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Putative Signal Peptide on the Small Subunit of the Periplasmic Hydrogenase from *Desulfovibrio vulgaris*

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We sequenced the NH_2 terminus of the large and small subunits of the periplasmic hydrogenase from the sulfate-reducing bacterium *Desulfovibrio vulgaris* (Hildenborough) and found that the small subunit lacks a region of 34 NH_4-terminal amino acids coded by the gene for the small subunit (G. Voordouw and S. Brenner, Eur. J. Biochem. 148:515–520, 1985). We suggest that this region constitutes a signal peptide based on comparison with known procaryotic signal peptides.

A significant fraction of the hydrogenase activity of sulfate-reducing bacteria belonging to the genus *Desulfovibrio* is localized in the periplasmic space (2, 18, 19). Two types of periplasmic hydrogenase have been isolated and characterized: a nickel- and nonheme iron-containing hydrogenase (nickel-iron hydrogenase) composed of two subunits (8, 13) and an exclusively nonheme iron (three Fe_4S_4 clusters)-containing hydrogenase (iron hydrogenase) of higher specific activity (11, 19). Voordouw et al. (22) were the first to provide evidence that the iron hydrogenase is also composed of two subunits (45.8 and 13.5 kilodaltons [kDa]). They cloned and sequenced a DNA fragment encoding a protein reactive with polyclonal antibody to the periplasmic iron hydrogenase of *D. vulgaris* (Hildenborough). The following conclusions were drawn based on the nucleotide sequence (23). The hydrogenase operon is composed of two genes, separated by 14 base pairs and coding for 46- and 13.5-kDa polypeptides, i.e., the large and small subunits. The NH_2-terminal amino acid sequence of the large subunit is colinear with the AUG translational start of the gene, suggesting the absence of a signal peptide and leading to speculation that the gene might code for a cytoplasmic hydrogenase. A high degree of homology between the NH_2-terminal region of the large subunit and the amino acid sequences of the eight iron ferredoxins substantiates the biochemical information, indicating that two of the three iron clusters are of the ferredoxin type (7, 11). As 10 of the remaining 11 cysteine residues were located on the large subunit, it was suggested that the third Fe_4S_4 cluster is also located on the large subunit; however, the possible involvement of the single cysteinyl residue on the small subunit as a ligand for an iron-sulfur cluster could not be eliminated.

Because of the existence of multiple molecular forms of hydrogenase (1, 23), we were interested in establishing whether the periplasmic hydrogenase of *D. vulgaris* isolated by our purification procedure was identical to that encoded by the hydrogenase gene cloned by Voordouw and Brenner (21) and, if so, whether the small subunit of the iron hydrogenase is encoded by the gene immediately downstream from the gene for the 46-kDa protein. In this note we report that the NH_2-terminal amino acid sequence of the large subunit of the iron hydrogenase isolated by our purification procedure corresponds to the nucleotide sequence of the gene (21) coding for the large subunit but that the small subunit lacks a hydrophobic NH_2-terminal amino acid sequence encoded by the gene for the small subunit. The missing NH_2-terminal sequence of amino acids derived from the nucleotide sequence has the general characteristics of a signal peptide.

Cells of *D. vulgaris* (Hildenborough) were grown, and the hydrogenase was purified as described previously (11). The hydrogenase small subunit was separated in a 15% sodium dodecyl sulfate-polyacrylamide gel and electroeluted with an ISCO model 1750 electrophoretic concentrator by the protocol described by Hunkapiller et al. (10). Amino-terminal sequence analyses were performed with an Applied Biosystems model 470A protein sequencer equipped with an online 120A PTH analyzer. Amino acid analyses of samples hydrolyzed under vacuum for 24, 48, or 72 h were performed with a Beckman model 119CL analyzer. Cysteine content was determined as described by Hirs (9), and tryptophan content was determined as described by Matsubara and Sasaki (15).

The sodium dodecyl sulfate gel electrophoresis (15% polyacrylamide) pattern of the iron hydrogenase purified by our procedure is shown in Fig. 1. The pattern is similar to that reported previously by Voordouw et al. (22), with the large subunit having an apparent molecular mass of 46 kDa and the small subunit having an apparent molecular mass of 13.5 kDa. From NH_2-terminal amino acid sequence data presented in this note, the calculated molecular mass of the small subunit is 10 kDa, which is considerably less than the molecular mass of the small subunits of the nickel-iron hydrogenases.

