respiration with H_2 as electron donor and did not contain hydrogenase activity demonstrating that the HydABC complex is the only hydrogenase in *W. succinogenes* (16). A genetic system allowing expression of mutated *hydABC* genes in *W. succinogenes* has been established (16).

Isolated hydrogenase from *W. succinogenes* obtained by chromatofocusing of the membrane extract as described by Dross *et al.* (17) contained about equimolar amounts of HydABC and HydAB complexes separable by isoelectric focusing (4). The HydABC form contained 1.6 mol of heme b/mol of nickel and catalyzed DMN reduction by H_2 in contrast to the HydAB form, which lacked heme b and DMN reduction activity. These results indicated that HydC is essential for quinone reactivity and that the HydABC complex carries two heme b groups. The midpoint potentials of the two heme b groups were determined as −240 and −100 mV relative to the standard hydrogen electrode (4).

The heterotrimeric formate dehydrogenase complex (FdhABC) of *W. succinogenes* is homologous to the *E. coli* FdnGHI complex (11, 12). As in NiFe-hydrogenase, the hydrophilic part of *W. succinogenes* formate dehydrogenase has been shown to be oriented toward the periplasmic side of the membrane (18). The generation of Δp by either H_2 or formate oxidation with DMN as electron acceptor has been demonstrated experimentally using intact cells of *W. succinogenes*, inverted membrane vesicles, or proteoliposomes (11, 19, 20). The H^+/e ratio for both reactions was determined to be close to 1.0 thus strongly supporting the redox loop mechanism of Δp generation (20).

Based on the HQNO-occupied site in *E. coli* FdnI, site-directed modification of *W. succinogenes* HydC was performed to investigate its menaquinone binding site. Furthermore the predicted heme b-ligating histidine residues were replaced in HydC carrying a Strep-tag II. This allowed one-step purification of wild-type and variant HydC proteins and the determination of their heme b content. A sophisticated model of the membrane-bound part of *W. succinogenes* NiFe-hydrogenase is presented and compared with other membrane-bound diheme cytochromes *b* that are part of respiratory electron transport chains.

EXPERIMENTAL PROCEDURES

Bacteria and Growth Conditions—*W. succinogenes* was grown with H_2 or formate as electron donor and with fumarate or nitrate as electron acceptor as described previously (21, 22). For growth with H_2, the cultures were stirred under an atmosphere of H_2 (150 kilopascals). Kanamycin (25 mg/liter) and chloramphenicol (12.5 mg/liter) were added when indicated. *E. coli* XL-1 Blue cells were grown aerobically at 37 °C in NZYM medium (23). Ampicillin (100 mg/liter), kanamycin (50 mg/liter), and chloramphenicol (25 mg/liter) were added to the medium as appropriate.

Cell Fractionation and Enzyme Activities—*W. succinogenes* cells harvested in the exponential growth phase were suspended (10 g of cell protein/liter) in an anoxic buffer (pH 8.0) containing 50 mM Tris-HCl and 1 mM dithiothreitol. The suspension was passed through a French press at 130 megapascals and at a flow rate of 10 ml/min. The resulting cell homogenate was centrifuged for 1 h at 100,000 × *g* to yield the membrane fraction (sediment) and the soluble fraction. The reduction of benzyl viologen or DMN by H_2 was recorded photometrically as described previously (17, 19). Electron transport with H_2 as electron donor and fumarate as electron acceptor was recorded photometrically using intact bacteria as described previously (24). One unit of enzyme activity was equivalent to the consumption of 1 μmol of H_2/min at 37 °C. Protein was measured using the Biuret method with KCN (25).

Purification of W. succinogenes NiFe-hydrogenase by Strep-Tactin Affinity Chromatography—The membrane fraction (20 g of protein/liter) of cells grown with formate and fumarate was suspended in a buffer (pH 7.3) containing 50 mM Tris acetate and Triton X-100 (1 g/g of protein). The suspension was stirred for 1 h at 4 °C. After centrifugation (1 h at 100,000 × *g*) the membrane extract (supernatant) was applied to a Strep-Tactin Sepharose column (IBA, Göttingen, Germany) that was equilibrated with the same buffer containing 0.05% (v/v) Triton X-100. After washing the column with equilibration buffer, Strep-tagged proteins were eluted with a buffer (pH 7.3) containing 50 mM Tris acetate, 0.05% (v/v) Triton X-100, and 5 mM desthiobiotin.

Separation of Strep-tagged HydC and HydAB—The HydABC complex from *W. succinogenes* HydC-Strep was subjected to isoelectric focusing as described previously (4) with the following modifications. The pH range of the ampholytes was 6–9 (Servalyt 6–9), and dodecyl maltoside (0.01%) and decyl maltoside (0.1%) were added to the Ampholine solution. The gel bands containing HydAB (at pH ~7.5) and Strep-tagged HydC (at pH ~8.5) were cut out. The gel material and the Ampholine were removed from the samples as described previously (4).

