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INVOLVEMENT OF HISTIDINE RESIDUES IN THE CATALYTIC MECHANISM OF HYDROGENASES

Isabelle Mus-Veteau* and Françoise Guerlesquin

Laboratoire de Chimie Bactérienne, Centre National de la Recherche Scientifique (CNRS)
BP71, 13277 Marseille Cedex 9, France

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In spite of their structural and amino acid sequence differences, Fe-only and Ni-containing hydrogenases achieved the same catalytic reactions. A chemical modification of histidine residues using a highly specific reagent (pentaammineruthenium II) has been carried out on *Desulfovibrio vulgaris* Hildenborough Fe-hydrogenase and *Desulfovibrio desulfuricans* Norway Ni-Fe-Se-hydrogenase. The preliminary results obtained suggest the existence of a general mechanism involving histidine residues in the two groups of hydrogenases. These residues may be part of the histidine-containing motive shown to be present in both Fe- and Ni-Fe-hydrogenase sequences by Hydrophobic Cluster Analysis. This analysis also allows us to suggest a functional role for the small subunit of *Desulfovibrio vulgaris* Hildenborough Fe-hydrogenase. © 1994 Academic Press, Inc.

Hydrogenases are iron-sulfur enzymes that catalyze the reversible oxidation of molecular hydrogen : $H_2 <=> 2H^+ + 2e^-$. This reaction results of at least two consecutive reactions : the activation of molecular hydrogen, that takes place at the active site (H center), followed by an electron transfer to the acceptor binding site (A center). Although the A center is invariably beleived to be constituted of (Fe-S) clusters, the H center is a special iron-sulfur cluster in Fe-hydrogenases (1) or a Ni atom in Ni-Fe- and Ni-Fe-Se-hydrogenases (2-3). In Fe-hydrogenases, the number of iron atoms ranges from 10 to 16 per molecule (1) organized in two ferredoxin like (4Fe-4S) clusters probably involved in the A center and an additional iron-sulfur cluster thought to be involved in the H center. EPR experiments suggest that this additional cluster would be a (6Fe-6S) cluster or a pecular (4Fe-4S) cluster (4). Cloning and sequencing *Desulfovibrio vulgaris* Hildenborough Fe-hydrogenase gene have given evidence that the protein is composed of two subunits (Mr 46000 and Mr 13000). The presence of conserved cysteine residues on the large subunit leads to propose that this subunit has the main importance in the binding of (Fe-S) clusters. A signal peptide sequence was found on the small subunit : the role of this small subunit has been proposed as inducing the translocation of the molecule to the periplasm (5). Ni-containing hydrogenases are composed

*Corresponding author. Fax: 93 76 60 17.

