A Glutamate Is the Essential Proton Transfer Gate during the Catalytic Cycle of the [NiFe] Hydrogenase*

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Kinetic, EPR, and Fourier transform infrared spectroscopic analysis of Desulfovibrio fructosovorans [NiFe] hydrogenase mutants targeted to Glu-25 indicated that this amino acid participates in proton transfer between the active site and the protein surface during the catalytic cycle. Replacement of that glutamic residue by a glutamine did not modify the spectroscopic properties of the enzyme but cancelled the catalytic activity except the para-H₂/ortho-H₂ conversion. This mutation impaired the fast proton transfer from the active site that allows high turnover numbers for the oxidation of hydrogen. Replacement of the glutamic residue by the shorter aspartic acid slowed down this proton transfer, causing a significant decrease of H₂ oxidation and hydrogen isotope exchange activities, but did not change the para-H₂/ortho-H₂ conversion activity. The spectroscopic properties of this mutant were totally different, especially in the reduced state in which a non-photosensitive nickel EPR spectrum was obtained.

How do all these steps take place in hydrogenases? These proteins are metalloenzymes that all contain iron, and in many cases, also nickel. The crystallographic structures of several [Fe] hydrogenases (4, 5) and [NiFe] hydrogenases (6, 7) have been obtained by x-ray diffraction studies. In both types of enzymes, the active site is a deeply buried bimetallic center, in which the metals are bridged by thiol groups and have CO and CN⁻ as ligands. This type of coordination favors the binding of molecular hydrogen or hydride to the active site (1, 8). The crystal structures also indicate that Fe–S clusters are located between the active site and the protein surface, which are thought to form the intramolecular electron pathway in the H₂ production/oxidation mechanism (9). In [NiFe] hydrogenases, one nickel and one iron atom form the bimetallic center. The nickel is coordinated to four cysteine ligands via their thiol groups. Two of them are terminal ligands, and the other two are bridging ligands that also coordinate the iron atom (7). One CO and two CN⁻, which are detected by FTIR,¹ are also ligands of the iron atom (10, 11). In addition, an oxygen species also bridges both metals in the inactive oxidized states (10), whereas it disappears in the active reduced states and is likely replaced by a hydride bridge (8, 12–14).

The redox chemistry of [NiFe] hydrogenases is very rich, and at least seven redox states of the active site are detected by FTIR (15). Three of these states are EPR-active due to paramagnetic properties of the nickel. These states are named Ni-A or unready (an inactive oxidized state that needs a long activation period to catalyze H₂ oxidation), Ni-B or ready (an in-active oxidized state that can be quickly activated upon reduction), and Ni-C or actice (an active reduced state) (16, 17). The EPR-silent states are named SI (an unready reduced state), SI (a silent active state, which has two species in acid-base equilibrium), and R (the super-reduced state) (15).

As indicated before, the identification of the electron transfer pathway in hydrogenases is quite straightforward from the crystallographic structure. In standard [NiFe] hydrogenases, it is formed by two [4Fe4S] clusters and one [3Fe4S] cluster (7), but the relative value of their midpoint potential raised questions about their influence on the electron transfer kinetics (18, 19). However, the distances between these redox clusters are adequate for fast electron transfer through the protein (9). A hydrophobic channel that connects the protein surface with the active site for H₂ transport has also been detected by x-ray diffraction studies in Desulfovibrio fructosovorans [NiFe] hydrogenase (20). There is much less evidence regarding the proton transport pathway. Protons are transported inside pro-

