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Cloning and Sequencing of the Genes Encoding the Large and Small Subunits of the Periplasmic (NiFeSe) Hydrogenase of *Desulfovibrio baculatus*

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The genes coding for the large and small subunits of the periplasmic hydrogenase from *Desulfovibrio baculatus* have been cloned and sequenced. The genes are arranged in an operon with the small subunit gene preceding the large subunit gene. The small subunit gene codes for a 32 amino acid leader sequence supporting the periplasmic localization of the protein, however no ferredoxin-like or other characteristic iron-sulfur coordination sites were observed. The periplasmic hydrogenases from *D. baculatus* (an NiFeSe protein) and *D. vulgaris* (an Fe protein) exhibit no homology suggesting that they are structurally different, unrelated entities.

Hydrogenase was first described by Stephenson and Strickland (31) as an enzyme that catalyzed the reversible activation of dihydrogen according to the reaction $H_2 \leftrightarrows 2H^+ + 2e$. They proposed that the enzyme was responsible for both the production and utilization of hydrogen in biological systems. Subsequently, hydrogenase activity was shown to be present in a large number of anaerobic and aerobic procaryotes (8, 20) as well as some eucaryotes such as algae (9), green plants (35), and protozoa (19). By biochemical and genetic investigations, it has been demonstrated that a number of bacteria contain two or more discrete hydrogenases which may play different physiological roles (2, 10, 15, 20, 27, 28). These hydrogenases can be broadly grouped into two classes by their metal contents; the exclusively nonheme iron hydrogenases and the nickel-containing hydrogenases. However, each of these broad classes may encompass different molecular forms of hydrogenase (6, 16, 33a).

The sulfate-reducing bacteria belonging to the genus *Desulfovibrio* have been shown to contain three types of hydrogenases by their physical properties, NH_2-terminal amino acid sequences, and immunological reactivities (B. C. Prick, S. H. He, C. Li, N. K. Menon, E.-S. Choi, A. E. Przybyla, D. V. DerVartanian, H. D. Peck, Jr., G. Fauque, J. Le Gall, M. Teixeira, I. Moura, J. J. G. Moura, D. Patil, and B. H. Huynh, unpublished observations). All three hydrogenases catalyze the same reaction, are composed of two subunits of various sizes, and contain nonheme iron but exhibit different metal contents and redox center compositions. They may be designated (Fe) hydrogenases, containing two (Fe_4S_4) clusters plus an atypical (Fe_4S_4) or (Fe_3S_x) center (15, 22, 25), (NiFe) hydrogenases containing a redox-active nickel, two (Fe_4S_4) clusters, and a three-iron center (34), and (NiFeSe) hydrogenases containing nickel, selenium, and two (Fe_4S_4) nonheme iron centers (24, 33a). The latter hydrogenase has a molecular mass of 85 kilodaltons (kDa) and is composed of a large and a small subunit (56.9 and 30.8 kDa, respectively).

The genes coding for the large and small subunits of the periplasmic (Fe) hydrogenase from *D. vulgaris* have been cloned in *Escherichia coli*, and proteins immunoreactive with antibody against purified (Fe) hydrogenase are produced (40). The cloned hydrogenase from *D. vulgaris*, however, is enzymatically inactive and lacks the full complement of nonheme iron clusters (39). Even though the enzyme is inactive, this observation and the complementation of a *pyrF E. coli* mutant with DNA from *D. vulgaris* indicate that many promoters from the sulfate-reducing bacteria are recognized by *E. coli* transcriptional enzymes (8, 18).

In this paper, we report the cloning and sequencing of the genes coding for the large (57.7-kDa) and small (30.8-kDa) subunits of the periplasmic (NiFeSe) hydrogenase from *D. baculatus* and compare their derived amino acid sequences with those of the periplasmic (Fe) hydrogenase from *D. vulgaris*.

