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Carboxyl-terminal processing may be essential for production of active NiFe hydrogenase in *Azotobacter vinelandii*

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The NiFe hydrogenase from *Azotobacter vinelandii* is a membrane-bound $\alpha\beta$ heterodimer that can oxidize H_2 to protons and electrons and thereby provide energy. Genes encoding the α and β subunits, *hoxG* and *hoxK* respectively, followed by thirteen contiguous accessory genes potentially involved in H_2 oxidation, have been previously sequenced. Mutations in some of these accessory genes give rise to inactive enzyme containing an α subunit with decreased electrophoretic mobility. Mass spectral analysis of the subunits demonstrated that the α subunit had a molecular weight 1,663 Da less than that predicted from *hoxG*. Since the N-terminal sequence of the purified α subunit matches the sequence predicted from *hoxG* we suggest this difference is due to removal of the C-terminus of the α subunit which may be an important step linked to metal insertion, localization, and formation of active hydrogenase.

Azotobacter vinelandii; Hydrogenase subunit; Mass spectrometry; C-Terminal processing; Nickel

1. INTRODUCTION

Azotobacter vinelandii is an aerobic Gram-negative bacterium able to fix N_2. This organism expresses a NiFe hydrogenase system that can recycle electrons from H_2 produced as a byproduct of N_2 fixation. The enzyme is membrane bound and can be isolated after solubilization with detergents [1,2]. The purified hydrogenase is a heterodimer consisting of a small (β) and large (α) subunit with molecular weights of 31 and 67 kDa, respectively, as estimated by SDS-PAGE. Metal analyses indicate that the active enzyme probably contains 1 mole Ni and at least seven moles of Fe per mole of heterodimer [2]. The purified enzyme is inactivated by O_2, which presumably destroys the iron–sulfur centers.

The structural genes, *hoxK* and *hoxG* encoding the β and α subunits of hydrogenase, as well as thirteen contiguous accessory genes involved in O_2-dependent hydrogenase activity, have been cloned and sequenced [3–6] (Fig. 1). The N-terminal amino acid sequence of the β subunit [7] matches that predicted for the *hoxK* gene product, with a presumed 45 amino acid signal sequence removed. The predicted molecular weight of the processed β subunit is 34,232 Da. The N-terminal amino acid sequence of the α subunit [7] matches that predicted for the *hoxG* gene product with the N-terminal methionine removed. The deduced molecular weight of this subunit is 66,605 Da.

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Mutations in some of the accessory genes result in loss of whole-cell, O_2-dependent hydrogenase activity and production of a form of the α subunit with altered electrophoretic mobility [4]. Two classes of mutations were described: mutations in *hoxZ,O* and *Q* result in two forms of the α subunit, one with electrophoretic mobility equivalent to the wild-type α subunit, and a second with a mobility slightly lower than wild-type α subunit, while mutations in *hoxM* and *L* result in a single form with lower than wild-type mobility.

Defining the chemical difference between the two forms of the hydrogenase α subunit is therefore essential to establish the functions of the accessory gene products. Since modification of the N-terminus of the α subunit apparently did not explain the two forms, we decided to test the hypothesis that the increased mobility of the wild-type relative to the mutant α subunit is due to a C-terminal cleavage event. Since C-terminal processing events have been reported for several eukaryotic and prokaryotic proteins [8–10] it would not be surprising if C-terminal processing were responsible for the observed differences in electrophoretic mobility. Our results suggest that these multiple forms of the NiFe hydrogenase α subunit are due to inhibition of a C-terminal processing event.

2. MATERIALS AND METHODS

2.1. *Bacterial strain and growth conditions*

Azotobacter vinelandii CA cultures were grown aerobically under N_2-fixing conditions in Burks medium [11].

2.2. *Purification of the β and α subunits*

Hydrogenase was purified as previously described [2]. Purified hy-

drogenase was separated into its component subunits and away from a few remaining contaminating proteins, under denaturing conditions, by repeated reverse-phase chromatography on a Brownlee RP300 (C-8) guard column using a gradient elution from 0.1% TFA to 0.085% TFA in 80% acetonitrile at a flow rate of 0.2 ml/min. For each chromatographic run, 100–200 pmol of purified hydrogenase was diluted with an equal volume of deionized water and loaded onto the column. The β and α subunits eluted at about 45% and 48% acetonitrile, respectively. Fractions were collected and stored at −20°C.