The amino-terminal amino acid sequence determined for the first 40 residues of the isolated small subunit shows that they correspond to residues 35 to 75 in the sequence deduced from the gene. Thus, the complete amino acid sequence for the small subunit deduced from the nucleotide sequence is likely to represent a preprotein from which a 34-residue amino-terminal signal peptide must be co- or posttranslationally cleaved to generate the mature protein. The amino acid composition of the isolated small subunit was also determined in our laboratory, and it is consistent with the finding that the gene codes for a 34-residue amino-terminal signal sequence. The presence of a single cysteinyl residue within the signal peptide region conflicts with our data indicating a cysteinyl residue within the mature (processed) small subunit (Table 1). However, the experimental error inherent in the technique does not preclude the possibility that the

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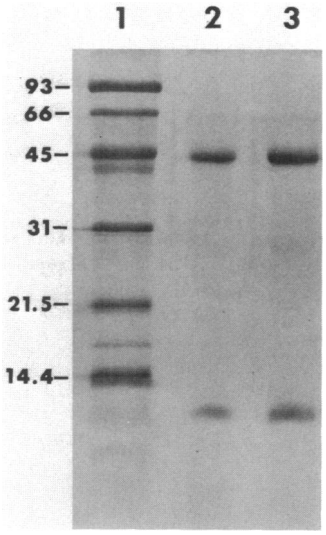

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified hydrogenase on a 15% gel. Lanes: 1, protein standards with molecular mass indicated in kilodaltons; 2 and 3, 2 μg and 5 μg of hydrogenase, respectively.

small subunit contains no cysteine, in agreement with the nucleotide sequence. These amino acid sequencing and composition results demonstrate that the small subunit of the iron hydrogenase of *D. vulgaris* is encoded by the second gene in the hydrogenase operon as postulated by Voordouw and Brenner (21).

The NH_2-terminal amino acid sequence (40 residues) of the small subunit deduced from the nucleotide sequence of the gene is as follows: Met-Gln-Ile-Ala-Ser-Ile-Thr-Arg-Arg-Gly-Phe-Leu-Lys-Val-Ala-Cys-Val-Thr-Thr-Gly-Ala-Ala-Leu-Ile-Gly-Ile-Arg-Met-Thr-Gly-Lys-Ala-Val-Ala ⋮ Ala-Val-Lys-Gln-Ile-Lys. Residues 34 and 35 (dashed line) constitute a classic cleavage point for signal peptidases (6), and the 34-amino-acid sequence includes the sole cysteine residue in the small subunit. The latter observation clearly excludes the small subunit as a participant in the formation of nonheme iron centers. The amino acid sequence has many of the properties of a procaryotic signal peptide (6). As shown by the hydropathic profile (Fig. 2), the 34 NH_2-terminal amino acid residues encoded by the gene are generally hydrophobic and arranged in two minor and one major hydrophobic cluster following the NH_2-terminal basic region. Downstream from the first 34 amino acids, there is no indication of hydrophobic clusters. This NH_2-terminal region lacks glutamyl, tryptophanyl, and aspartyl residues but contains five basic amino acids: two lysyl and three arginyl residues, with two of the latter being adjacent. The carboxyl terminus of the putative signal peptide is an alanine, and within the major hydrophobic domain of 16 amino acid residues there is a single threonine residue and two glycyl residues. The putative signal peptide is longer than most signal peptides found in gram-negative bacteria (6) and exhibits a short hydrophobic region upstream from the NH_2-terminal basic region. The properties of the putative signal peptide are also within the limits of variation for signal peptides as outlined by von Heijne (20). Owing to the unusual length, it would be considered an extreme sequence according to his criteria.

The mechanism by which the large and small subunits of *D. vulgaris* hydrogenase are translocated into the periplasm is unknown. Our results indicate only that the small subunit is encoded by the hydrogenase operon and undergoes modification between the time of translation and its localization in the periplasm. However, in the interest of defining the most plausible mechanism the following three schemes should be considered. The first of these involves cotranslation of the hydrogenase operon as a single precursor polypeptide chain and the generation of two subunits during the translocation process. This mechanism is unreasonable ow-

TABLE 1. Amino acid composition of the small subunit of *D. vulgaris* hydrogenase

Amino acid	No. of residues	No. of residues from sequence of cloned gene[a]	
		With signal peptide[b]	Without signal peptide[c]
Cysteine	1	1	0
Aspartic acid + asparagine	9–10	9	9
Threonine[d]	4	8	4
Serine[d]	4	6	5
Glutamic acid + glutamine	10	9	8
Proline	7–8	6	6
Glycine	8	10	6
Alanine	9–10	13	7
Valine	8	8	5
Methionine	0–1	3	1
Isoleucine	2–3	6	2
Leucine	9	10	8
Tyrosine	5	6	6
Phenylalanine	3	4	3
Histidine	3–4	4	4
Lysine	11	13	11
Tryptophan	0–1	1	1
Arginine	3	6	3

[a] From reference 21.
[b] Molecular mass, 13.8 kDa.
[c] Molecular mass, 10.3 kDa.
[d] Values extrapolated to zero time.

