Volume 331, number 1,2, 91–95 FEBS 13011 September 1993

Carboxy-terminal processing of the large subunit of [NiFe] hydrogenases

Nanda K. Menon, Jeff Robbins, Marie Der Vartanian, Daulat Patil, Harry D. Peck Jr., Angeli L. Menon, Robert L. Robson, Alan E. Przybyla*

Department of Biochemistry, University of Georgia, Athens, GA 30602-7229 USA

Received 23 July 1993

Two electrophoretic forms of the large subunit of the soluble periplasmic [NiFe] hydrogenase from *Desulfovibrio gigas* have been detected by Western analysis The faster moving form co-migrates with the large subunit from purified, active enzyme. Amino acid sequence and composition of the C-terminal tryptic peptide of the large subunit from purified hydrogenase revealed that it is 15 amino acids shorter than that predicted by the nucleotide sequence. Processing of the nascent large subunit occurs by C-terminal cleavage between His^{576} and Val^{537}, residues which are highly conserved among [NiFe] hydrogenases. Mutagenesis of the analogous residues, His^{582} and Val^{583}, in the *E coli* hydrogenase-1 (HYD1) large subunit resulted in significant decrease in processing and HYD1 activity.

Hydrogenase large subunit; C-Terminal processing; Mutagenesis

1. INTRODUCTION

Hydrogenases can be classified into two major groups (i) [Fe] hydrogenases and (ii) [NiFe] hydrogenases. [NiFe] hydrogenases are widely occurring and have been reported in anaerobic, facultative anaerobic and aerobic bacteria [1]. To date, nucleotide sequence information of 18 different [NiFe] hydrogenases has been published [1,2]. One common element, among this diverse family of multimeric enzymes is the presence of a large subunit with conserved N- (RXCXXC) and C- (DPCXXCXXH) terminal motifs.

Synthesis of active [NiFe] hydrogenase is a complex process which involves insertion of nickel and iron–sulfur clusters, post-translational processing of the two subunits, and membrane translocation.

Processing of the small subunit involves removal of the signal peptide. This has been inferred from a comparison of the N-terminal protein sequence and the nucleotide-derived amino acid sequence from the gene encoding the small subunit [3–7]. The nascent and mature forms of the small subunit have been detected immunologically in *E. coli* and *Alcaligenes eutrophus* [7,8]. Additionally the N-terminal 40–45 amino acids of the small subunit of the [NiFe] hydrogenase from *Desulfovibrio vulgaris*, have been shown to function as a signal peptide [9].

Two electrophoretically different forms of the large subunit of membrane-bound hydrogenases have also been reported [7,8,10–12]. A larger form of the protein (nascent large subunit, NLS) is processed to a faster moving form (processed large subunit, PLS) which co-migrates with the large subunit of active hydrogenase. The N-terminal amino acid sequence of the purified large subunit is co-linear with the nucleotide-derived N-terminal sequence [3,5,10]. Consistent with this, and based on electrospray mass spectrometry of the large subunit from the *Azotobacter vinelandii* [NiFe] hydrogenase, it has been suggested that the PLS arises as a result of C-terminal cleavage of the NLS [13]. Carboxyl end processing of proteins has been reported for other prokaryotic and eukaryotic proteins [14,15].

In this paper we present the amino acid sequence of the C-terminal tryptic peptide of the large subunit isolated from active *Desulfovibrio gigas* hydrogenase and mutational analysis of the proposed processing site of the *hyab* gene encoding the large subunit of *E. coli* HYD1.

2. MATERIALS AND METHODS

Poros QP II anion-exchange resin was purchased from Poros Inc., Boston, MA. Sequencing grade trypsin was purchased from Sigma Chemical Co., St. Louis, MO. Phenomenex SEC3000 was from Phenomenex, Torrance, CA, and RP302 was from Bio-Rad Labs, Richmond, CA. Anhydrotrypsin agarose was purchased from Takara Biochemicals Inc., Berkeley, CA.

2.1. *Purification of* D gigas *hydrogenase*

D gigas [NiFe] hydrogenase was purified from 120 g of cells using a protocol which included Poros II QP anion exchange chromatography, preparative native polyacrylamide gel-electrophoresis, and hydroxylapatite chromatography. The resulting hydrogenase was pure as judged by silver staining of SDS-polyacrylamide gels. The specific activity of the enzyme was 550 μmol of hydrogen evolved/mg protein/min using methyl viologen as electron acceptor. The total yield of enzyme was 30 mg. Protein was stored at −70°C at a concentration of 5–6 mg/ml.