2.3. *Derivatization of cysteines*

Cysteines were reduced and derivatized with 4-vinylpyridine as described [12].

2.4. *Preparation of β subunit samples for mass spectral analysis*

The β subunit was concentrated 5–10 times using Amicon Centricon 3 microconcentrators centrifuged at 7,000 × g for 4 h. Concentrated fractions were stored at −20°C.

2.5. *Resolubilization of the α subunit*

Samples were dried under vacuum using a Speed-vac concentrator and re-solubilization was attempted using a variety of solvents, including 5% acetic acid, 0.1 M ammonium hydroxide, 40–95% ethanol, 50% methanol, and combinations of 0.5–5% acetic acid in 40–60% ethanol or in 50% methanol. 20 μl of each solvent were added to dried samples and the ingredients were mixed vigorously for 1 min using a vortex mixer. The mixture was then transferred to a fresh tube and dried. Laemmli sample buffer was added to the samples, which were heated at 100°C for 2 min, and then analyzed by SDS-PAGE [13]. Band intensities were compared by eye.

2.6. *Preparation of α subunit samples for mass spectral analysis*

Samples for mass spectral analysis were dried under vacuum using a Speed-vac concentrator and re-solubilized for analysis in 20 μl of 0.5% acetic acid in methanol. About 2 pmol of each subunit were analysed by matrix-assisted laser desorption mass spectrometry, and about 200 pmol were analysed by electrospray mass spectrometry.

2.7. *Mass spectral analysis*

Matrix assisted laser desorption mass spectrometry was performed using a Finnegan Lasermat Mass Spectrometer [14]. Each sample was combined with a matrix consisting of 7 mg of sinapinic acid (Aldrich Chemical Co.) in 1.0 ml of 0.1% TFA in 70% acetonitrile in water in a 1-to-10 ratio. 1 μl was loaded onto a lasermat target and dried under incandescent light. The mass spectrometer was single-point calibrated using recombinant human growth hormone (Genentech Inc.) (M+H=22,126). Laser power was set at 75% of full power. Data from 10 to 15 laser pulses was averaged.

Electrospray mass spectral analysis was performed using a Finnegan Mat TSQ 700 Mass Spectrometer at a sample flow rate of 1 μl/min and a source voltage differential of −3,700 V [15].

3. RESULTS

Purified hydrogenase was separated into the component subunits and away from contaminating proteins, under denaturing conditions, by reverse-phase chromatography. Both subunits eluted as peaks with shoulders, possibly indicating the presence of impurities, however, when analyzed by SDS-PAGE the material in one shoulder and main peak both gave a major band at the molecular weight of the β subunit. Similarly, the material in the other shoulder and main peak both gave a major band at the molecular weight of the α subunit. Fractions for further analysis were pooled to include the shoulder and main peak for both samples. Concentration and re-application of the pooled β subunit fractions resulted in a single symmetrical peak. Concentration of the pooled large subunit fractions resulted in loss of the sample. For a final check of the homogeneity of these samples, cysteines in the purified β and α subunits were reduced and 4-ethylpyridine derivatives were prepared [12]. Homogeneity of the derivatized samples was established by reverse-phase chromatography, which yielded a single symmetrical peak for each sample. Therefore, the initial heterogeneous elution of both subunits is probably due to incomplete denaturation under the conditions used for purification. Mass spectral analysis was performed on non-derivatized samples.