¹ The abbreviations used are: FTIR, Fourier transform infrared; MES, 4-morpholineethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid.
teins via motions of water molecules and amino acid residues with acid-base properties, such as histidine, glutamic acid, and aspartic acid (21). Several routes have been proposed for [NiFe] hydrogenases, and most likely, proton transport during catalysis does not stick to a single one (7, 14, 22, 23). A great deal of experimental data support a general agreement in considering that the proton acceptor group after heterolytic cleavage of $H_2$ in the active site is one of the terminal cysteine ligands of the nickel atom (22–32). Therefore, most probably, this cysteine is the starting point, or the ending point, in the case of $H_2$ production, of the proton transport pathway. A glutamic acid residue conserved in [NiFe] hydrogenases (Glu-25 in D. fructosovorans and Glu-18 in Desulfovibrio gigas hydrogenases) is H-bonded to the mentioned terminal cysteine bound to nickel. Thus, it is a potential candidate for the next step of the proton transport pathway (14, 22, 23). In this work, we report the spectroscopic and kinetic characterization of mutants produced at the Glu-25 position of D. fructosovorans [NiFe] hydrogenase, demonstrating that this residue has an important role in hydrogenase catalysis.

**EXPERIMENTAL PROCEDURES**

_Bacterial Strains, Plasmids, and Growth Conditions—_Escherichia coli strain DH5a, F−, endA1, hsdR17 (rK− mK−), supE44, thi−, recA1, gyrA96, relA1, Δ(lacF−lacZYA)U169, λθdowZ2M15 was used as a host in the cloning of recombinant plasmids. The bacterium was routinely grown at 37 °C in LB medium. Ampicillin at 100 μg/ml or gentamycin at 20 μg/ml was added when cells harbored pUC18 or pBGF4 derivatives, respectively. The pBGF4 plasmid, which is a new shuttle vector of the pBM family, reporting the gentamycin resistance gene (33), was used in this study to carry the [NiFe] hydrogenase operon from _D. fructosovorans_ as described previously (19).

_D. fructosovorans_ strain MR400 (hyn::npt) carrying a deletion in the [NiFe] hydrogenase operon (34) was grown anaerobically at 37 °C in SOS medium (33). Large culture volumes were performed as described previously (19). Kanamycin at 50 μg/ml was present routinely, and 50 μg gentamycin/ml were added only when cells harbored the plasmid pBGF4.

_Site-directed Mutagenesis—_The QuikChange™ XL site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands) was used to generate point mutations in the large subunit gene _hynB_. The PstI-AatII fragment from pBGF4 was subcloned into pUC18 to generate pUCBn that was used as a template in mutagenesis experiments. After mutagenesis, the PstI-AatII fragment was fully sequenced and inserted in the PstI-AatII-digested pBGF4. The recombinant plasmid was introduced into _D. fructosovorans_ MR400 by electroporation (33).

**Protein Purification**—The Strep tag II sequence (IBA Gmbh, Gottingen, Germany) was introduced in the hydrogenase genes, and the tagged protein was purified in one step on a Strep-Tactin® column (IBA Gmbh). The yield of purification was in a range between 0.3 and 0.5 mg of pure hydrogenase/liter of culture.

**Activity Measurements**—$H_2$ uptake activity was measured with 1 mM sodium dithionite in the same buffer. The reductive titration was conducted by stepwise additions of small quantities of 20 mM sodium dithionite in the same buffer.

**Biochemical Properties**—The activity of the mutants was measured by three different assays, which give complementary information. $H_2$ uptake in the presence of a redox dye involves the steps of $H_2$ splitting at the active site as well as the steps of hydron and electron transfer. Isotopic exchange reactions involve both the splitting of $H_2$ (or its isotopes) and the hydron transfer steps but not the electron transfer step. Finally, conversion of _para_-$H_2$ to _ortho_-$H_2$ involves only the step of splitting and recombination of $H_2$ at the active site. Fig. 1 shows the pH dependence of the $H_2$ uptake activity values for wild type hydrogenase and the mutants E25D and E25Q. E25D has a similar pH profile to wild type enzyme, and its activity is roughly half of the native hydrogenase activity. Instead, the other mutant, E25Q has only residual activity (less than 0.1% of the wild type one).