*Corresponding author. Fax: (1) (706) 542-1738, Email: Przybyla@BCHIRIS.biochem uga.edu.

2.2. *Separation of large and small subunits*

Aliquots containing 200 μg of purified hydrogenase were adjusted to 1% SDS and β-mercaptoethanol, heated at 65°C for 10 min and loaded on a Phenomenex Biosep SEC-3000 molecular sieve HPLC column. The column was eluted isocratically with Tris-HCl buffer (pH 6.8, 50 mM, 0.01% SDS) at 0.3 ml/min The large and small subunits were resolved into two distinct peaks. Fractions from the first peak containing pure large subunit (as judged by SDS-PAGE) were pooled and concentrated to 2 mg/ml using a Millipore Ultrafree microconcentrator (10,000 NMWL).

2.3. *Tryptic digestion of large subunit*

300 pmol of purified large subunit (20 μg) derivatized with iodoacetamide was digested with trypsin (enzyme:substrate ratio of 1:20, 12 h at 37°C) according to described protocols [16]. Digests were routinely monitored for completion by reverse phase chromatography (Fig. 2a).

2.4 *Purification of C-terminal peptide*

600 pmol of trypsin-digested large subunit (500 μl total volume, pH adjusted to 5.0) was loaded onto a 1 ml anhydrotrypsin agarose column. Unbound peptides were eluted at 3 ml/h and 4°C with acetate buffer (50 mM, pH 5.0, containing 20 mM $CaCl_2$) and 0.2 ml fractions were collected. Each fraction was analyzed by reverse-phase chromatography (Fig. 2b).

2 5. *Amino acid sequencing and analysis*

Amino acid sequence and composition analyses were performed at the University of Georgia Molecular Genetics Facility with an Applied Biosystem gas-phase sequenator and an Applied Biosystem amino acid analyzer.

2 6. *Site-directed mutagenesis*

Site-directed mutagenesis of the *hyab* gene at His^{582} and Val^{583} [17], was performed using a Promega Altered Site Mutagenesis kit, as per manufacturers protocol. All mutations were confirmed by sequencing of a 340 bp *NruI–NsiI* fragment encompassing the mutated residues which was ligated into the corresponding region of the parent *hya* operon cloned into the single copy plasmid, pLC-682 [18]. The plasmids containing the mutations were transformed into an *hya* deletion strain, AP1 [7].

2.7. *Hydrogenase-specific assays*

Activity staining of *E. coli* HYD1 in native polyacrylamide gels was done as previously described [7]. Western analysis of SDS-PAGE-fractionated HYD1 large subunit was performed with antibodies specific to the *Bradyrhizobium japonicum* [NiFe] hydrogenase large subunit. Large subunit of *D gigas* hydrogenase was detected using antibodies against *D gigas* native hydrogenase

3. RESULTS AND DISCUSSION

We have previously observed two forms of the *E. coli* HYD1 large subunit while analyzing expression of the *hya* operon [7]. The smaller form co-migrated with the large subunit of the purified HYD1. The N-terminal protein sequence of the large subunit matched the N-terminal of the *hyab* translation product (less the N-terminal methionine), thus ruling out the possibility that processing involved an N-terminal cleavage. This project was initiated to determine if the maturation of the HYD1 large subunit involved a C-terminal cleavage event. However, low yields of purified HYD1, as well as the tendency of the purified large subunit to precipitate during trypsin digestion in the absence of SDS, precluded the use of this protein. As the proposed processing site was well conserved in [NiFe] hydrogenases [1,13], we decided to use the periplasmic [NiFe] hydrogenase from *D. gigas*. This protein was readily purified at high yields and remained soluble through all protocols employed in this study. Additionally, it has also been shown that the N-terminus of the large subunit of *D. gigas*, as determined by protein sequencing, as well as predicted by the gene, are co-linear [4]. A Western blot of *D. gigas* crude cell extract, purified enzyme and recombinant large subunit expressed in *E. coli* (pALM35. Table I) demonstrated that the large subunit of this enzyme underwent a 'processing' step analogous to that reported for membrane-bound [NiFe] hydrogenases, and that the PLS co-migrated with the large subunit from purified hydrogenase (Fig. 1). The large subunit of the recombinant *D. gigas* hydrogenase, however, is not processed in *E. coli*.