The HPLC solvent used for elution of the subunits was compatible with both laser desorption and electrospray mass spectrometry. The β subunit fractions were concentrated and analyzed directly. The α subunit precipitated from pooled fractions over a day or two whether the samples were stored at room temperature or at −20°C and could not be re-solubilized in aqueous buffers. Inability to re-solubilize the α subunit in aqueous buffers ruled out enzymatic techniques for C-terminal analysis, and led us toward mass spectral analysis. A variety of solvents, chosen to be compatible with the planned mass spectral analysis, were tested for their ability to re-solubilize the α subunit. The solvents tested included water, 5% acetic acid, 0.1 M ammonium hydroxide, 40–95% ethanol, 50% methanol, and combinations of 0.5–5% acetic acid in 40–60% ethanol or in 50% methanol. 90–100% of the α subunit was re-solubilized using combinations of 0.5–5% acetic acid in 40–60% ethanol or in 50% methanol. The subunit was insoluble in 0.1 M ammonium hydroxide, slightly soluble in water, and partially soluble in 40–60% ethanol or in 50% methanol. Samples for mass spectral analysis were dried under vacuum using a Speed-vac concentrator and re-solubilized for mass spectral analysis using 0.5% acetic acid in 50% methanol.

Mass spectral analysis of the individual subunits was performed using both Matrix Assisted Laser Desorption Time of Flight [14] and Electrospray [15] Mass Spectrometry. The laser desorption technique yielded a molecular weight of 34.5–34.7 kDa for the β subunit.

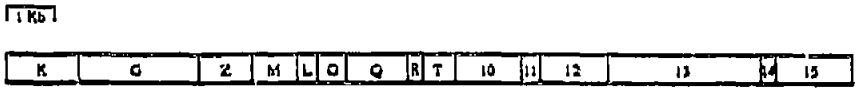

Fig. 1. Physical and genetic map of the NiFe hydrogenase (*hox*) gene cluster from *Azotobacter vinelandii*. Genotypic notations are depicted by capital letters. Additional potential genes have been re-numbered [6].

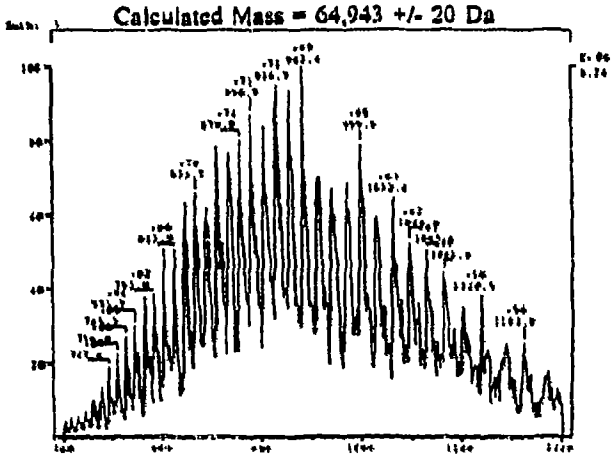

Fig. 2. Electrospray mass spectrum of the α subunit (HOXG).

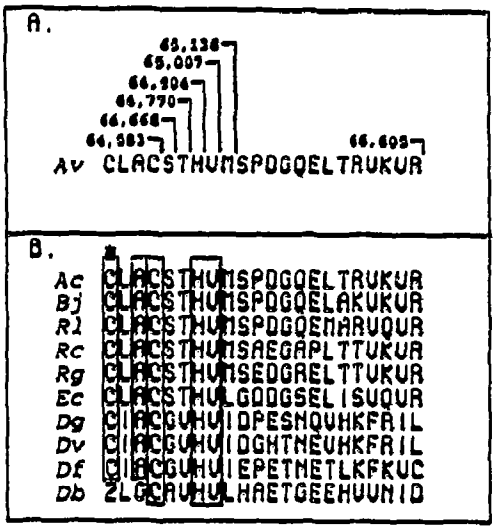

Fig. 3. Predicted mass for HOXG α subunit C-terminal cleavage products and C-terminal homologies. (A) The final 22 amino acids encoded by *hoxG* are shown, along with masses (Da) predicted for various cleavage products. (B) Comparison of C-terminal sequences of membrane-bound or periplasmic NiFe hydrogenases. The asterisk denotes the Ni-liganding cysteine residue. *Av, Azotobacter vinelandii* [3]; *Ac, Azotobacter chroococcum* [7]; *Bj, Bradyrhizobium japonicum* [16]; *Rl, Rhizobium leguminosarum* [17]; *Rc, Rhodobacter capsulatus* [18]; *Rg, Rhodocyclus gelatinosus* [19]; *Ec, Escherichia coli* [20]; *Dg, Desulfovibrio gigas* [21]; *Dv, Desulfovibrio vulgaris* [22]; *Df, Desulfovibrio fructosovorans* [23]; *Db, Desulfovibrio baculatus* [21].