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of two subunits (Mr 60000 and Mr 30000) (6) and contain a Ni atom involved in the H center and two or three (Fe-S) clusters (2). The small subunit contains a N-terminal signal sequence such as in Fe-hydrogenases, but some cysteine residues are highly conserved in both the small and the large subunits. Until now, three-dimensional structure informations on hydrogenases are lacking. Three Ni-hydrogenases have been crystallized in forms suitable for analysis by X-ray diffraction (7,8) but no structure of Fe- or Ni-hydrogenases has yet been solved. In an attempt to obtain a model for the nickel site of hydrogenases, a nickel-substituted-rubredoxin from *D. vulgaris* Hildenborough has been synthetized (9). The similarity of its EPR spectrum with that of the Ni center of Ni-containing hydrogenases indicated the possible involvement of 4 cysteines in a distorted tetrahedral geometry as Ni ligands in Ni-Fe-hydrogenases, in accordance with the recent results observed with the Ni-substituted rubredoxin from *P. furiosis* (10). These 4 cysteines are thought to be the conserved cysteine residues located on the large subunit of Ni-containing hydrogenases (6, 9). However, it is to be underlined that, although the Ni-rubredoxin shows spectroscopic and redox properties similar to that found in Ni-containing-hydrogenases, this substituted protein was not able to catalyze the oxidoreduction of molecular hydrogen. In opposite, a slight hydrogenase activity has been observed with the Ni-substituted rubredoxin from *D. desulfuricans* (11). Various spectroscopic studies carried out on Ni-Fe-hydrogenases reveal the presence of a nitrogen atom, probably from a histidine residue, in the proximal environment of the Ni atom (12-13). Coremans and coll. (14) have recently shown that H_2-uptake activity of Ni-Fe-hydrogenases depends on an ionizable basic group. In the same way, the protonation state of adjacent groups of binding site in *M. elsdenii* hydrogenase have been shown to affect the rate of molecular hydrogen cleavage or formation (15). The lack of histidine in Ni-substituted rubredoxin from *D. v. H.* could justify the absence of molecular hydrogen oxidoreduction observed in the substituted protein. In another hand, various enzymatic reactions have been described to be mediated by histidine residues such as p-hydroxybenzoate hydroxylase (16) or cytochrome c peroxidase (17). All these data prevailed us on to propose that histidine residues could be involved in the catalytic process of hydrogenases. To study the hypothetical role of histidine residues, we have carried out chemical modifications on Ni-Fe-Se-hydrogenase from *Desulfovibrio desulfuricans* Norway and Fe-hydrogenase from *Desulfovibrio vulgaris* Hildenborough, using pentaammineruthenium II. A comparative study of both modified enzymes is described and a structural comparison of Fe- and Ni-hydrogenases is proposed on the basis of the protein sequences.

Materials and methods

Protein purification : Hydrogenases were purified from two *Desulfovibrio* species : *Desulfovibrio desulfuricans* Norway strain (DdN) and *Desulfovibrio vulgaris* Hildenborough strain (DvH). A fast purification procedure has been used in both cases : after a periplasmic extraction, the proteins were loaded onto a DEAE cellulose column and eluted using a Tris-HCl/NaCl buffer gradient. After a concentration step on an ultrafiltration cell (Amicon), the last step was performed with an high performance liquid chromatography column (HPLC) (LKB, Ultropac column TSK G2000SW). The proteins were estimated to be pure by SDS gel using a phast system (Pharmacia-LKB).

Protein modification : The method applied for chemical modification is the same as previously described by Yocom et al (18). $(Ru(NH_3)_5H_2O)^{2+}$ was generated by the reduction of a solution of $[Ru(NH_3)_5Cl]Cl_2$ (10mM in 150mM phosphate, pH 7.0) over a Zinc/mercury amalgam (19, 20). The hydrogenase from DdN was at 0.63mg/ml concentration, in 20mM phosphate buffer pH 7.6. DvH hydrogenase was at 0.074mg/ml concentration in 0.2M phosphate/1M NaCl buffer pH 7.6 (NaCl was used to stabilize the enzyme). The hydrogenase contained in a dialysis bag was added to the ruthenium solution. After 24h for DdN hydrogenase and 10h for DvH hydrogenase, the dialysis bag was removed. The solution was then washed with the appropriate buffer in an ultrafiltration cell (Amicon), in order to remove unreacted ruthenium reagent. Under air atmosphere, the pentaammineruthenium II-histidine complexes thus formed are oxidized to the more stable trivalent state of ruthenium.

In vitro activity measurements of the proteins : hydrogenase activity was routinely measured by the H_2 evolution assay (21). Each experiment has been carried out by the mean of three repetitive measurements.