A computer-generated tryptic peptide map of the *D. gigas* large subunit, as derived from the DNA sequence of the gene, predicted that the C-terminal fragment would not terminate in an Arg or Lys residue. Hence, this fragment would not bind to anhydrotrypsin agarose, and would be eluted in the void volume. Analysis of the unbound eluate from the anhydrotrypsin column showed 4 major peaks (Fig. 2b). Appropriate controls demonstrated that peaks A and B were chemical constituents of the reaction mixture and peak T was trypsin. Peak 1 was the only tryptic fragment which did not bind to anhydrotrypsin and its amino acid sequence is shown (Fig. 3). The sequence data demonstrated that the carboxyl end of the purified large subunit was 15 amino acids shorter than that deduced from the gene, and that the C-terminal cleavage occurred after His^{536}. To ensure that the last amino acid at the cleavage site was accurately identified, amino acid composition of the peptide was determined. The results of this analysis (Table II)

Table I

List of plasmids used in this study

Plasmid	Description	Reference
pALM35	9 kb *bglII–BamHI* fragment from pG4D [4] cloned into pTZ18R	This work
pLC1682	Single copy plasmid containing Inc FII group of R-100 origin	[18]
pLC47	*hya* operon inserted into *EcoRI–BamHI* site of pLC1682 and transformed into *hya*⁻ strain AP1	[7]
pHL582	His^{582} of *hyab* mutated to Val. Mutation inserted into pLC47	This work
pVW583	Val^{583} of *hyab* mutated to Trp Mutation inserted into pLC47	This work

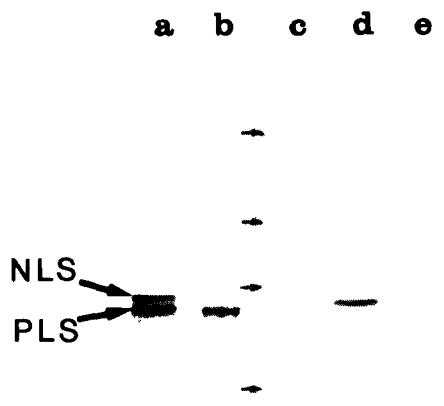


Fig. 1. Western analysis of *D. gigas* [NiFe] hydrogenase. Lanes: a, *D gigas* crude extract; b, purified enzyme; c, standard marker; d, crude extract of an *E. coli* strain carrying pALM35; e, crude extract of *E coli* carrying pTZ18R. Samples were fractionated on a 10% denaturing gel. The western blot was screened with antibodies raised against purified *D. gigas* hydrogenase.

confirmed that His^{536} was the terminal amino acid of the peptide as there were only two Val residues in the fragment. If cleavage had occurred beyond His^{536}, the peptide would have contained additional valine residues.

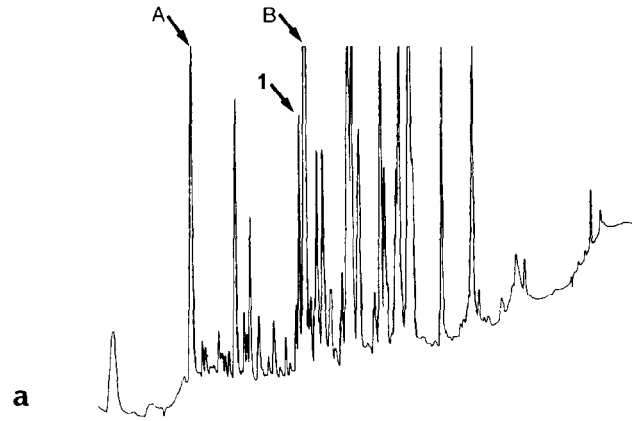
Electrospray mass spectrometry of the large subunit isolated from purified hydrogenase of *A. vinelandii* has shown that it is 1,633 Da smaller than that predicted by the hoxG gene [13,19]. Based on this data, and comparative analysis of gene sequence of the large subunit of various [NiFe] hydrogenases it has been proposed that the large subunit is cleaved at the conserved His residue