MATERIALS AND METHODS

Bacteria and growth media. *D. baculatus* DSM1743 was originally isolated from the consortium "*Chloropseudomonas ethylica*" (4) and identified as *Desulfovibrio* sp. strain 9974. The bacterium contains desulforubidin as its bisulfate reductase, and the amino acid composition of its cytochrome c_3 is very similar to that of the cytochrome c_3 of *D. desulfuricans* (Norway 4) (B. Prickril and J. Le Gall, unpublished observations). These observations, together with our data on hydrogenase reported in this paper, indicate that *D. baculatus* and *D. desulfuricans* are closely related, if not the same species. *D. baculatus* was grown on a lactate-sulfate medium at 37°C and harvested as described previously (7). The periplasmic (NiFeSe) hydrogenase was eluted from intact cells by repeated freeze-thaw cycling and purified as described by Teixeira et al. (33a). The purified soluble (NiFeSe) hydrogenase of *D. desulfuricans* Norway 4 was a gift of Daulat S. Patil.

Chemicals. All restriction enzymes and DNA ligase were purchased from Boehringer-Mannheim and used as suggested by the manufacturer. Reagents for sequencing and deletion cloning were from Pharmacia or Amersham.

Library construction. A *D. baculatus* genomic library was constructed from size-fractionated (8 to 12 kilobases [kb]) *Sau*3A partial digest fragments. Fragments ligated into the

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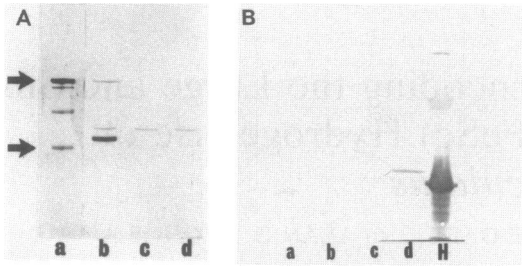

FIG. 1. (A) Antibody directed against *D. baculatus* periplasmic hydrogenase was used to identify hydrogenase-specific peptides synthesized by *E. coli* cells transformed with *D. baculatus* DNA. Lanes: (a) native periplasmic hydrogenase, (b to d) cell extracts from cells transformed with (b) PC4, (c) PC6, or (d) HB101. Arrows indicate the large and small subunits of the native hydrogenase (56.9 and 30.8 kDa, respectively). (B) Antibody directed against the small subunit of *D. desulfuricans* soluble hydrogenase was used to identify hydrogenase-specific peptides synthesized by *E. coli* cells transformed with *D. baculatus* DNA. Lanes: Cell extracts from cells transformed with (a) SC1, (b) PC6, (c) HB101, or (d) PC4; H, partially purified hydrogenase from *D. baculatus* fractionated by SDS-PAGE.

*Bam*HI site of pBR322 were transformed into *E. coli* HB101. The library consisted of 6,600 recombinant clones.

Antibody screening. Antibodies directed against *D. baculatus* periplasmic hydrogenase were used to screen about 800 recombinant clones for the presence of a hydrogenase-specific gene(s). Seven positive clones were identified, and their plasmid-encoded peptides were characterized by Western blot (immunoblot) analysis. The cells from 1.5 ml of an overnight culture were collected, suspended in 200 μl of Laemmli sample buffer, boiled for 2 min, and fractioned by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% acrylamide). The proteins were electroblotted overnight onto nitrocellulose, and uncomplexed nitrocellulose was blocked with milk protein (Blotto) for 1.5 h prior to reaction with antibodies. The filters were incubated in antibody solution for 2 h and washed three times for 10 min each in TBST buffer (0.05 M Tris, pH 8.0, 0.16 M NaCl, 0.05% Tween). The blots were then incubated with a 1:3,000 dilution of goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase for 1 h at 22°C. After the filters were washed twice for 10 min each in TBST, they were treated with coloring reagent (0.33 mg of nitroblue tetrazolium and 0.165 mg of 5-bromo-4-chloro-indolyl phosphate per ml in 100 mM Tris hydrochloride, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) for 10 min and washed with distilled water.

Protein purification and sequencing. In initial experiments, we found that the periplasmic hydrogenase from *D. baculatus* cross-reacted with antibody raised against the native soluble hydrogenase from *D. desulfuricans* Norway 4. Since a very pure sample of the latter hydrogenase was available, it was used to obtain protein sequence data. The soluble hydrogenase fraction from this species was fractionated by SDS-PAGE (10% acrylamide), and the areas containing the large and small subunits were cut from the gel and electroeluted overnight. The eluted proteins were further purified by fractionation, eluted by SDS-PAGE (8% acrylamide), and processed for NH_2-terminal sequencing (13). A gas phase sequenator yielded 40 NH_2-terminal residues of the small subunit and 20 residues of the large subunit.