Hydrophobic cluster analysis (HCA) : this analysis has been performed using computer treatment (Doriane S.A., HCA Plot V2) (22)

Results

Chemical modification of the Desulfovibrio desulfuricans Norway Ni-Fe-Se hydrogenase

Ruthenium modification of various metalloproteins have been already reported (18, 23-24). Reactions between the ruthenium reagent and the model compounds of imidazole and histidine involve pentaammineruthenium (III) and N-3 position of imidazole sidechain (25, 26). The reaction of Ni-Fe-Se-hydrogenase with $(Ru(NH_3)_5H_2O)^{2+}$ occurs readily under the same conditions. The optical spectra of native and modified hydrogenases from DdN at pH 7.6 display an increase in the absorbance of the pentaammineruthenium-enzyme around 300nm due to charge-transfer transitions between imidazole ligand and ruthenium, owing to the modification of 2 or 3 histidine residues (Table I). The wavelength of the charge-transfer transitions depends upon the state of the protonation of the N-1 nitrogen of the modified histidine. The protonated form has two characteristic absorptions at 304 and 450nm and the deprotonated form at 365 and 600nm (27). In our experiments, at pH 7.6, modified histidines from DdN hydrogenase are probably in the protonated state. The absorption ratio of the protein (A390/A280) remains unchanged, indicating that the protein structure and the iron-

Table I : Optical properties and specific activities of modified Fe- and Ni-Fe-hydrogenases depending on the pH value

Hydrogenases	*Ni-Fe-*			*Fe-*	
pH values	***5.5***	***7.6***	***8.3***	***4.5***	***7.6***
RA304nm	+	+	-	+	-
RA365nm	-	-	+	-	+
RSA %	**20**	**20**	**80**	**10**	**80**

RA304nm, RA365nm : optical differences between native and modified hydrogenases at 304 and 365 nm (RA = relative absorption).
RSA : relative specific activity (100% = specific activity of the native hydrogenase).

sulfur clusters are not affected by the chemical modification. The catalytic activity of the modified DdN hydrogenase has been tested and compared to that of the native hydrogenase. These experiments show that, at pH 7.6, the presence of ruthenium reagent in DdN hydrogenase brings to the loss of about 80% of H_2 oxidoreduction (Table I).

Chemical modification of the Desulfovibrio vulgaris Hildenborough Fe-hydrogenase

We have performed the same chemical modification on Fe-hydrogenase. Modified Fe-hydrogenase optical spectrum does not present the specific absorption at 304nm at pH 7.6, but a bump is observed around 365nm (Table I). This absorption property is interpreted to be due to the $[Ru(NH_3)_5^{3+}]$-histidine residues in which the N-1 nitrogen is in a deprotonated state. The activity tests show a slight decrease of about 20% of the H_2 evolution indicating that the DvH hydrogenase is still active after the modification of some histidine residues in the conditions in which the modified histidines are deprotonated. Optical spectra suggest that, at the same pH value, the $[Ru(NH_3)_5^{3+}]$-histidines of DdN and DvH hydrogenases are not in the same protonation state. At pH 7.6, $[Ru(NH_3)_5^{3+}]$-histidines from DdN hydrogenase would be protonated and those from DvH hydrogenase deprotonated. We have followed the absorption evolution at 304 and 365nm according to different pH values. These experiments indicate that the pKa of modified histidines in both hydrogenases are different : about 8.3 for DdN hydrogenase and 6.6 for DvH hydrogenase. We have tested the activity of both modified hydrogenases at different pH values (Table I) and we have observed the loss of H_2 activity of DvH modified hydrogenase at pH values lower than 6.6. This experiment indicates that, in both hydrogenases, when the $[Ru(NH_3)_5^{3+}]$-histidines are in the protonated form, the enzymes lose their catalytic activity.