Construction of W. succinogenes hyd Mutant Strains—The *hydC* mutant strains were generated by transforming *W. succinogenes* Δ*hydABC* with derivatives of pHydcat (16). This plasmid contains the entire *hydABC* operon and integrates into the genome of *W. succinogenes* Δ*hydABC* by homologous recombination (16). Derivatives of pHydcat were constructed using the QuikChange site-directed mutagenesis kit (Stratagene) and specifically synthesized oligonucleotides (Table I). Modified pHydcat plasmids were sequenced to confirm the mutations. Nitrate-grown cells of *W. succinogenes* Δ*hydABC* were used for transformation as described previously (13, 26). Transformants were selected on agar plates containing a medium with formate and nitrate as energy substrates as well as kanamycin (25 mg/liter) and chloramphenicol (12.5 mg/liter). The integration of pHydcat into the genome of *W. succinogenes* Δ*hydABC* was confirmed by Southern blot analysis as described previously (16).

RESULTS

The Proposed Menaquinone Reduction Site of HydC—The HQNO molecule found in the crystal structure of *E. coli* formate dehydrogenase-N apparently occupies the menaquinone reduction site within the FdnI subunit (6). HQNO is located on the cytoplasmic side of the membrane accepting a hydrogen bond from His-169, which axially ligates the iron atom of the distal heme b group. Further residues of FdnI (Asn-110, Gly-112, Gln-113, Met-172, and Ala-173) situated on transmembrane helices III and IV are in van der Waals contact with the hydroxyquinoline ring of HQNO. Except Gly-112, these residues are conserved in HydC of *W. succinogenes* NiFe-hydrogenase and other members of the HydC/FdnI family (Table II, see also the model of HydC in Fig. 1). The corresponding residues of HydC were replaced by site-directed modification to determine whether the site of menaquinone reduction on HydC is similar to that proposed for *E. coli* FdnI. Table III summarizes the properties of the resulting mutants. Mutants N128D, Q131H, and Q131L did not grow by fumarate respiration with H_2 as electron donor. The membrane fraction of these mutants grown with formate and fumarate catalyzed benzyl viologen reduction by H_2 with wild-type specific activities, whereas the specific activities of reduction of either fumarate or DMN by H_2 were drastically inhibited. The heme b groups were present in these variant HydC proteins, and their reduction by H_2 was not impaired by the mutations as seen from the wild-type amount of heme b reduced by H_2 after the addition of Triton X-100 and fumarate to the membrane fraction of the mutants (Table III). For all three mutants the apparent K_m value for DMN was found to be similar to that of the wild type (13 μM). The cell homogenate of the mutants was applied in the $H_2 \rightarrow$ DMN assay for the measurements. Replacement of Met-203 and Ala-

TABLE I

Oligonucleotide primers used for site-directed mutagenesis of the hydC gene

A pair of complementary primers was used for each modification of which only the forward primer is shown. The altered nucleotides are printed in bold, and the modified codons are underlined. For the introduction of the Strep-tag II-encoding nucleotide sequence of 30 base pairs, three consecutive rounds of mutagenesis were necessary using the shown Strep-tag primers thereby inserting five, two, and three codons, respectively. Inserted nucleotides are underlined, and the stop codon of HydC is printed in bold. The numbers drawn at the 5′-ends of the sequences correspond to the nucleotide positions given in the EMBL, GenBank™, and DDBJ data base entry with accession number X65189.

Modification in HydC	Forward primer sequence (5′ → 3′)
D88N	^{3524}CCTCTTTATCTTT**AA**CAAAGGGTGCAGGG
E94Q	^{3543}GGGTGCAGGGTT**C**AGCGAGCCTCTTTTTGGG
D100N	^{3557}GCGAGCCTCTTTTTGG**A**ATCTCATCAATCC
Y114F	^{3601}GGCAACTAAGAAACT**T**TATGCTTCTTGG
G125L	^{3635}CCCCCATCTCAAA**TT**GGTTTACAATCCCG
Y127A	^{3637}CCCATCTCAAAGGGGTT**GC**CAATCCCGTTCAGC
Y127H	^{3637}CCCATCTCAAAGGGGTT**C**ACAATCCCGTTCAGC
Y127F	^{3637}CCCATCTCAAAGGGGTTT**T**CAATCCCGTTCAGC
P129A	^{3648}GGGGTTTACAAT**G**CCGTTCAGCTAGC
Q131H	^{3649}GGGTTTACAATCCCGTTCA**C**CTAGCAGCCTATATGGG
H200A	^{3848}GGCGTTTGTGATTTTTATTCCTGTC**GC**CATCTATATGGCG
Y202F	^{3867}CCTGTCCACATCT**T**TATGGCGACATGG
M203I	^{3867}CCTGTCCACATCTATAT**C**GCGACATGGAACTCC
A204F	^{3867}CCTGTCCACATCTATATG**TTC**ACATGGAACTCCGCG
Strep-tag1	^{3950}CCACAAAAAACACTATCCTCAATTTGAGAAA**TAA**TCTTCTTTAAAGAAACAGTG
Strep-tag2	^{3950}CCACAAAAAACACTATAGCCACCCTCAATTTGAG
Strep-tag3	^{3950}CCACAAAAAACACTATAGCGCTTGGAGCCACCCTCAATTTGAG