A portion of the same small subunit sample was also used to immunize an 8-week-old rabbit, and antiserum specific for the small subunit was produced. This antibody was finally used for analyzing the proteins expressed in recombinant *E. coli* cells.

Southern blot analysis. Plasmid digests were routinely subjected to electrophoresis on 0.8 or 1.2% agarose gels in Tris-borate buffer and transferred overnight to nitrocellulose in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (30). The filters were baked at 80°C for 2 h and prehybridized with 0.5% Blotto in 8× SSC for 6 h at 65°C. The filters were hybridized in 0.5% Blotto in 6× SSC for 12 h at 37°C with 10^6 cpm of small subunit probe or 2×10^6 to 2.5×10^6 cpm of large subunit probe. The probes were end labeled with [γ-^{32}P]ATP with T4 polynucleotide kinase (21) and purified by elution from a DEAE-Sephacel column in 0.5 M NaCl. The filters hybridized against the small subunit probe were washed four times for 20 min each with 2× SSC at room temperature. Filters hybridized with the large subunit probe were washed three times for 20 min each in 4× SSC at room temperature.

M13 cloning and sequencing. Two fragments (0.82 and 1.0 kb) of an 8-kb recombinant carrying genes for the small and large subunits, respectively, were subcloned into the mp18 derivative of M13. Sequencing was done by the procedure of Sanger et al. on 0.5-mm gradient gels (26). Overlaps were generated by deletion cloning with T4 DNA polymerase, by sonication or by subcloning of *Sau*3A digests into the *Bam*HI site of the mp18 polylinker. Regions of compression were resolved by using c^7-deaza-dGTP in place of dGTP. Data were analyzed by the Beckman Microgenie program.

RESULTS

Seven clones which showed strong hybridization signals with antibodies directed against native *D. baculatus* periplasmic hydrogenase were isolated from about 800 recombinants and processed for further screening. When cell extracts of the positive clones were gel fractionated, transferred to nitrocellulose, and reacted with the same antibody, two clones (PC4 and PC6) contained peptides which reacted with the antibody (Fig. 1A). Four immunologically reactive peptides were observed when purified *D. baculatus* hydrogenase was treated with immunoglobulins directed against the enzyme (lane a). Two of the polypeptides indicated by arrows are the large (56.9 kDa) and small (30.8 kDa) subunits of the enzyme. The other two immunostained polypeptides represent minor contaminants of the purification procedure whose presence appeared to be amplified during the antibody production and immunostaining procedures. The level of this contamination varied between preparations and could be due to degradations. PC4 coded for a protein which was about 3 kDa larger than the small subunit of authentic *D. baculatus* periplasmic hydrogenase (lane b). PC6 encoded a protein which was identical in size to the small subunit (lane c).

As preliminary experiments demonstrated that the periplasmic hydrogenase from *D. baculatus* cross-reacted with antibody directed against *D. desulfuricans* Norway 4 soluble hydrogenase, the PC4 and PC6 gene products were tested for cross-reactivity with *D. desulfuricans* antibody. This antibody only reacted with the peptide coded for by PC4 (Fig. 1B). The signal obtained from PC6 with *D. baculatus* antibody thus could also have been due to an impurity in the *D. baculatus* enzyme preparation used for immunization. Because of this finding, PC4 was chosen for further analysis.

Two oligonucleotide probes specific for the amino terminals of the large and small subunits of *D. desulfuricans*

Norway 4 hydrogenase were used to verify the antibody screens. Southern blots (35) of plasmids purified from PC4 and digested with various restriction enzymes were hybridized sequentially to both oligonucleotide probes. An *Eco*RI digest of PC4 yielded 2.6- and 1.0-kb fragments which hybridized to the small and large subunit probes, respectively (Fig. 2). Consistent with the antibody screens, neither probe hybridized to PC6. When the 2.6-kb *Eco*RI fragment (SC1) was subcloned into pBR322, it coded for a peptide which reacted with hydrogenase small subunit antibody and was about 3.0 kDa smaller than the small subunit of native hydrogenase. This indicated that the 2.4-kb subclone did not carry the entire small subunit gene and that there was an *Eco*RI site towards the end of the gene (Fig. 1B). Further analysis showed that an 800-bp *Eco*RI-*Eco*RV subclone contained all of the small subunit coding information for the hydrogenase gene carried in the 2.4-kb fragment (data not shown).