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**RESULTS**

**Biochemical Properties**—The activity of the mutants was measured by three different assays, which give complementary information. $H_2$ uptake in the presence of a redox dye involves the steps of $H_2$ splitting at the active site as well as the steps of hydron and electron transfer. Isotopic exchange reactions involve both the splitting of $H_2$ (or its isotopes) and the hydron transfer steps but not the electron transfer step. Finally, conversion of _para_-$H_2$ to _ortho_-$H_2$ involves only the step of splitting and recombination of $H_2$ at the active site. Fig. 1 shows the pH dependence of the $H_2$ uptake activity values for wild type hydrogenase and the mutants E25D and E25Q. E25D has a similar pH profile to wild type enzyme, and its activity is roughly half of the native hydrogenase activity. Instead, the other mutant, E25Q has only residual activity (less than 0.1% of the wild type one).

Rather similar results were obtained when comparing the $D_3H^+$ exchange activity of the hydrogenase mutants with the wild type one (Fig. 2). Virtually no activity could be detected with E25Q, whereas E25D had activity values that were 30–60% of those of the wild type at acidic and neutral pH. In all measurements, the rate of evolution of the double exchange product ($H_2$) was significantly lower than the rate of evolution of the single exchange product (HD), which is the typical behavior of standard [NiFe] hydrogenases (40). An interesting result is that at basic pH, the $D_3H^+$ exchange activity of E25D drops down, whereas the $H_2$ uptake activity maintains high values at pH 8 and 9. To determine whether this discrepancy was due to an isotopic effect or to a change in a rate-limiting step at high pH, the $D_3H^+$ uptake activities of the wild type and E25D mutant were compared, and the $H_2$ production activity of the wild type and mutant enzyme was tested at pH 9.0. The $D_2$ uptake activity values of 63 and 36 units/mg, respectively, were very similar to those obtained with $H_2$ uptake activity, but the $H_2$ production activity of the mutant exhibited a 80% decrease at pH 9.0 relative to the wild type enzyme (not shown). The results indicate that the different pH profiles for the $D_3H^+$ exchange probably originate from the $H_2$ production step becoming rate-limiting at high pH in the E25D mutant. The
E25Q mutant had negligible activity in these assays.

The ortho-para hydrogen conversion experiments show that the mutations had little effect on this activity assay (Fig. 3). After a 200-min reaction, the two mutants and wild type hydrogenase reached similar levels of hydrogen conversion, whereas the blank measurement hardly gave hydrogen conversion (O2 traces cause a small increase of the ortho-H2/para-H2 ratio).