TABLE II

Residues replaced in W. succinogenes HydC

HydC residue (for location see Fig. 1)	Corresponding residue in *E. coli* FdnI[a]	Property as derived from the *E. coli* FdnI crystal structure (6)	Degree of conservation in members of the HydC/FdnI family[a]
His-25	His-18	Axial heme iron ligand of distal heme b group	Completely conserved
His-67	His-57	Axial heme iron ligand of proximal heme b group	Completely conserved
Asp-88	None		Not conserved
Glu-94	None		Often present in HydC homologs but absent in FdnI homologs
Asp-100	None		Not conserved
Tyr-114	None		Completely conserved in HydC homologs but absent in FdnI homologs
Gly-125	Gly-107		Completely conserved
Tyr-127	Tyr-109		Histidine in HydC homologs or phenylalanine in FdnI homologs are frequently present
Asn-128	Asn-110	Possibly hydrogen bonding the OH group of HQNO	Completely conserved except in *Shewanella oneidensis* HydC where glycine in found
Pro-129	Ala-111		Proline completely conserved in HydC homologs; FdnI homologs contain alanine, leucine, or phenylalanine
Gln-131	Gln-113	van der Waals contact with HQNO ring	Histidine or arginine rarely found in HydC homologs
His-186	His-155	Axial heme iron ligand of proximal heme b group	Completely conserved
His-200	His-169	Axial heme iron ligand of distal heme b group, forms hydrogen bond with *N*-oxide ring of HQNO	Completely conserved
Tyr-202	Tyr-171	Oη atom forms hydrogen bond to His-275 from FdnH, which corresponds to His-305 in *W. succinogenes* HydA	Completely conserved
Met-203	Met-172	van der Waals contact with HQNO ring	Alanine, valine, leucine, isoleucine, or threonine also found
Ala-204	Ala-173	van der Waals contact with HQNO ring	Valine or glycine rarely present

[a] See Ref. 3 for an alignment of *E. coli* formate dehydrogenase-N FdnI, *W. succinogenes* HydC, and similar sequences.

204 in *W. succinogenes* HydC led to mutants that had essentially wild-type properties (Table III).

Further conserved residues of HydC located near Asn-128 and Gln-131 were replaced, and the resulting mutants were characterized (Tables II and III). Strains G125L, Y127H, Y127F, and P129A grew with H_2 and fumarate as energy substrates and showed nearly wild-type specific activities of fumarate or DMN reduction by H_2. In contrast, substitution of Tyr-127 by alanine abolished growth with H_2 as electron donor, and the specific activity of H_2-dependent DMN reduction was inhibited by more than 90% as compared with the wild type. All mutants resembled wild type in heme b reduction by H_2 indicating the presence of an intact HydABC complex.

The crystal structure of *E. coli* FdnI suggests the presence of a proton pathway leading from the cytoplasmic membrane surface to the HQNO site. This contains several water molecules maintained by hydrogen bonds to the polar residue Glu-100, located in the loop between helices II and III, and to His-197 near the C terminus (6, 10). As both residues are not conserved in HydC homologs, variants of *W. succinogenes* HydC were produced containing modified residues that might be involved in a corresponding proton transfer pathway, namely Asp-88, Glu-94, Asp-100, and the conserved Tyr-114 (Table II). The mutants D88N, E94Q, D100N, and Y114F were, however, not affected in growth by fumarate respiration with H_2 as electron donor or in specific electron transport or hydro-