The genes coding for the small and large subunits of the putative periplasmic (NiFeSe) hydrogenase of *D. baculatus* were completely sequenced (Fig. 3). The genes were separated by 15 nucleotides, and the small subunit gene preceded the large subunit gene. A ribosome-binding site (AGGAG GA) (37) was centered at −7 bp (with respect to the translational initiator ATG codon of the small subunit), and a Pribnow sequence (12) was centered 13 bp upstream from the ribosome-binding site (Fig. 3). The 15-bp region after the TAA termination codon of the small subunit gene also contained a perfect ribosome-binding site (33) but lacked a Pribnow sequence, indicating that this 15-bp region does not contain a functional promoter.

The first amino acid residue of the mature small subunit protein as determined by peptide sequencing was located at +96 bp from the translational initiator (ATG) identified in the gene sequence. The amino acid sequence derived from the cloned gene after nucleotide +96, however, was identical to the protein-derived sequence. The first 96 bp code for a 32-amino-acid signal peptide which is cleaved from the mature protein. The calculated molecular mass of the signal peptide (3,399 Da) is consistent with the difference in size of authentic small subunit and the peptide synthesized by clone 4 (Fig. 1). Figure 4 compares the leader sequences of the periplasmic hydrogenase and cytochrome *c* of *D. vulgaris* with the periplasmic hydrogenase of *D. baculatus*. Figure 5 shows the hydropathy profile of the signal peptide of the (NiFeSe) hydrogenase small subunit. The 32 NH_2-terminal amino acids encoded by the gene were generally hydrophobic following the NH_2-terminal basic amino acids.

The initiator codon for the large subunit gene was determined by amino acid and nucleotide sequencing to be GTG. The first 18 amino acids coded by the gene were in perfect agreement with the results obtained from NH_2-terminal amino acid sequencing, indicating the absence of a signal peptide.

The amino acid compositions of the large and small subunits calculated from the derived amino acid sequences are presented in Tables 1 and 2. The molecular mass of the large subunit was calculated to be 57,673 Da, a value in close agreement with the 56,400 Da obtained by SDS-PAGE. The molecular mass of the mature small subunit was calculated to be 30,844, which compared well with the molecular mass of 28,600 Da determined by SDS-PAGE.

The codon usage for the large and small subunits is presented in Table 3. Although the sample size was limited, it was evident that all codons were used at least once in *D. baculatus* genes. The limited sample from *D. vulgaris*, on the other hand, showed that 16 codons were not used at all in the periplasmic hydrogenase subunit genes. Some of the codons used infrequently in *D. baculatus*, such as CTA, ACA, GGG, and ATA, are also used infrequently in *E. coli* genes (11).

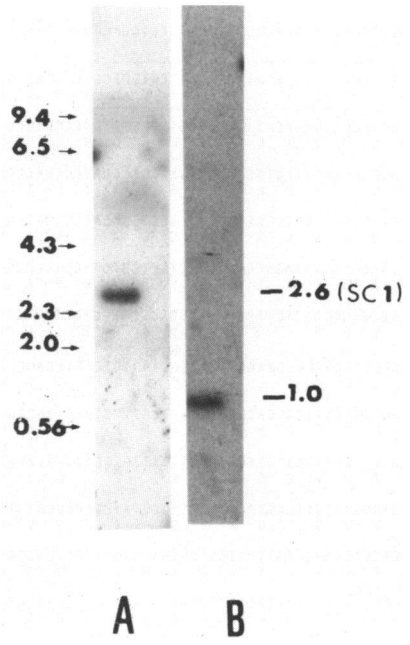

FIG. 2. Southern analysis of PC4 restricted with *Eco*RI and hybridized with oligonucleotide probes. Lanes: (A) PC4 hybridized with oligonucleotide probe homologous to the small subunit; (B) PC4 hybridized with oligonucleotide probe homologous to the large subunit. Hybridization and washing condition were as described in Materials and Methods.