Table II

Amino acid composition of the C-terminal tryptic peptide of *D gigas* [NiFe] hydrogenase

Amino acid	Experimental value	Expected value
Aspartic acid	1.017 (1)	1.0
Glutamic acid	0.408 (0)	0.0
Serine	1.411 (1)	1.0
Glycine	1.899 (2)	1.0
Histidine	1.755 (2)	2.0
Threonine	0.952 (1)	1.0
Alanine	1.361 (1)	1.0
Proline	1.000 (1)	1.0
Tyrosine	0.824 (1)	1.0
Valine	1.614 (2)	2.0
Isoleucine	0.9 (1)	1.0
Leucine	0.205 (0)	0.0

The figures in parentheses are the experimental values rounded up or down to the closest complete integer. Cysteine residues were not estimated.



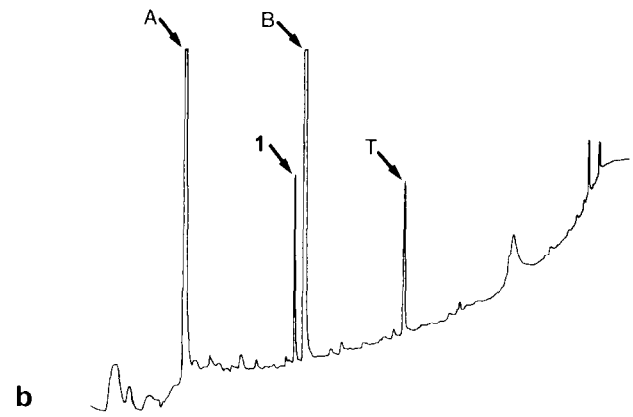


Fig. 2. Elution profile of a tryptic digest of the large subunit of *D. gigas* (a) and the unbound fraction eluted from an anhydrotrypsin agarose column (b) Samples were eluted from a reverse-phase (RP302) column with an acetonitrile gradient ((buffer A) 0.1% TFA and (buffer B) 0.08% TFA and 80% acetonitrile) at 0.7 ml/min. Peaks A and B were chemical peaks of constituents of the reaction mixture. Peak T was trypsin. Peak 1 corresponded to the C-terminal fragment of the large subunit.

analogous to His^{536} of *D. gigas* [4] and His^{582} of *E. coli* [17].

A comparison of the nucleotide-derived amino acid sequence of the large subunit of several [NiFe] hydrogenases showed that the proposed His/Val cleavage site formed part of the conserved motif at the C-terminal end of the large subunit. The His residue is conserved in all but HYD3 of *E. coli*, whereas the Val residue is conserved to a lesser extent. Since the recombinant *D. gigas* hydrogenase was unprocessed and inactive, and there is no genetic system presently available to assess the effect of site-directed mutagenesis in *D. gigas*, we decided to mutagenize the analogous His^{582} and Val^{583}, at the C-terminal cleavage site of the large subunit of *E. coli* HYD1. Both $His^{582}Val$ and $Val^{583}Trp$ mutations resulted in significant but not total loss of HYD1 activity (Fig. 4A). Western analysis of the membrane frac-

D. gigas peptide	R^{522}	T V H S Y D P C I A C G V H^{536}
D. gigas sequence	R^{522}	T V H S Y D P C I A C G V H V I D P E S N Q V H K F R I
E. coli sequence		T L H S F D P C L A C S T H V L G D D G S E L I S V Q V R

▲

Fig. 3. Comparison of protein-derived and nucleotide-derived amino acid sequences of *D. gigas* and *E. coli* [NiFe] hydrogenases. The top line shows the amino acid sequence of the carboxyl tryptic peptide of *D. gigas* [NiFe] hydrogenase large subunit. The middle line shows the nucleotide-derived amino acid sequence of the carboxyl end of the large *D. gigas* subunit. The bottom line shows the nucleotide derived amino acid sequence of the carboxyl end of the *E. coli* HYD1 large subunit. Underlined residues are conserved in the large subunit of all [NiFe] hydrogenases. Arg^{522} is the site of trypsin digestion. ▲ indicates the C-terminal processing site between His^{537} and Val^{538}.

tion showed significantly lower levels of PLS than that found in wild-type cells (Fig. 4B). This indicated that the recognition of the cleavage site is not solely dependent on the His^{582} or Val^{583} residues. Interestingly, in the case of HYD3 in *E. coli*, which also shows processing of the large subunit [11], the His and Val residues are substituted by Arg and Met, even though other residues of the carboxyl end motif are conserved [20].