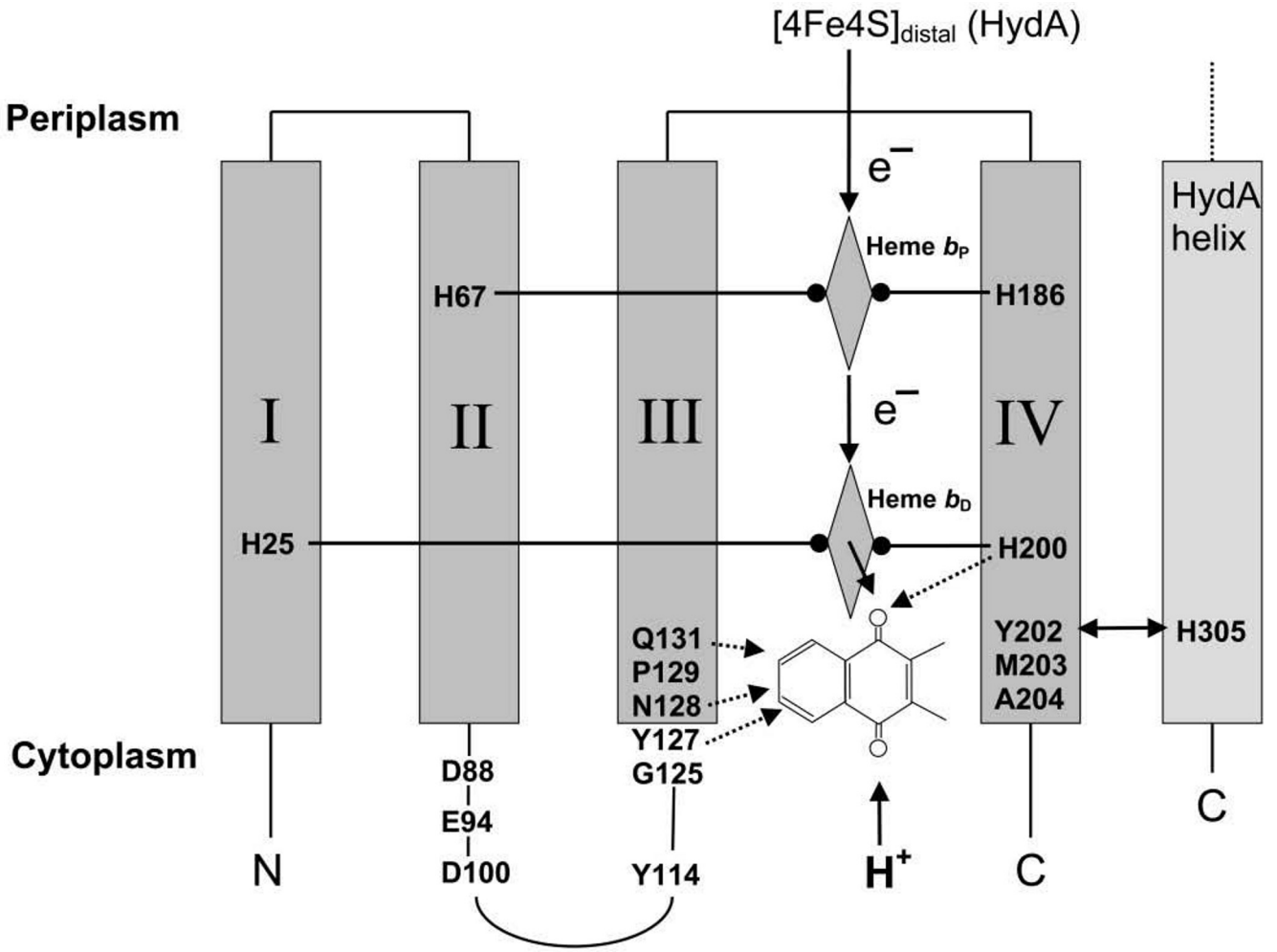

FIG. 1. **Topology model of the membrane-integral part of *W. succinogenes* NiFe-hydrogenase.** The HydC protein forms four transmembrane helices, while HydA is anchored in the membrane by its C-terminal helical segment. Heme b groups are shown as *diamonds*. *Heme* b_P and *Heme* b_D denote the proximal and distal heme b group, respectively. DMN is shown to occupy the proposed menaquinone binding site near the cytoplasmic side of the membrane. All HydC residues indicated were modified in this study. A *dashed arrow* designates a probable direct or indirect interaction of a residue and bound menaquinone. Pathways of electrons and protons are indicated by *solid arrows*. The nature of the proton pathway to the menaquinone reduction site is not known. The *double-headed arrow* indicates the interaction of helix IV of HydC and the HydA helix.

TABLE III
Properties of W. succinogenes strains producing modified HydC proteins
Cells used for activity measurements were grown in medium containing formate and fumarate as energy substrates.

Strain	Doubling time with H_2 and fumarate	Electron transport activity, $H_2 \rightarrow$ fumarate	Specific hydrogenase activity			Cytochrome *b* reduced by H_2[b]
			$H_2 \rightarrow$ DMN in cell homogenate	$H_2 \rightarrow$ BV[a] in membrane fraction	$H_2 \rightarrow$ BV in soluble fraction	
	h	*units/mg of protein*		*units/mg of protein*		*μmol/g of protein*
Wild type	1.8	3.5	4.5	4.2	0.19	0.35
D88N	1.7	2.9	4.4	3.9	0.09	0.29
E94Q	1.9	2.8	4.0	4.5	0.11	0.29
D100N	1.9	2.4	4.0	4.0	0.05	0.34
Y114F	1.8	2.4	3.9	4.4	0.18	0.33
G125L	1.8	2.1	3.1	3.9	0.17	0.40
Y127A	∞	0.12	0.4	4.0	0.17	0.38
Y127H	2.1	2.2	3.2	4.4	0.17	0.35
Y127F	1.9	2.6	4.2	4.9	0.20	0.30
N128D[c]	∞	≤0.05	0.25	4.9	0.15	0.29
P129A	1.9	2.1	3.9	4.7	0.16	0.35
Q131L[c]	∞	≤0.05	0.08	3.9	0.10	0.31
Q131H	∞	≤0.05	0.17	4.2	0.12	0.32
Y202F	∞	≤0.05	≤0.01	4.3	0.11	≤0.02
M203I	1.7	2.4	4.9	4.6	0.19	0.35
A204F	2.0	2.8	3.8	4.3	0.13	0.35

[a] BV, benzyl viologen.
[b] Determined using a Triton X-100 extract of the membrane fraction as described by Gross *et al*. (4).
[c] Data in part taken from Ref. 20.

TABLE IV
Properties of W. succinogenes strains producing (modified) Strep-tagged HydC proteins
Cells used for activity measurements were grown in medium containing formate and fumarate as energy substrates.