DISCUSSION

The DNA coding for the large and small subunits of the periplasmic (NiFeSe) hydrogenase of *D. baculatus* has been cloned and sequenced. The derived amino acid sequences of these hydrogenase subunits and those of the (Fe) hydrogenase subunits exhibit no structural or sequence homologies, indicating that the two hydrogenases must be regarded as discrete enzymatic entities. The genes for the large and small subunits of both hydrogenases appear to constitute an operon; however, the small subunit initiates the operon in *D. baculatus*, while the large subunit precedes the small one in *D. vulgaris*.

The NH_2-terminal amino acid sequences of the small subunits of *D. vulgaris* and *D. baculatus* hydrogenases are not colinear with the ATG translational start of the nucleotide sequence, which indicates the presence of a leader sequence. The mature subunit protein of the (Fe) hydrogenase lacks 34 NH_2-terminal amino acids encoded by the gene (23), and the mature subunit protein of the (NiFeSe) hydrogenase lacks 32 NH_2-terminal amino acids encoded by the gene. The peptide synthesized in *E. coli* transformed with the *D. baculatus* periplasmic hydrogenase gene was 3,400 Da larger than the native mature subunit, indicating that *E. coli* synthesizes the preprotein of the small subunit but is unable to process the leader sequence.

The properties of the signal peptides on the small subunits of both enzymes were within the limits of variation for signal