The significance of C-terminal processing of the large subunit in the biosynthesis of [NiFe] hydrogenases could be manifold. It is generally thought that the processing event is influenced by the availability of nickel and its subsequent liganding to the large subunit. Processing, in *E. coli*, is affected by mutations in genes required for nickel uptake and incorporation [21], as well as by mutations in putative conserved nickel-liganding residues of the large subunit. Thus a mutation in the *hypb* gene of *E. coli*, which is a prerequisite for the incorporation of nickel into all three *E. coli* hydrogenases [22], resulted in the accumulation of NLS of HYD1 [7] and HYD3 [11] in the soluble fraction. Addition of 400 μM nickel suppressed the mutation and yielded active, membrane-bound HYD1, reinforcing the role of this gene product in nickel incorporation. Recently, in our attempts to identify putative Ni-binding residues in *E. coli* HYD1 [1] we have observed that mutagenesis of 5 residues in the large subunit (Arg^{74}, Cys^{76}, Asp^{574}, Cys^{576} and Cys^{579}) which are conserved without exception in all [NiFe] hydrogenases, inhibited nickel liganding to the nascent peptide and led to complete loss of enzymatic activity. The large subunits in all five mutants were unprocessed and in the soluble fraction of the cell.

These studies suggested that nickel-binding is one of the requirements for the C-terminal cleavage of NLS. The carboxy extension on NLS, which contains several charged residues (see Fig. 3) might facilitate nickel liganding to the active site, and its cleavage may result in the folding of the protein in a stable, active conformation. Whether the protein is translocated across the membrane before the processing event, or after nickel liganding and processing, is an open question. However, in the absence of a recognizable signal peptide, a role for the carboxy end of the protein in the translocation process cannot be ruled out. In this regard, it has been shown that at least 10% of the C-terminus of β-lactamase is required for membrane translocation in *E. coli* even though the protein has an amino terminal signal peptide [23].

The biosynthesis of hydrogenase is not the only example of post-translational processing associated with the incorporation of a metal ion into a metalloenzyme. Two forms of DMSO reductase, a molybdenum-requiring enzyme, have been identified in *Rhodobacter sphaeroides* f.sp. *denitrificans* [24]. A larger precursor form accumulated in cells when the enzyme was induced by

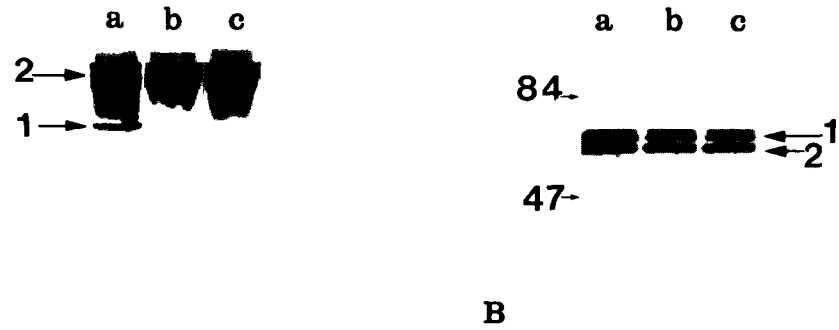


Fig. 4. (A) Activity staining of *E coli* membrane extracts, after electrophoresis on a 7% non-denaturing polyacrylamide gel. Lane a, *E coli* strain TG1; lane b, AP1 transformed with pHL582; lane c, AP1 transformed with pVW583. 1 indicates HYD1 specific activity band, and 2 indicates HYD2 activity. (B) Western analysis of membrane extract of (lane a) TG1, (lane b) pHL582, (lane c) pVW583. 1 indicates the two forms of the large subunit of HYD1, and 2 indicates the large subunit of HYD2.

DMSO in the absence of molybdenum. The molybdenum-free precursor remained in the cytoplasmic or membrane fraction and was not translocated to the periplasm. On addition of molybdenum, the precursor form was processed to a smaller form and transported into the periplasm. It is not clear, however, whether the conversion of the precursor form represents a processing step at the amino or carboxyl end of the protein.