close to the predicted value of 34,232 Da, and 65–66 kDa for the α subunit, less than the predicted value of 66,605 Da. Electrospray mass spectrometry yielded a mass of 64,942 ± 20 Da for the α subunit (Fig. 2). This value is 1,663 Da lower than the value of 66,605 Da deduced from the sequence of *hoxG*. We confirmed that the α subunit N-terminus corresponded to that predicted for the *hoxG* gene product, minus the N-terminal methionine, and thereby concluded that a peptide of about fifteen amino acids had been removed from the C-terminus of the α subunit (Fig. 3) during maturation of the enzyme. Interpretable electrospray mass spectra was not obtained for the β subunit.

4. DISCUSSION

Our evidence suggests that a C-terminal processing event is an essential step in the maturation of the *A. vinelandii* NiFe hydrogenase. The mass determined for the α subunit, 64,942 ± 20 Da, indicates that the site of cleavage is located within a few residues of the Cys-Leu-Ala-Cys sequence (Fig. 3), which has been previously defined as the Ni-binding site by analogy to the NiFeSe hydrogenase from *Desulfovibrio baculatus* [24]. The mass spectral data is consistent with cleavage occurring after the histidine in the sequence, His-Val-Met (Fig. 3). It is interesting to note that the last two completely conserved residues in all examples of membrane-bound or periplasmic NiFe hydrogenases are the His and Val at this proposed cleavage site. This strict conservation strongly suggests that these two residues are involved in C-terminal processing and are essential for production of active hydrogenase.

It is likely that this processing is required for production of other active hydrogenases, since electrophoretically retarded forms of the α and β subunits from NiFe hydrogenases have been reported in hydrogenase locus mutants of *Bradyrhizobium japonicum* [25] and *Escherichia coli* [20,26–28]. In *B. japonicum*, a chromosomal deletion downstream from the NiFe hydrogenase structural genes resulted in disappearance of wild-type forms of the α and β subunits and appearance of an electrophoretically retarded form of each subunit. In *E. coli* three NiFe hydrogenase systems, designated HYD1, HYD2, and HYD3 [28], are encoded by the *hya* [20], *hyb* (Menon, N.K. and Przybyla, A.E., personal communication), and *hyc* [29] operons, respectively. A fourth operon containing the *hyp* genes [26] is required for the activity of all three enzymes. An *E. coli hya* operon deletion mutant complemented with various portions of the *hya* operon produced two membrane-bound forms of the HYD1 large (α) subunit [27]. Two membrane-bound forms of the HYD2 large subunit have also been observed in deletion mutants of the *hyb* operon [27]. Furthermore, an in-frame deletion in *hycH* results in the production of a larger, unprocessed form of the HYD3 large subunit [29]. In addition, two forms of the NiFe hydrogenase large subunit from *Desulfovibrio gigas* (personal communication, Dr. A.L. Menon) and from *D. baculatus* [30] have been observed when cloned structural genes encoding these enzymes are expressed in *E. coli*.

N-Terminal sequences have been determined for the large subunit of HYD1 from *E. coli* [31], and the NiFe hydrogenases from *B. japonicum* [16] and *D. gigas* [32]. The *E. coli* sequence matches the amino acid sequence predicted from *hyaB*, minus the N-terminal methionine

[20]. The *B. japonicum* (see [16]) and *D. gigas* [32] N-terminal sequences match the predicted translation product sequence from the NiFe hydrogenase large subunit genes. Since N-terminal processing is not responsible for the multiple forms observed for the large subunit of these NiFe hydrogenases, we propose that a C-terminal processing event also occurs during the maturation of these hydrogenases, and that this is a general event occurring during processing of membrane-bound NiFe hydrogenases.