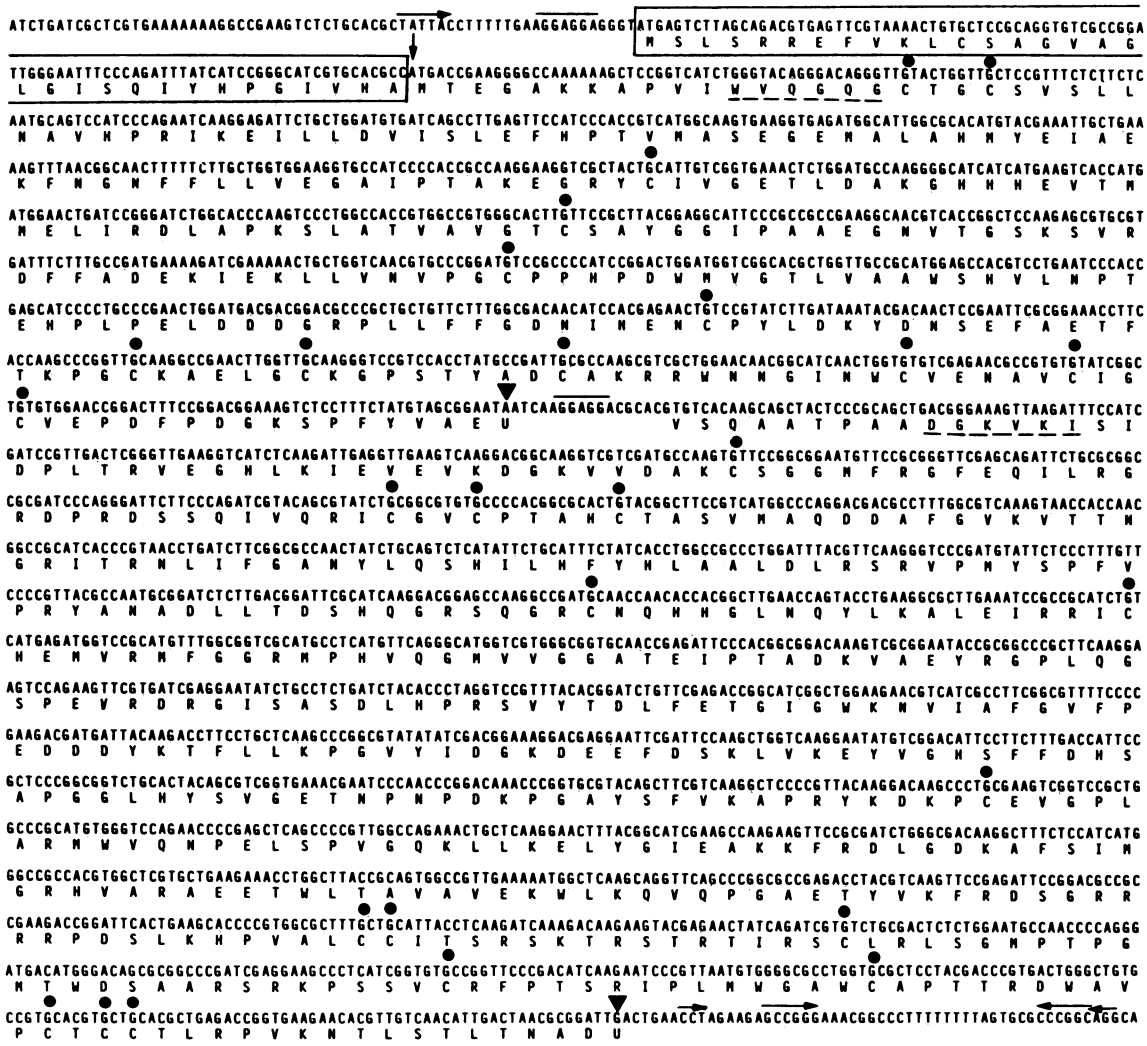

FIG. 3. Nucleotide and derived amino acid sequences for the small and large subunits of *D. baculatus* periplasmic hydrogenase. Sequences underlined with dashed lines represent nucleotide probe sequences for the two subunits. The leader sequence is enclosed in a box. ⟶, possible Pribnow box. Solid lines over nucleotides indicate probable ribosome-binding sites. ⟷, represent inverted repeats which could be a component of a transcriptional terminator. ↓, Beginning of protein as deduced by amino acid analysis. ▼, Termination codon.

peptides as outlined by von Heijne (36). Because of their exceptional length, however, they should be considered by his criteria to be extreme signal sequences, similar to those reported for *Bacillus subtilis* (29). The signal peptidase cleavage sites, however, are different, Ala-Ala for the (Fe) hydrogenase and Ala-Met for the (NiFeSe) hydrogenase. The presence of signal peptides confirms enzymological data indicating that both hydrogenases are localized in the periplasm. Voordouw and Brenner (38) have reported that the nucleotide sequence encoding the tetraheme cytochrome c_3 of *D. vulgaris*, the redox partner of the hydrogenase, is not colinear with the amino acid sequence of the mature protein and have proposed that the cytochrome c_3 is synthesized as a preprotein with a signal peptide consisting of 21 amino acid residues. They also suggested that "hydrogenase may thus be separated from its putative redox substrate by a lipid bilayer"; however, the presence of signal peptides on the small subunits of both the (Fe) and (NiFeSe) hydrogenase confirms the enzymological localization studies and indicates that their suggestion is not valid.

Although significant homology exists between the signal peptides of the small subunits of the (Fe) and (NiFeSe) hydrogenases, there does not appear to be any homology between the amino acid sequences of their mature small subunits. The occurrence of cysteinyl residues in these proteins is of particular interest because of their roles as ligands for metal redox centers. In contrast to the mature small subunit of the (Fe) hydrogenase, which contains no

FIG. 4. Amino acid homology of the leader sequences from (a) *D. vulgaris* c_3, (b) *D. baculatus* periplasmic hydrogenase, and (c) *D. vulgaris* periplasmic hydrogenase. Protein alignment was done by the Beckman Microgenie program. Arrow indicates signal peptidase cleavage sites; boxes enclose neutral amino acid changes, and shaded boxes enclose conserved amino acids.

cysteinyl residues, the mature small subunit of the (NiFeSe) hydrogenase contains 12 cysteinyl residues, 7 of which were grouped in the carboxy terminus of the protein. There was no obvious pattern in the arrangement of the cysteinyl residues, but their presence suggests different functions for the small subunits of the hydrogenases. Two of the cysteinyl residues were found towards the NH_2 terminus in the sequence Cys-Thr-Gly-Cys-Ser, which also occurs in the NH_2-terminal amino acid sequences of the (NiFe) hydrogenases of *D. gigas* and *D. multispirans* (Prickril et al., unpublished observations). A second important difference was their molecular masses, 10,300 Da for the small subunit of the (Fe) hydrogenase and 30,844 Da for the small subunit of the (NiFeSe) hydrogenase.