Nickel binding, C-terminal cleavage, membrane translocation, and localization, may take place within a very short period of time. Development of an in vitro processing system may greatly aid in understanding and determining the sequence of events leading to the biosynthesis of active hydrogenase.

Acknowledgments: We wish to thank Dr. John Wunderlich, manager of the University of Georgia Molecular Genetics Facility, for his suggestions and assistance in preparing C-terminal tryptic peptides for analysis, as well as for performing amino acid composition and sequence analyses. This work was supported by NSF MCB 9005734 to A.E P. and H.D P.Jr., NIH GM34903-08 to A.E.P. and H.D.P.Jr., and DOE DE-FG09-86ER13614 to H.D.P.Jr. and A.E.P.

REFERENCES

[1] Przybyla, A.E., Robbins, J., Menon, N.K. and Peck Jr., H.D. (1992) FEMS Microbiol. Rev. 88, 109–136.
[2] Wu, L.F. and Mandrand, M.A. (1993) FEMS Microbiol. Rev. 104, 243–270.
[3] Francis, K., Patel, P., Wendt, J.C. and Shanmugam, K.T. (1990) J. Bacteriol. 172, 5750–5757.
[4] Li, C , Peck Jr., H.D., LeGall, J. and Przybyla, A.E. (1987) DNA 6, 539–551.
[5] Voordouw, G., Menon, N.K., Legall, J., Choi, E.S., Peck Jr., H.D. and Przybyla, A.E. (1989) J. Bacteriol. 171, 2894–2899.
[6] Ford, C.M , Garg, N., Garg, R P., Tibelius, K.H. and Yates, M.G. (1990) Mol. Microbiol. 4, 999–1009.
[7] Menon, N.K., Robbins, J., Wendt, J.C., Shanmugam, K.T. and Przybyla, A.E. (1991) J. Bacteriol. 173, 4851–4861.
[8] Kortluke, C. and Friedrich, B. (1992) J. Bacteriol. 174, 6290–6293.
[9] Niviere, V., Wong, S.L. and Voordouw (1992) J. Gen. Microbiol 138, 2173–2182.
[10] Menon, A.L., Mortenson, L.E. and Robson, R.L. (1992) J. Bacteriol 174, 4549–4557.
[11] Jacobi, A., Rossmann, R. and Böck, A.E. (1992) Arch. Microbiol. 158, 444–451.
[12] Fu, C. and Maier, R.J. (1992) Abstract No. N-80, American Society for Microbiology, 92nd General Meeting, May 26–30, New Orleans.
[13] Gollin, D.J., Mortenson, L.E. and Robson, R.L. (1992) FEBS Lett. 309, 371–375.
[14] Nagasawa, H., Sakagami, Y., Suzuki, A., Suzuki, H., Hara, H and Hirota, Y. (1989) J. Bacteriol. 171, 5890–5893.
[15] Diner, B.A , Ries, F.D., Cohen, B.N. and Metz, J.G. (1988) J. Biol. Chem. 263, 8972–8980.
[16] Stone, K.L., LoPresti, M B., Crawford, M.J., Deangilis, R., and Williams, K.R. (1989) in: A Practical Guide to Protein and Peptide Purification for Microsequencing (Matsudaira, P. ed.) pp. 31–47, Academic Press, New York.
[17] Menon, N K., Robbins, J., Peck Jr., H.D., Chatelus, C.Y., Choi, E.S. and Przybyla, A.E. (1990) J Bacteriol. 172, 1969–1977.
[18] Froehlich, B.J. and Scott, J.R. (1991) Gene 98, 99–101.
[19] Menon, A.L., Stultz, L.W., Robson, R.L. and Mortenson, L.E. (1990) Gene 96, 67–74
[20] Böhm, R., Sauter, M. and Böck, A. (1990) Mol. Microbiol. 4, 231–243.
[21] Waugh, R. and Boxer, D.H. (1986) Biochimie 68, 157–166.
[22] Maier, T., Jacobi, A., Sauter, M. and Böck, A. (1993) J. Bacteriol. 175, 630–635.
[23] Koshland, D. and Botstein, D (1980) Cell 20, 749–760.
[24] Yoshida, Y., Takai, M., Satoh, T. and Takami, S. (1991) J. Bacteriol. 173, 3277–3281.