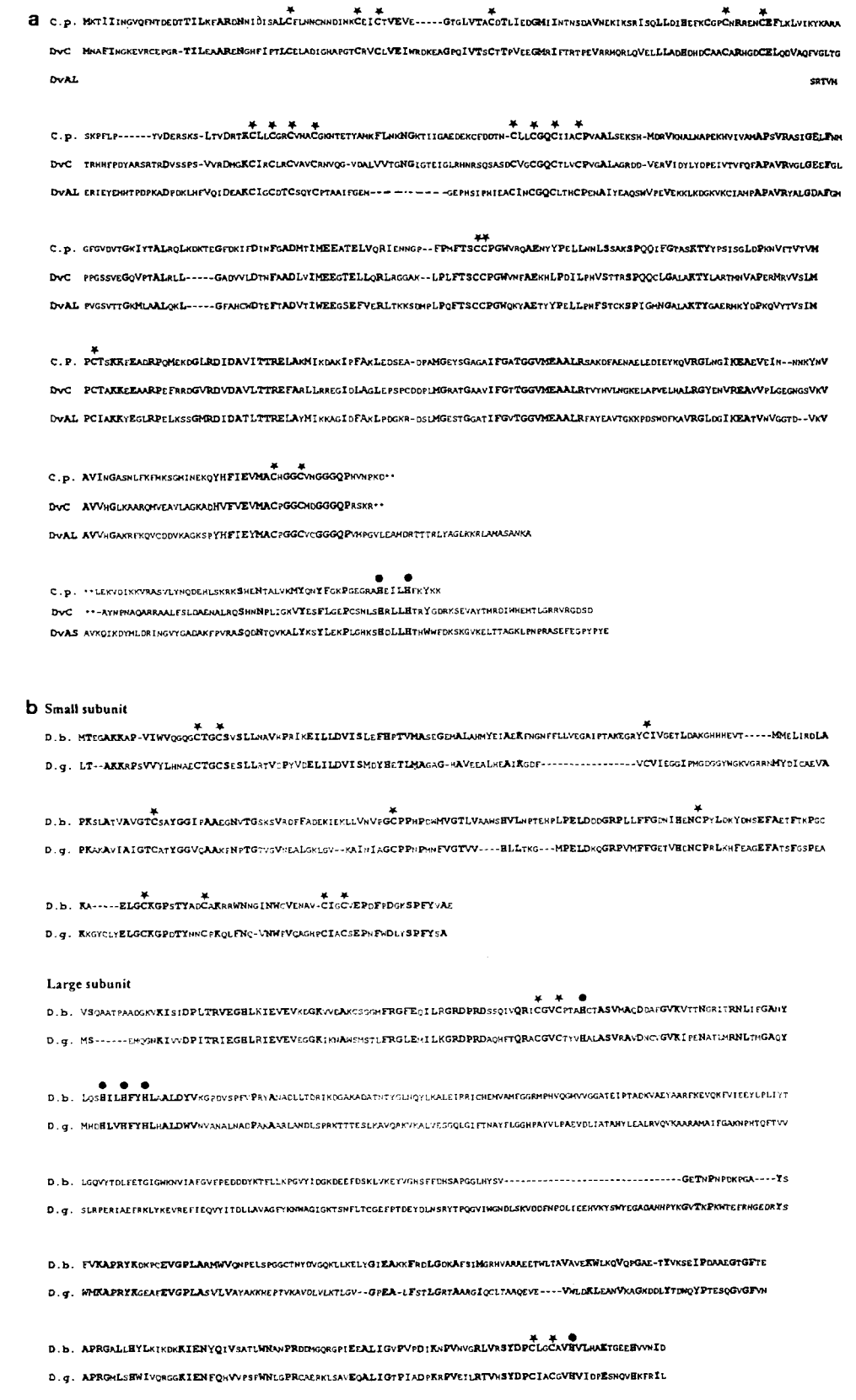
Discussion

The catalytic relevance of histidine residues in Fe- and Ni-Fe-Se-hydrogenases has been preliminarily investigated using chemical modifications. The experimental conditions used lead to a decrease of hydrogen oxidoreduction for both enzymes when the modified histidines are in the protonated state. These first results suggest that the modified histidines would be implicated more likely in the catalytic process than in the Ni coordination, assisting the Ni center in the activation of molecular H_2. The H_2 activity persistance observed on the modified Fe-hydrogenase at pH=7.6 lets us suppose that the N-1 nitrogen, when deprotonated, could play the role of the N-3 nitrogen (covalently bound to the ruthenium) and thus, could catalyse the heterolytic cleavage of molecular hydrogen. A similar involvement of histidine residues in both Fe-only and Ni-Fe-hydrogenases would be an interesting explanation for identical enzymatic activities in so structurally different proteins. In such a scheme, the H center would be expected to be a more or less powerful electrontrap.