EPR Spectroscopy—To investigate the influence of Glu-25 mutations on the active site of the enzyme, the various paramagnetic states of the [NiFe] center were studied by EPR spectroscopy. The two mutants were titrated anaerobically, and the [NiFe] EPR signals were compared with those given by the wild type enzyme poised at the same potentials. In the oxidized state, the EPR spectra of E25Q are composed of a mixture of the Ni-A (g = 2.31, 2.24, 2.01) and Ni-B (g = 2.33, 2.16, 2.01) signals identical to those of the wild type enzyme (Fig. 4, a and b). The total amount of Ni-A + Ni-B signals represents 0.8 spin/mol of enzyme in E25Q preparations, with a proportion of the Ni-B signal corresponding to 40% of the total nickel signal, a value slightly higher than those usually found for the wild type enzyme. In the oxidized E25D mutant, the nickel EPR signal is mainly composed of the usual Ni-A signal (50–55%), but a new species with g = 2.286, 2.238, 2.01 is also present (Fig. 4c). This species, which we termed Ni-A’, corresponds to about 40% of the total nickel signal, which represents typically 0.3–0.4 spin/mol of enzyme. The rest of the spectrum is composed of a minor fraction (5–10%) of Ni-B signal. In both mutants, all these nickel signals disappear upon reduction and are replaced by other nickel signals in the ~350 mV to ~400 mV potential range (Fig. 5). The reduced E25Q mutant gives the usual Ni-C signal (Fig. 5b), which is split at low temperature (<8 K) by the magnetic coupling with the proximal [4Fe-4S]1+ cluster (Fig. 5c) (41). Spin intensity measurements performed at 30 K showed that this Ni-C signal corresponds to 0.12 spin/mol of enzyme. A careful comparison of the split Ni-C signal in E25Q and in the wild type enzyme shows a small variation of the line splitting that likely arises from a small decrease of the intercenter exchange interaction (ΔJ/3J ≈ 10%) in the mutant. As in the wild type enzyme, the E25Q Ni-C signal is light-sensitive and is changed into the well known Ni-L2 and Ni-L1 signals (42) upon illumination (not shown). By contrast, the reduced E25D mutant exhibited very different spectroscopic features. At 30 K, this mutant gave a complex EPR spectrum (Fig. 5d), suggesting the presence of a mixture of several paramagnetic species or a marked change of the magnetic coupling with the proximal [4Fe-4S]1+ center or of hyperfine interactions. No change of this spectrum was observed when the E25D sample was prepared in D2O by varying the pH between pH 5 and 9. Moreover, the spectrum shape did not change upon variation of the microwave power and of the temperature in the 10–70 K range. These observations and the small variations of the line amplitude between preparations clearly indicated the presence of several nickel species in the reduced E25D mutant. By making differences between spectra given by various preparations, we identified...
three different species, one with g values at $g_X = 2.228$, $g_Y = 2.097$, $g_Z = 2.039$, the two others giving lines at $g_X = 2.242$ and $2.193$, $g_Y = 2.09$ and $2.071$, $g_Z = 2.048$ and $2.027$, but the two sets of g components could not be attributed. The total amount of these nickel signals corresponds to 0.1 spin/mol. It is worth noting that these species are not photosensitive, even after a long time illumination (>1 h). Interestingly, at very low temperature (< 4 K), this complex EPR spectrum exhibits additional splitting (Fig. 5e), which reveals the magnetic coupling between the [NiFe] center and the proximal [4Fe-4S]$^{1-}$ center. This coupling is much smaller than that observed for the split Ni-L species where the intercenter exchange interaction with the [4Fe-4S]$^{2+}$ cluster is cancelled, and the magnetic coupling results from dipolar coupling only (42). Thus, the new nickel species found in the reduced E25D mutant are reminiscent of the Ni-L species; they show a similar g-tensor rhombicity and weakness of the spin-coupling with the proximal [4Fe-4S]$^{1-}$ cluster. For the two Glu-25 mutants, the EPR signals of the FeS clusters are unaffected by mutation (not shown).

**FTIR Spectroscopy**—Infrared measurements were also performed to complete the characterization of the active site of Glu-25 mutants (Fig. 6). The spectra of the oxidized samples of both mutants show the intense band at 1947 cm$^{-1}$, corresponding to the stretching vibrational mode of the CO ligand in the Ni-A state of D. fructosovorans [NiFe] hydrogenase (39). At 2096 and 2084 cm$^{-1}$, the less intense bands appear due to the CN$^-$ ligands in the same state. The shoulders at 2091 and 2081 cm$^{-1}$ correspond without doubt to the CN$^-$ bands of the Ni-B state. These later ones are more evident for the E25Q mutant, which is in agreement with the EPR results. The frequencies of the bands are equal to those of wild type enzyme (39). The shoulder that is clearly observed at 1953 cm$^{-1}$ for E25D could correspond to the CO band of the Ni-A$^-$ state or to an EPR-silent state, which is in high proportion in the oxidized state, as the EPR spectrum indicates. This shoulder often appears, although in lower proportion, in some native samples of [NiFe] hydrogenases (15, 43). Thus, it probably corresponds to an EPR-silent state that is sometimes present in their oxidized samples. Fig. 6 also shows the FTIR spectra of the mutants after the reductive treatment necessary for activation of [NiFe] hydrogenases. The spectrum of E25Q shows the bands corresponding to the Ni-C (1938, 2060, and 2074 cm$^{-1}$) and Ni-R (1938, 2060, and 2074 cm$^{-1}$) states of the wild type enzyme (37). Instead, the spectrum of activated E25D has different features. At least five CN$^-$ bands were observed, and their frequencies do not correspond to those of the Ni-C and Ni-R states. Moreover, an additional broad CO band appears at 1925 cm$^{-1}$. Thus, the FTIR spectra of this reduced mutant are also in agreement with the spectral heterogeneity found by EPR.