The large subunit of the (Fe) hydrogenase contained 17 cysteinyl residues, and 8 of the cysteinyl residues were towards the NH_2 terminus, arranged as in a characteristic eight-iron ferredoxin structure (38). It was postulated that these eight cysteinyl residues in the ferredoxin domain serve as ligands for the two ferredoxin-type $[Fe_4S_4]$ clusters found in the (Fe) hydrogenase. Some of the remaining nine cysteinyl residues are presumably involved as ligands for the unique three- or four-iron cluster. Following the ferredoxin domain, there was a stretch of 33 amino acid residues which exhibited many of the properties of a signal peptide (36), and it has been proposed that this hydrophobic region might represent an internal signal sequence originating from the fusion of a gene encoding the eight-iron ferredoxin with a gene encoding a four-iron hydrogenase plus its signal peptides (23).

The large subunit of the (NiFeSe) hydrogenase exhibited no structural or sequence homology with the large subunit of the (Fe) hydrogenase. The large subunit of the (NiFeSe) hydrogenase of *D. baculatus* has GTG (Val) instead of ATG as an initiator codon. It is known that in 8 to 10% of *E. coli* genes, GTG replaces ATG as an initiator codon (32). It has also been documented that in some of these cases, protein production is 20% of the amount observed when ATG is the initiator (32), and this may explain in part the stoichiometric discrepancy in the expression of antigenically reactive *D. baculatus* large and small subunits in transformed *E. coli* cells (Fig. 1). There are 15 cysteine residues in the large subunit of the (NiFeSe) hydrogenase, and they are grouped in two clusters: one towards the amino terminus and one towards the carboxy terminus. It should be noted that the derived amino acid sequence did not reveal any ferredoxin-type pattern of the cysteinyl residues but did contain two Cys-Cys arrangements. It was not possible to infer anything about the liganding of the iron or nickel from the arrangement of the cysteinyl residues in the two subunits. From the results of Voordouw and Brenner (37) and the data presented in this paper, it is concluded that the (Fe) hydrogenase and the (NiFeSe) hydrogenase must be regarded as discrete enzymes and there is no evidence that they are even distantly related.

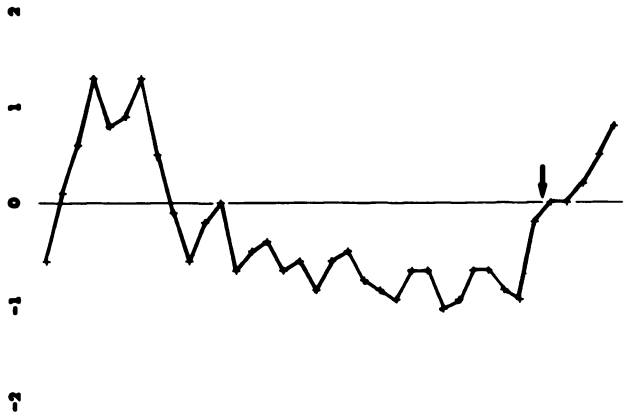

FIG. 5. Hydrophilicity plot of the leader sequence of *D. baculatus* periplasmic hydrogenase small subunit. The vertical axis represents hydrophobicity values of various amino acids. Hydrophobic amino acids have negative values. Arrow points to the signal peptidase cleavage site.

TABLE 1. Amino acid composition of *D. baculatus* hydrogenase small subunit[a]

Amino acid	No. of residues	Amino acid	No. of residues
Ala	25	Leu	22
Arg	7	Lys	16
Asn	13	Met	7
Asp	15	Phe	12
Cys	12	Pro	20
Gln	2	Ser	12
Glu	24	Thr	14
Gly	25	Trp	5
His	10	Tyr	7
Ile	13	Val	22

[a] Total amino acids, 284 does not include the 32-amino-acid leader sequence. Molecular weight, = 30,844 calculated from the amino acid composition.

The (NiFeSe) hydrogenase is characterized by having selenium in amounts equivalent to nickel and producing H_2 in excess of hydrogen-deuterium in the deuterium exchange reaction (17, 24). Selenium has been reported to be present as selenocysteine in a hydrogenase from *Methanococcus vanielii* (41), and selenium has been found in a hydrogenase from *Methanococcus voltae* (S. B. Woo and H. D. Peck, unpublished data). Selenocysteine is also found in the formate dehydrogenase of *E. coli* and has been reported to be inserted into the protein cotranslationally (42). The UAG codon appears between short inverted repeats for the addition of selenocysteine at the UAG position in the formate dehydrogenase. The (NiFeSe) hydrogenase gene does not contain a UAG codon, and therefore it can be concluded that a different nucleotide sequence codes for selenocysteine, selenium is present in a form other than selenocysteine, or the selenium is added posttranslationally.