This last decade, structural knowledge on Fe- and Ni-Fe-hydrogenases have been improved by protein sequence determination using genetic techniques. Within each group of enzymes, the similarities in the primary structure have been extensively studied (6, 28), but a comparative analysis of both groups has never been approached. Concerning Fe-

hydrogenases, a ferredoxin-like sequence has been found in the N-terminal part of all sequences and is supposed to be the (4Fe-4S) cluster binding site (Fig. 1a). The others highly conserved cysteine residues in the middle of the protein sequence are probably involved in the H center coordination. The absence of cysteine residue in the small subunit of *Desulfovibrio vulgaris* Hildenborough hydrogenase is probably the reason why a functional role of this subunit has been ruled out. In Ni-containing-hydrogenases, no characteristic ferredoxin-like sequence can be observed. All large subunit sequences contain a motive R-x-C-x-x-C-x-x-x-H in the N-terminal part of the polypeptide chain and a motive D-P-C-x-x-C-x-v-H in the C-terminal part (Fig. 1b). These highly conserved motives have been proposed to provide the ligands required for the binding of the redox active Ni (29). The cysteines contained in the small subunit are supposed to be involved in the (Fe-S) clusters coordination. Although the cysteine distribution has been extensively discussed in the litterature, the histidine role has not been taken in account. In Ni-Fe-hydrogenases, it has been reported from site directed mutagenesis experiments, that the highly conserved histidine residues, found into the motives -C-x-x-C-x-x-(x)-H in the N- and C-terminal parts of the large subunit, have no determinant role in the catalytic activity of the enzyme (30). We used an Hydrophobic Cluster Analysis (HCA) (22) to estimate the location of the different metal centers in the protein sequence. This sequence representation clearly shows a high similarity of some hydrophobic clusters of the small subunit of *Desulfovibrio vulgaris* Hildenborough hydrogenase and the C-terminal part of the *Clostridium pasteurianum* hydrogenase I sequence. One of these conserved motives contains two histidines in a hydrophobic environment (Fig. 1a) which appears to be very similar to an other special hydrophobic cluster, containing three histidine residues, highly conserved in the large subunits of Ni-Fe-hydrogenases (Fig. 1b). These histidine-containing motives could be one of the few homology points between Fe- and Ni-Fe-hydrogenases and, consequently, could be expected to provide the nitrogen atom supposed to be involved in the heterolytic cleavage of molecular hydrogen. This preliminary study allows us to invision the development of this programm using spectroscopic technics like EPR to controle the Ni and (Fe-S) clusters integrity after modification. The replacement by site directed mutagenesis of the histidine residues contained in the common conserved hydrophobic motives underlined by HCA representation, would provide evidence on their involvement in the catalytic mechanism of hydrogenases. The verification of our present results would allocate a particular functional role to the *Desulfovibrio vulgaris* Hildenborough Fe-hydrogenasesmall subunit.

Figure 1 : Sequence alignment deduced from HCA study.
Fe-only hydrogenases : Cp, monomeric *Clostridium pasteurianum* hydrogenase I; DvC, *Desulfovibrio vulgaris* Hildenborough monomeric polypeptide chain encoding by gene C; DvA, periplasmic hydrogenase from *Desulfovibrio vulgaris* Hildenborough encoding by gene A (DvAL : large subunit, DvAS : small subunit).
Ni-containing-hydrogenases : Db, *Desulfovibrio baculatus*, Dg, *Desulfovibrio gigas*
Conserved cysteines (*) and histidines (●) are marked.
Cp hydrogenase I and DvC sequences were divided in two parts; ** indicates where the sequences are cut.


a
C.p.
DvC
DvAL
b
Small subunit
D.b.
D.g.
Large subunit
D.b.
D.g.

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