Strain	Doubling time with H_2 and fumarate	Electron transport activity, $H_2 \rightarrow$ fumarate	Specific hydrogenase activity			Cytochrome *b* reduced by H_2[b]
			$H_2 \rightarrow$ DMN in cell homogenate	$H_2 \rightarrow$ BV[a] in membrane fraction	$H_2 \rightarrow$ BV in soluble fraction	
	h	*units/mg of protein*	*units/mg of protein*			*μmol/g of protein*
Wild type	1.8	3.5	4.5	4.2	0.19	0.35
HydC-Strep	1.5	2.9	4.0	3.9	0.17	0.39
H25A-Strep	∞	≤0.05	≤0.01	4.0	0.11	≤0.02
H67A-Strep	∞	≤0.05	≤0.01	3.9	0.08	≤0.02
H186A-Strep	∞	≤0.05	≤0.01	3.5	0.09	≤0.02
H200A-Strep	∞	≤0.05	≤0.01	4.8	0.20	≤0.02

[a] BV, benzyl viologen.
[b] Determined using a Triton X-100 extract of the membrane fraction as described by Gross *et al*. (4).

genase activity indicating that neither hydroxyl group of the four residues is essential for proton transfer (Table III).

The contact of *E. coli* FdnI and the iron-sulfur subunit FdnH is mediated by a hydrogen bond between the side chains of Tyr-171 in helix IV of FdnI and His-275 of FdnH. The latter residue is situated near the cytoplasmic end of the C-terminal membrane-spanning helix of FdnH. Both residues are strictly conserved within the HydC/FdnI family (Table II). The *W. succinogenes* mutant having the equivalent Tyr-202 of HydC replaced by phenylalanine showed negligible specific activities of fumarate or DMN reduction but wild-type activity of benzyl viologen reduction by H_2 (Table III). Heme b was not reduced by H_2 in this mutant, although a wild-type amount of HydC was detected in the membrane fraction by Western blot and enzyme-linked immunosorbent assay analysis (not shown). This suggests that electron transfer from the iron-sulfur subunit HydA to HydC was interrupted in mutant Y202F. Mutant *W. succinogenes* H305M producing HydA with His-305 (corresponding to His-275 of FdnH) replaced by methionine was found previously to have properties similar to those of mutant Y202F (4).

Purification of the W. succinogenes HydABC Complex by Strep-Tactin Affinity Chromatography—A Strep-tag II sequence (SAWSHPQFEK) with the N-terminal serine and alanine residues serving as linker was attached to the C terminus of HydC. The corresponding mutant, *W. succinogenes* HydC-Strep, showed wild-type properties with respect to growth with H_2 and specific hydrogenase activity indicating that the tag affects neither the total amount nor the assembly of the HydABC complex (Table IV). The hydrogenase was purified from the Triton X-100-solubilized membrane fraction by Strep-Tactin affinity chromatography as detailed under "Experimental Procedures." The activities of both DMN and benzyl viologen reduction by H_2 eluted together from the affinity column (not shown). The specific activity of DMN reduction by H_2 was 32 times greater in the eluate than in the bacterial membrane fraction (Table V). The total activity of the eluate amounted to 18% of that present in the membrane fraction. The preparation mainly consisted of three polypeptides with sizes corresponding to those of HydA, -B, and -C as judged from SDS-PAGE (Fig. 2*A*). These proteins were identified as the respective hydrogenase subunits using appropriate antisera in Western blot and enzyme-linked immunosorbent assay analysis (not shown). The hydrogenase preparation contained 1.6 mol of heme b/mol of nickel based on heme b reduction by either H_2 or dithionite (Table VI). Attachment of the Strep-tag II to HydC therefore enabled easy and straightforward isolation of the HydABC complex from the membrane of *W. succinogenes* avoiding chromatofocusing and isoelectric focusing used in previous purification procedures (4, 17, 27). The Strep-tag II is also superior to the use of a hexahistidine tag at the C-terminal end of HydC

TABLE V
Purification of W. succinogenes NiFe-hydrogenase carrying Strep-tagged HydC
Cells (35 g wet weight) were grown in medium containing formate and fumarate as energy substrates. Hydrogenase was purified using Strep-Tactin affinity chromatography. The Strep-Tactin eluate was concentrated by ultrafiltration using a 50-kDa filter (Amicon).

Step	$H_2 \rightarrow$ DMN	$H_2 \rightarrow$ DMN
	units	*units/mg*
Membrane fraction	25,300	13
Membrane extract	13,530	13
Strep-Tactin eluate	4,550	420

(28). The corresponding mutant, strain HydC-His_6, merely contained one-third of the wild-type specific activity of DMN reduction by H_2. Furthermore only about 20% of the membrane proteins that were eluted with imidazole from a nickel affinity chromatography column consisted of hydrogenase (28).