In addition to the periplasmic enzyme in *D. baculatus*, hydrogenases have also been purified from cytoplasmic and

TABLE 2. Amino acid composition of *D. baculatus* hydrogenase large subunit[a]

Amino acid	No. of residues	Amino acid	No. of residues
Ala	38	Leu	37
Arg	42	Lys	31
Asn	12	Met	12
Asp	31	Phe	19
Cys	15	Pro	34
Gln	16	Ser	35
Glu	23	The	31
Gly	42	Trp	8
His	16	Tyr	15
Ile	21	Val	40

[a] Total amino acids, 519; molecular weight, 57,673 (calculated from the amino acid composition).

TABLE 3. Codon distribution comparison[a]

Codon	aa	No. of uses		Codon	aa	No. of uses		Codon	aa	No. of uses		Codon	aa	No. of uses	
		D. baculatus	*D. vulgaris*			*D. baculatus*	*D. vulgaris*			*D. baculatus*	*D. vulgaris*			*D. baculatus*	*D. vulgaris*
TTT	F	9	0	TCT	S	8	0	TAT	Y	9	4	TGT	C	11	2
TTC	F	23	28	TCC	S	18	9	TAC	Y	14	18	TGC	C	17	17
TTA	L	2	0	TCA	S	8	0	TAA	U	1	0	TGA	U	1	0
TTG	L	8	2	TCG	S	4	7	TAG	U	0	2	TGG	W	13	6
CTT	L	10	4	CCT	P	4	2	CAT	H	16	9	CGT	R	11	5
CTC	L	8	15	CCC	P	29	22	CAC	H	12	12	CGC	R	17	13
CTA	L	1	0	CCA	P	3	0	CAA	Q	5	0	CGA	R	6	0
CTG	L	33	13	CCG	P	19	8	CAG	Q	14	14	CGG	R	4	3
ATT	I	12	2	ACT	T	7	1	AAT	N	4	0	AGT	S	4	0
ATC	I	24	21	ACC	T	22	27	AAC	N	22	8	AGC	S	9	8
ATA	I	1	2	ACA	T	4	0	AAA	K	11	2	AGA	R	9	0
ATG	M	20	20	ACG	T	12	8	AAG	K	37	47	AGG	R	4	0
GTT	V	13	2	GCT	A	13	4	GAT	D	25	4	GGT	G	17	9
GTC	V	28	23	GCC	A	31	32	GAC	D	21	25	GGC	G	34	35
GTA	V	6	0	GCA	A	10	10	GAA	E	33	18	GGA	G	15	1
GTG	V	18	10	GCG	A	12	9	GAG	E	15	15	GGG	G	5	5

[a] Codon usage in genes coding for the large and small subunits of *D. baculatus* hydrogenase were determined in this study. Codon usage in the genes coding for the large and small subunits of *D. vulgaris* hydrogenase were determined by Voordouw et al. (36). aa, Amino acid.

membrane fractions (33a). Initially, both of these hydrogenases also appear to be (NiFeSe) hydrogenases in that they are composed of two nonidentical subunits with similar molecular masses, contain nonheme iron plus selenium and nickel in equivalent amounts, and, although they exhibit variable nickel(III) EPR spectra as isolated, all show a "$g = 2.19$" EPR signal after reduction with hydrogen (33a). Recently, we have also purified a membrane-bound (NiFe) hydrogenase by extraction with Triton X-114 (5). This conclusion is based on the observations that the purified membrane-bound hydrogenase cross-reacts with antibody to the (NiFe) hydrogenase of *D. gigas* (3) but not with antibody against the periplasmic (NiFeSe) hydrogenase of *D. baculatus* and from plasma emission studies showing the presence of Ni and Fe. With these findings in mind, the gene coding for the small subunit of the (NiFeSe) hydrogenase was used to probe *D. baculatus* genomic DNA for an additional hydrogenase gene(s) having significant homology to the periplasmic (NiFeSe) hydrogen gene. We were unable to locate a second gene with homology for the small subunit of the periplasmic (NiFeSe) hydrogenase. These findings suggest that the membrane and cytoplasmic hydrogenases must be encoded by different genes.

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