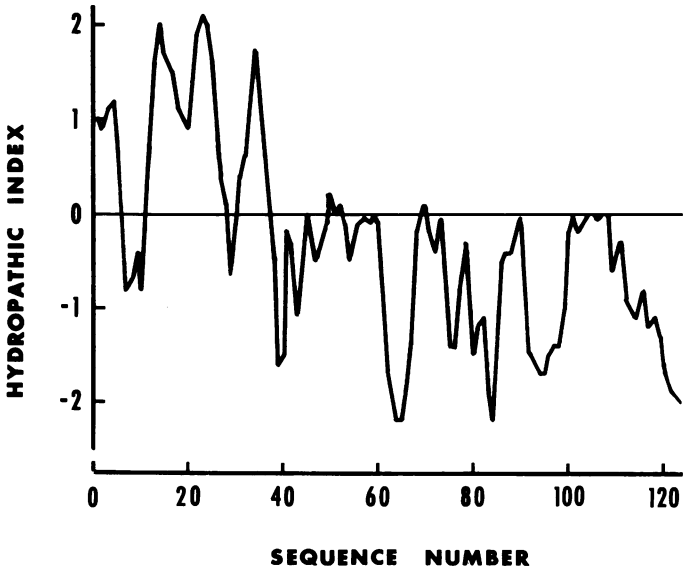

FIG. 2. Hydropathic profile of the hydrogenase small subunit. Residue positions are indicated starting from the NH_2-terminal sequence of the small subunit deduced from the cloned gene (22). A window of six residues was used in the Intelligenetics program based on reference 14.

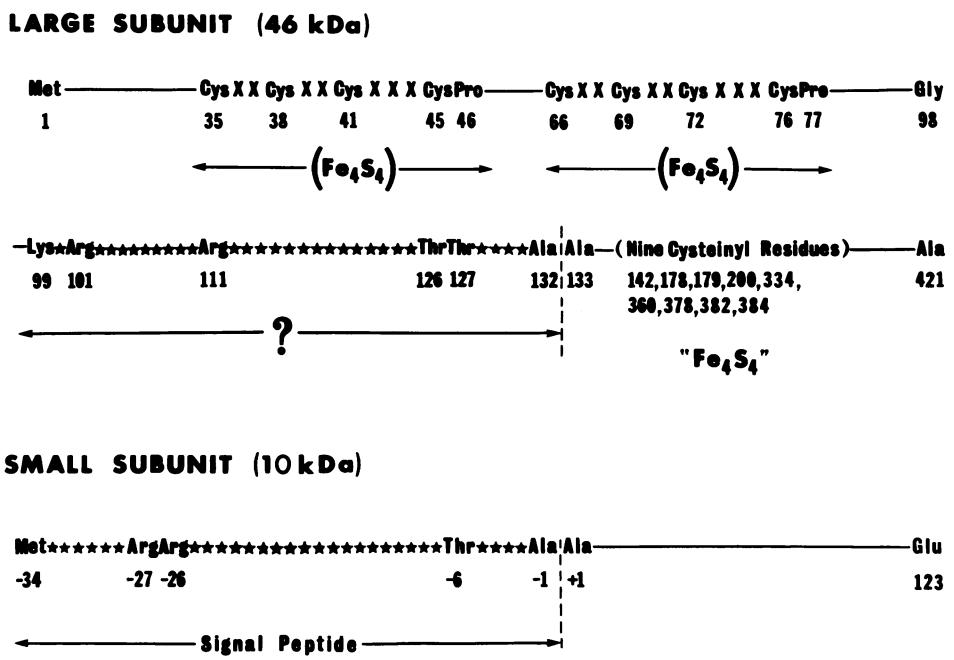

FIG. 3. Putative signal sequences in the unprocessed hydrogenase from *D. vulgaris*. The large subunit contains a ferredoxinlike region (residues 26 to 85) and a hydrophobic region which resembles an amino-terminal signal sequence (residues 99 to 132). Symbols: ★, a residue within a signal sequence; ×, residues within (Fe_4S_4) regions. The single cysteine of the small subunit preprotein is at position −19. The large-subunit carboxy-terminal region marked "Fe_4S_4" contains potential binding sites for one or more additional iron-sulfur clusters.

ing to the presence of an amber stop codon and a ribosome binding site upstream from the initiator codon for the small subunit. Alternatively, the hydrogenase may be posttranslationally translated across the membrane with the small subunit acting as a carrier for the large subunit. In the absence of any experimental precedent for one subunit acting as a carrier for a second subunit for posttranslational translocation, this possibility is also improbable. A third possibility is the presence of an internal signal peptide encoded in the large subunit, allowing for segregated translocation of the two hydrogenase subunits into the periplasm, thereby necessitating or even ensuring assembly of the active hydrogenase in the periplasm. This third mechanism appears more plausible than either of the first two possibilities, especially because an internal signal sequence has previously been observed (16). We therefore determined the hydropathic profile of the entire large subunit (data not shown) and found a hydrophobic region located between residues 99 and 132, which has the general properties of an amino-terminal signal peptide and is similar to the signal peptide of the small subunit (Fig. 3). The location of this possible signal peptide between the ferredoxin-related domain and the remainder of the large subunit is consistent with the idea that the iron hydrogenase evolved through the fusion of two genes: one coding for a four-iron hydrogenase and the other for an eight-iron ferredoxin.

In recent years, a number of different microbial redox enzymes have been identified as periplasmic or peripheral membrane proteins (3–5, 12, 17); however, their mechanisms of translocation across the cytoplasmic membrane have not been studied. The results obtained with the small subunit of the iron hydrogenase suggest that the mechanism of translocation of these complex redox proteins is analogous to the well-studied translocation of the other enzymes and proteins.

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