Interestingly the HydABC preparation obtained by Strep-Tactin affinity chromatography was found to separate during isoelectric focusing into a reddish-brown and a greenish protein band, a behavior not observed with the wild-type HydABC complex. The greenish band comprised the HydA and HydB proteins, whereas the reddish-brown band consisted merely of HydC. Strep-tagged HydC contained 1.9 mol of heme b (reduced by dithionite) and 1.9 mol of iron/mol of HydC. The iron content was determined by total reflection x-ray fluorescence spectrometry (29). The cytochrome *b* visible light absorption spectrum of HydC was identical to that of the HydABC complex. Only 53% of the heme b could be reduced by $DMNH_2$ in Strep-tagged HydC as was found for the HydABC complex (Table VI). The results clearly demonstrate that the HydC protein binds two heme b groups independently of the presence of HydAB.

Modification of Conserved Histidine Residues in Strep-tagged HydC—The crystal structure of *E. coli* FdnI strongly suggests that the two heme b groups of *W. succinogenes* HydC are axially ligated by the four conserved histidine residues indicated in Table II and Fig. 1. His-67 and His-186 of HydC are predicted to ligate the proximal heme b group, while His-25 and His-200 are likely to ligate the distal heme b group. To examine the function of the heme b groups, His-25, His-67, and His-186 of *W. succinogenes* HydC had been replaced previously by alanine or methionine (4). The resulting mutants did not catalyze DMN reduction by H_2, and heme b was not reduced by H_2 in membrane extracts. The mutant enzymes were still bound to the membrane, and the membrane fraction contained HydC in wild-type amounts. It was, however, not possible to elucidate whether the variant HydC proteins contained heme b since attempts to purify the modified enzymes failed. Here all four predicted heme b-ligating histidine residues of HydC were

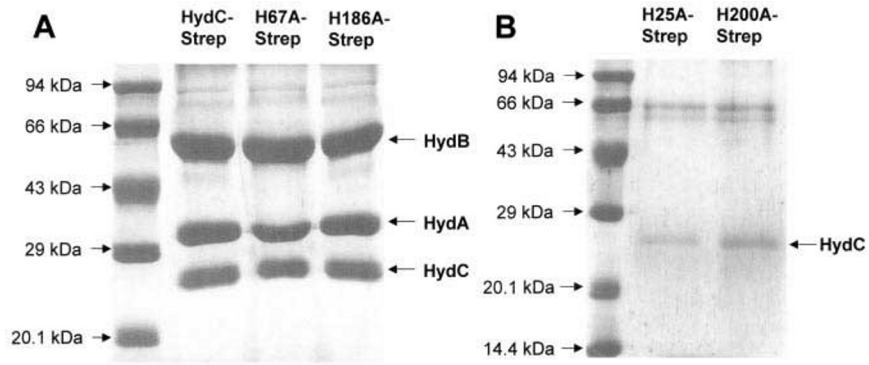

FIG. 2. **SDS-PAGE of *W. succinogenes* hydrogenase preparations obtained by Strep-Tactin affinity chromatography from the indicated strains.** SDS-PAGE and staining with Coomassie Blue were performed as described by Gross *et al.* (16). The amount of protein applied to each lane was 40 μg (*A*) and 10 μg (*B*). The protein of the faint band designated as HydC in *B* was identified by Western blot and enzyme-linked immunosorbent assay using an anti-*W. succinogenes* HydC serum (4).

TABLE VI
Amount of heme b reduced in purified NiFe-hydrogenases from different W. succinogenes strains

The amount of heme b reduced by hydrogen, $DMNH_2$, or dithionite was calculated from the absorbance difference between the reduced and the oxidized sample at 565 and 575 nm using the molar extinction coefficient of 23.4 mM^{-1} cm^{-1} (44). Cytochrome *b* visible light absorption spectra were identical for all three preparations with the maximum at 562 nm. Nickel was determined using total reflection x-ray fluorescence spectrometry (4, 29).

Strain	Heme b reduced by		
	Hydrogen	$DMNH_2$	Dithionite
	mol/mol of nickel		
HydC-Strep	1.6	0.86	1.6
H67A-Strep	≤0.02	0.68	0.73
H186A-Strep	≤0.02	0.68	0.75

replaced by alanine in HydC carrying the Strep-tag II. The resulting mutants (H25A-Strep, H67A-Strep, H186A-Strep, and H200A-Strep) did not grow by fumarate respiration with H_2 as electron donor (Table IV). All four mutants grew with formate instead of H_2 but did not catalyze H_2 oxidation by fumarate or DMN, whereas wild-type activity of benzyl viologen reduction by H_2 was retained in the membrane fraction (Table IV). Heme b was not reduced by H_2 in membrane extracts of the mutants. Strep-tagged hydrogenases were isolated from the mutants using Strep-Tactin affinity chromatography. Heterotrimeric hydrogenase preparations were obtained from strains H67A-Strep and H186A-Strep (Fig. 2*A*). The purified enzymes did not catalyze heme b reduction by H_2 in contrast to hydrogenases isolated from strain HydC-Strep or from the wild type (Table VI). The total amount of heme b reduced by dithionite in hydrogenase preparations from strains H67A-Strep and H186A-Strep is only half of that measured with hydrogenase isolated from strain HydC-Strep (Table VI). Heme b is almost fully reduced by $DMNH_2$ in hydrogenases from strains H67A-Strep and H186A-Strep in contrast to hydrogenase from strain HydC-Strep (Table VI). The substitution of either histidine ligand of the distal heme b group in mutants H25A-Strep and H200A-Strep led to preparations containing comparatively small amounts of HydC but no HydA or HydB (Fig. 2*B*). The preparations did not catalyze benzyl viologen reduction by H_2 and did not contain heme b.