**DISCUSSION**

X-ray diffraction data (14, 23, 29, 30), x-ray absorption spectroscopy (28), EPR (27, 32), model complexes chemistry (25, 26), and theoretical calculations (24, 31) support that one of the terminal cysteine ligands of the active site nickel atom (Cys-543 in D. fructosovorans numeration) participates in the holoenzyme splitting of molecular hydrogen by acting as the base for proton binding. A conserved glutamic residue (Glu-25 or Glu-18 in D. fructosovorans or D. gigas numeration, respectively) has been proposed as the next step in the proton transfer pathway between the active site and the protein environment in [NiFe] hydrogenases because its carboxylic group forms a hydrogen bond with the mentioned terminal cysteine residue, and carbonyl groups have an adequate $pK_a$ for proton transport inside proteins (14, 22, 23). In addition, this amino acid residue together with Cys-543 are the most disordered of the
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Fig. 7. Crystallographic structure (wild type (Wt)) (19) and simulations (E25Q and E25D) of the active site of the [NiFe] hydrogenase from D. fructosovorans. The substitution simulations and the H-bond calculations were performed using DeepView 3.7. H-bonds are represented as blue dotted lines, and solid lines visualize the measured distances, which are indicated by numbers.

active site, suggesting a possible motion that might presume of an active role during the proton transfer (22, 23).

FTIR and EPR studies conducted on the E25Q mutant revealed that this protein has identical spectroscopic properties to those of the native enzyme, in both the oxidized and the reduced states and after illumination. These results suggest that the redox properties and the architecture of the mutant active site remained unchanged, as these two techniques are very sensitive to small variations of the active site geometry and redox properties (36). This assumption is confirmed by simulations, in which only a 0.3-Å extension of the O–S distance between the Glu-25 amide and the Cys-72/Cys-543 thiol was calculated, as compared with the O–S distance of the Glu-25 carboxylate and the Cys-72/Cys-543 thiol in the native enzyme (Fig. 7). However, in a most interesting way, this apparent similarity of the structural and redox properties of the mutant with the native enzyme hides a complete alteration of the catalytic properties. The replacement of Glu-25 by Gln abolished the hydrogen uptake and the proton transfer that does not participate in this conversion activity, which involves heterolytic rupture and recombination of molecular hydrogen. As EPR studies also indicated that the iron-sulfur clusters were unaffected by the mutation, the catalytic properties of E25Q can only be explained by the impairing of the fast proton transfer between active site and the protein environment because of the presence of the amide group.

It is worth noting that this work provides a better understanding on previous observations performed in Ralstoniaeutropha-soluble [NiFe] hydrogenase, in which a similar mutant to E25Q had lost its H2 uptake and D2/H+ activities (45). The authors explained this result as a consequence of the breaking of a hydrogen bond involved in the concerted proton/electron transfer for the oxidation of the [3Fe-4S] cluster. In the case of the E25Q mutant, IR and EPR spectroscopies have shown that the Fe–S clusters and the [NiFe] active site can be chemically reduced, indicating that the electron transfer toward the active site was still possible. However, we cannot yet speculate on the electron transfer rate between the active site and the proximal cluster. Thus, the E25Q replacement has modified the hydrogen bond network between the terminal Cys ligands of the nickel ion and the Thr-18 residue (Fig. 7) and has specifically impaired the proton transfer required for the enzyme activity (Fig. 7).