Interestingly, two forms of the molybdenum-containing DMSO reductase from *Rhodobacter sphaeroides* f. sp. *denitrificans* are present during induction with DMSO [33]. The higher molecular weight form is a precursor of the active periplasmic form and is found in cytoplasmic and membrane fractions, but not in periplasmic fractions. In contrast, the active form is only found in the periplasm. Maturation of DMSO reductase was inhibited when cells were induced with DMSO in molybdenum-free media. Addition of 1 mM molybdate to the cultures grown in molybdenum-free medium resulted in processing of the precursor to the mature form. Molybdate is therefore essential for processing and localization of this enzyme. Similarly, uptake of Ni or assembly and insertion of Ni or iron-sulfur centers are likely to be essential for localization and processing of the NiFe hydrogenase.

In *E. coli*, mutations in *hydB* [34], which is equivalent to *hypB* [26], have been shown to be overcome by high nickel concentrations. In addition, the production of active HYD1 is dependent on nickel insertion, which is apparently required for membrane localization and processing of the larger forms of both subunits into the mature forms [27]. Complementation of a *hya* operon deletion mutant with a plasmid containing *hyaA-E* resulted in 30% wild-type HYD1 activity. Addition of high concentrations of nickel to the culture media restored wild-type levels of activity, i.e. the *hyaF* gene product can be phenotypically replaced with high concentrations of Ni. Furthermore, there is a correlation between Ni incorporation, membrane localization, electrophoretic mobility, and activity [35]. Since *A. vinelandii hoxQ* is homologous to *hyaF* [4] we would expect high concentrations of Ni to phenotypically replace the *hoxQ* gene product, and in fact preliminary results support this supposition (personal communication, Dr. A.L. Menon).

Formation and functioning of NiFe hydrogenase in *A. vinelandii* is a complex process requiring at least fifteen different gene products. These gene products are potentially involved in metal uptake and insertion, formation of metal centers, and localization and processing of this enzyme, as well as the coupling of H_2 oxidation to O_2 reduction. One processing event, removal of the N-terminal 45 amino acid presequence from the small subunits of NiFe hydrogenases, has been shown to be dependent on nickel incorporation [24]. This presequence has been postulated to be a signal sequence involved in translocation of the NiFe hydrogenase across the plasma membrane. Our results suggest that removal of the C-terminus is a second processing event essential for production of active NiFe hydrogenase that appears to be correlated with Ni insertion. Nickel incorporation is apparently a crucial step in localization and processing, as well as in activation, of this NiFe hydrogenase. The proposed site of C-terminal cleavage of the α subunit is within a few residues of the Ni-liganding cysteine residue (Fig. 3) [24]. The C-terminal peptide may, therefore, function by holding the C-terminus in a conformation that allows Ni insertion. Removal of this peptide would then allow assembly of the Ni site.

Our future studies will focus on the correlation between Ni insertion, processing, and localization, and the role of hydrogenase accessory genes in these processes required for the production of active hydrogenase.