DISCUSSION

Topology Model of HydC and Location of the Menaquinone Binding Site—Fig. 1 shows a topology model of the membrane-integral part of *W. succinogenes* NiFe-hydrogenase consisting of HydC and of the C-terminal transmembrane segment of HydA. The model is based on the results presented here and elsewhere (4) as well as on the crystal structure of *E. coli* formate dehydrogenase-N (6). Although *W. succinogenes* HydC and *E. coli* FdnI clearly belong to the same protein family, it has to be kept in mind that the primary structures share only 18% identical residues. Notably there is currently no report on site-directed modification of either *E. coli* FdnI or any other cytochrome *b* subunit of formate dehydrogenases.

Regarding the crystal structure of FdnI, it is beyond doubt that the four strictly conserved histidine residues in HydC are the axial heme b iron ligands, whereas the conserved His-305 of HydA rather serves the stability of the HydABC complex (see below). His-158 and His-187 of HydC were previously excluded as heme b ligands (4). As suggested before (Fig. 2 in Ref. 4), His-67 and His-186 of *W. succinogenes* HydC ligate the proximal heme b near the periplasmic surface, while His-25 and His-200 ligate the distal heme b group near the cytoplasmic side of the membrane. This view is also supported by the data of Meek and Arp (5) who modified histidine residues of the HydC homolog HoxZ of *Azotobacter vinelandii*.

The properties of the mutants producing modified HydC proteins support the view that the menaquinone reduction site is located near the cytoplasmic side of the membrane and that its architecture is similar to that of the proposed quinone binding site of *E. coli* FdnI, which is occupied by an HQNO molecule in the crystal structure (6). The results suggest that Asn-128 and Gln-131 of HydC are directly involved in menaquinone binding (Fig. 1). Gln-131 cannot be functionally replaced by histidine, which is found at this position in HupC from *Rhodobacter capsulatus*. In contrast, the nearby Tyr-127 can be substituted by either histidine or phenylalanine (but not alanine) without affecting growth with H_2 as electron donor. Histidine at this position is frequently found in HydC homologs, whereas phenylalanine is commonly present in FdnI homologs, *e.g.* in the primary structures of *W. succinogenes* and *Campylobacter jejuni* FdhC (30, 31). Regarding the orientation of the side chain of the corresponding Tyr-109 in *E. coli* FdnI, Tyr-127 of *W. succinogenes* is expected to have an indirect influence on quinone reactivity. In *E. coli* FdnI, Tyr-109 is located at the N-terminal end of helix IV and is in van der Waals contact to both propionate side chains of the distal heme b. Replacement of tyrosine by a small residue like alanine might result in severe secondary structure changes, whereas replacement by bulky residues like histidine or phenylalanine might not. HoxZ of *A. vinelandii* contains a histidine (His-136) at the position corresponding to Tyr-127 of *W. succinogenes* HydC. The H136A mutant of *A. vinelandii* HoxZ catalyzed O_2-dependent H_2 oxidation at only 25% of the wild-type activity (5). It is conceivable that the heme b iron ligand His-200 of HydC may form a hydrogen bond to menaquinone as was found for the corresponding His-169 in the *E. coli* FdnI structure with bound HQNO (6). The conserved residues of helix IV, which make contact with HQNO in *E. coli* FdnI, seem not to be essential for quinone reactivity in *W. succinogenes* HydC (Fig. 1). The same holds true for residues located in the loop between helices II and III, namely Gly-125, Tyr-114, and His-122, the

latter residue having been previously replaced by alanine (4). The location of the menaquinone reduction site strongly suggests that two protons are taken from the cytoplasmic side of the membrane during menaquinone reduction, while two protons are generated in the course of H_2 oxidation on the periplasmic side of the membrane. Thereby a Δp is established by transmembrane electron transport from H_2 to menaquinone that is in accord with experimental results (see the Introduction). It is not known, however, which residues are involved in the putative proton pathway that connects the menaquinone reduction site of HydC with the cytoplasmic membrane surface. Since residues predicted to serve this function in *E. coli* FdnI are not conserved in HydC homologs, multiple pathways may exist with water molecules possibly bridging the relatively short distance from the aqueous phase to the menaquinone reduction site.