In the case of the E25D mutant, the spectroscopic and catalytic behaviors are the complete opposite of what was reported with the E25Q mutant. The FTIR and EPR spectra obtained in both the oxidized and the reduced states indicated that the electronic properties of the active site were significantly modified. Moreover, one of the most striking results of the E25D mutation was the loss of the photosensitivity properties of the reduced [NiFe] center, associated with the presence of spectroscopic features comparable with the NiL signals. This observation suggests that this mutation induced modifications that maintained the active site in a conformation close to that of the illuminated state. It is now commonly considered that the light sensitivity corresponds to the dissociation of hydrogenated species from the binuclear [NiFe] site (47). One of these species has been proposed to be a bridging hydride between the nickel and the iron ions in the Ni-C state (42). On the other hand, we have shown previously that a proton transfer involving the terminal Cys ligand of the nickel ion accompanied the photoprocess also. The cancellation of the exchange coupling with the proximal (4Fe-4S) cluster (47) suggested that the group that was losing the proton during this process was the Cys-72 thiol (31, 32).

These unexpected results obtained with the E25D mutant are likely to be due to an orientation change of the carboxylate strong) that are considered to play important roles in enzymatic catalysis, as their strength depends on their length, linearity, and the pKa match between the functional groups (44). The substitution of the glutamate carboxylate (wild type) by the glutamine amide (mutants) has likely induced a slight extension of the distance between the O and S atoms but has principally suppressed the acid-base properties of the residue involved in this link, prohibiting the proton transfer. The fact that the para-H2/ortho-H2 conversion activity of the mutant was almost not affected is the evidence that the binuclear center was well structured and fully functional. The mutation only affected the proton transfer that does not participate in this conversion activity, which involves heterolytic rupture and recombination of molecular hydrogen. As EPR studies also indicated that the iron-sulfur clusters were unaffected by the mutation, the catalytic properties of E25Q can only be explained by the impairing of the fast proton transfer between active site and the protein environment because of the presence of the amide group.

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These unexpected results obtained with the E25D mutant are likely to be due to an orientation change of the carboxylate
group of the aspartic residue relative to the nickel terminal cysteine ligands. The replacement of the glutamic residue by an aspartic residue shortened the lateral chain by one C–C bond, and our simulations suggested that the O–S distance between the Asp-25 carboxylate and the Cys-543 thiol was extended by 0.3 Å but was still at H-bond distance, whereas the O–S distance with the Cys-72 thiol was shortened by 0.5 Å (Fig. 7). The loss of photosensitivity of the E25D mutant suggests that Cys-72 remains unprotonated in the reduced state of the active site, at least under the conditions in which Ni-L signals are observed. The unprotonated state of Cys-72 might arise from the shortening of the thiol-carboxylate distance in this mutant, which brought the carboxylate close enough to the sulfur for protonation.

As Glu and Asp have similar pKₐ values (pKₐ is ~4), the 50% reduction of the E25D mutant H₂ uptake and D₂/H⁺ exchange activities might be due to the elongation of the H-bond with Cys-543 that would decrease the rate of proton transfer between the two amino acids (44). It is also possible that the lower mobility of aspartate lateral chain, as compared with that of the glutamate, might be responsible for this lower activity as the motion of the Glu-25 was thought to be important for proton transfer (14, 23). The activity for heterolotrophic cleavage/recombination of molecular hydrogen was once again not altered, indicating that substitutions in that position only modify activities in which proton transfer is involved and strengthening the hypothesis of the crucial role of Glu-25 in proton transfer.

In conclusion, the results presented in this study clearly show that a carboxylic group has to be present close to the active site, providing the transfer of protons. In case proton transfer is impaired, then no catalytic cycle will be possible. On the other hand, our experiments support the hypothesis in which deprotonation of the carboxylate and the Cys-543 thiol was extended by 0.5 Å (Fig. 7). The loss of photosensitivity of the E25D mutant suggests that Cys-72 remains unprotonated in the reduced state of the active site, at least under the conditions in which Ni-L signals are observed. The unprotonated state of Cys-72 might arise from the shortening of the thiol-carboxylate distance in this mutant, which brought the carboxylate close enough to the sulfur for protonation.
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