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REFERENCES

[1] Kow, Y.W. and Burris R.H. (1984) J. Bacteriol. 159, 564–569.
[2] Seefeldt, L.C. and Arp, D.J. (1986) Biochimie 68, 25–34.
[3] Menon, A.L., Stults, L.W., Robson, R.L. and Mortenson, L.E. (1990) Gene 96, 67–74.
[4] Menon, A.L., Mortenson, L.E. and Robson, R.L. (1992) J. Bacteriol. 174, 4549–4557.
[5] Chen, J.C. and Mortenson, L.E. (1992) Biochim. Biophys. Acta 1131, 122–124.
[6] Chen, J.C. and Mortenson, L.E. (1992) Biochim. Biophys. Acta 1131, 199–202.
[7] Ford, C.M., Garg, N., Garg, R.P., Tibelius, K.H., Yates, M.G., Arp, D.J. and Seefeldt, L.C. (1990) Mol. Microbiol. 4, 999–1009.
[8] Knight Jr., E., Fahey, D, Cordova, B., Hillman, M., Kutny, R., Reich, N. and Blomstrom, D. (1988) J. Biol. Chem. 263, 4520–4522.
[9] Smith, A.T., Santama, N., Dacey S., Edwards, M., Bray, R.C., Thorneley, R.N.F. and Burke, J.F. (1990) J. Biol. Chem. 265, 13335–13343.
[10] Hara, H., Nishimura, Y., Kato, J.I., Suzuki, H., Nagasawa, H., Suzuki, A. and Hirota, Y. (1989) J. Bacteriol. 171, 5882–5889.
[11] Newton, J.W., Wilson, P.W. and Burris R.H. (1953) J. Biol. Chem. 204, 445–451.
[12] Hawke, D. and Yuan, P. (1987) Applied Biosystems User Bulletin No. 28.
[13] Laemmli, U.K. (1970) Nature 227, 680–685.
[14] Tanaka, K., Waki, H., Ido, Y., Akita, S., Yoshida, Y. and Yoshida, T. (1988) Rapid Commun. Mass Spectrom. 2, 151–153.
[15] Carr, S.A., Hemling, M.E., Bean, M.F. and Roberts, G.D. (1991) Anal. Chem. 63, 2802–2824.
[16] Sayavedra-Soto, L.A., Powell, G.K., Evans, H.J. and Morris, R.O. (1988) Proc. Natl. Acad. Sci. USA 8395–8399.
[17] Hidalgo, E., Leyva, A. and Ruiz-Argüeso (1990) Plant Mol. Biol. 15, 367–370.

[18] Leclerc, M., Colbeau, A., Cauvin, B. and Vignais, P.M. (1988) Mol. Gen. Genet. 214, 97–107.
[19] Uffen, R.L., Colbeau, A., Richaud, P. and Vignais, P.M. (1990) Mol. Gen. Genet. 221, 49–58.
[20] 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.
[21] Voordouw, G., Menon, N.K., LeGall, J., Choi, E.S., Peck, Jr., H.D. and Przybyla, A.E. (1989) J. Bacteriol. 171, 2894–2899.
[22] Choi, E.S. (1990) Dissertation, University of Georgia, Athens, GA.
[23] Rousset, M., Dermoun, Z., Hatchikian, C.E. and Bélaich, J.P. (1990) Gene 94, 95–101.
[24] Przybyla, A.E., Robbins, J., Menon, N.and Peck Jr., H.D. (1992) FEMS Microbiol. Rev. 88, 109–136.
[25] Fu, C. and Maier, R.J. (1992) American Society for Microbiology. 92nd General Meeting, May 26–30, New Orleans, LA. Abstract No. N-80.
[26] Lutz, S., Jacobi, A., Schlensog, V., Böhm, R., Sawers, G. and Böck, A. (1991) Mol. Microbiol. 5, 123–135.
[27] Menon, N.K., Robbins, J., Wendt, J.C., Shanmugam, K.T. and Przybyla, A.E. (1991) 173, 4851–4861.
[28] Sawyers, R.G., Ballantine, S.P. and Boxer, D.H. (1985) J. Bacteriol. 164, 1324–1331.
[29] Sauter, M., Böhm, R. and Böck, D. (1992) Mol. Microbiol. 6, 1523–1532.
[30] Menon, N.K., Peck Jr., H.D., LeGall, J.,and Przybyla A.E. (1987) J. Bacteriol. 169, 5401–5407.
[31] Francis, K., Patel, P., Wendt, J.C. and Shanmugam, K.T. (1990) J. Bacteriol. 172, 5750–5757.
[32] Li, C., Peck Jr., H.D., LeGall, J. and Przybyla, A.E. (1987) DNA 6, 539–551.
[33] Yoshida, Y., Takai, M., Satoh, T. and Takami, S. (1991) J. Bacteriol. 173, 3277–3281.
[34] Waugh, R. and Boxer, D.H. (1986) Biochimie 68, 157–166.
[35] Przybyla, A.E., Menon, N.K., Robbins, J., DerVartanian, L. and Peck Jr., H.D. (1991) Third International Conference on Molecular Biology of Hydrogenases add. 18–22. Tròia, Portugal, July 29–August 1.