The results support the view that Tyr-202 of HydC and His-305 of HydA serve in the formation of a functional complex between HydAB and HydC as do the corresponding residues in *E. coli* FdnI. Both Tyr-202 of HydC and His-305 of HydA are essential for heme b reduction by H_2. It is likely that the HydABC complex is assembled in the corresponding mutants as the HydAB complex is bound to the membrane, *i.e.* to HydC, even in the absence of the HydA helix (16). On the other hand it also has to be considered that the HydAB dimer is bound to the membrane in the absence of HydC (16). It is tempting to speculate that the hydrogen bond between Tyr-202 and His-305 is necessary for the structurally correct assembly of HydC and HydAB allowing rapid electron transfer from the distal HydA iron-sulfur center to the proximal heme b group of HydC, which in turn is a prerequisite for menaquinone reduction. In contrast to *W. succinogenes* hydrogenase and *E. coli* formate dehydrogenase-N, there are other members of this enzyme family in which the helix of the iron-sulfur subunit apparently has been transferred to the cytochrome *b* subunit. An example is *W. succinogenes* FdhC, the cytochrome *b* subunit of the membrane-bound formate dehydrogenase, which is predicted to form five transmembrane helices with the first helix corresponding to that of *W. succinogenes* HydA or *E. coli* FdnH (3). The first helix of FdhC also contains a histidine residue that may be functionally equivalent to His-305 of *W. succinogenes* HydA or His-275 of *E. coli* FdnH. It is possible that five helices are necessary to allow electron transport from either H_2 or formate to a cytochrome *b* of the HydC/FdnI family. This hypothesis is supported by the fact that the *W. succinogenes* strain that lacks the HydA helix does not catalyze quinone reduction by H_2 (16).

The Functional Role of Two Heme b Groups in Electron Transfer to Quinone Reduction Sites in NiFe-hydrogenases and Other Respiratory Protein Complexes—The location of the menaquinone reduction site in *W. succinogenes* HydC implies that both heme b groups are involved in transmembrane electron transport from the iron-sulfur centers of HydA to menaquinone. This view is strongly supported by the properties of the mutants that contain HydC variants with modified axial heme b ligands. The lack of the proximal heme b group in HydC from mutants H67A-Strep and H186A-Strep obviously prevents electron transport from the iron-sulfur subunit HydA to the distal heme b group, while the formation of the HydABC complex is not affected. Individual modification of the histidine residues ligating the distal heme b group apparently prevents the formation of the HydABC complex from HydAB and HydC. Possibly the absence of the distal heme b results in structural rearrangements that prevent formation of the hydrogen bond between Tyr-202 of HydC and His-375 of HydA. These findings might generally hold true for membrane-bound NiFe-hydrogenases and formate dehydrogenases.

Membrane-bound subunits containing two heme b groups that are oriented to different sides of the membrane are also found in other respiratory enzyme complexes. Examples are membrane-bound nitrate reductase, the cytochrome bc_1 complex, and a distinct group of fumarate reductases and succinate dehydrogenases (for recent reviews, see Refs. 11 and 32–36). It is generally assumed for all these enzymes that both heme b groups are involved in electron transport to quinone binding sites. The center-to-center distances between the two hemes were determined to be in the range from 16 to 21 Å in crystal structures of representative enzymes (6, 37–39). Nevertheless the number of transmembrane helices, the location of the four heme b-ligating histidine residues, and the bioenergetic function of the enzyme complex differ considerably. The recently determined crystal structure of the membrane-bound nitrate reductase (NarGHI complex) from *E. coli* confirmed earlier models demonstrating that the membrane-bound diheme cytochrome *b* NarI spans the membrane five times with two heme b-ligating histidines located on helices II and V each (3, 39). Mechanistically similar to membrane-bound hydrogenases and formate dehydrogenases, quinol oxidation coupled to nitrate reduction by the NarGHI complex generates a Δp according to the redox loop mechanism (3, 32, 40). As the hydrophilic NarGH subunits face the cytoplasmic side of the membrane, the quinone binding site within NarI is expected on the periplasmic side of the membrane but could not be resolved in the crystal structure (39). Characterization of *E. coli* NarI proteins with altered heme b-ligating histidines confirmed the participation of both hemes in electron transport from quinol to the iron-sulfur subunit NarH (33). Like NarI, the diheme cytochrome *b* subunit of cytochrome bc_1 complexes possesses two helices (of eight) that contain two heme b-ligating histidine residues each. Electron transfer from ubiquinol to cytochrome *c*, however, generates a Δp by a protonmotive Q cycle involving both heme b groups and two quinone binding sites on different sides of the membrane (36). A group of succinate:quinone oxidoreductases are anchored to the membrane by diheme cytochrome *b* subunits with five transmembrane helices, *e.g.* by FrdC of *W. succinogenes* fumarate reductase or SdhC of *Bacillus subtilis* succinate dehydrogenase (11, 34, 35, 41). Here the heme b-ligating histidines are on four different helices. In addition to the cytochrome *b*, both enzymes contain two further hydrophilic subunits that are located at the inner surface of the membrane where the interconversion of fumarate and succinate is coupled to proton uptake or release. It was proposed that a menaquinone reduction site is located near the outer surface of the cytochrome *b* subunit in both enzymes implying that both heme b groups are essential for transmembrane electron transfer (41, 42). Assuming that protons involved in the redox reactions of menaquinone are exchanged at the outer side of the membrane, succinate oxidation by menaquinone as well as fumarate reduction by menaquinol would be electrogenic processes. While there is evidence that the former process is indeed catalyzed at the expense of Δp (11, 43), the latter process was experimentally shown to be electroneutral (20). Possibly menaquinol oxidation by fumarate is coupled to compensatory proton transport from the periplasm to the cytoplasm in *W. succinogenes* fumarate reductase (34).

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