Suplementary material

1. Protonation states – [NiFe] and [NiFeSe] Hydrogenases

The following table summarizes the different protonation states of the ionizable histidine residues present in the *Desulfovibrio gigas* [NiFe]-Hydrogenase.

Table S1 — Histidine protonation states from Desulfovibrio gigas [NiFe] Hydrogenase. The nomenclature utilized was: HISA stands for a proton in the ND1 atom of the histidine, HISB for a proton in NE2 and HISH on both.

HIS-10 Chain S	HIS-42 Chain S	HIS-51 Chain S	HIS-58 Chain S	HIS-158 Chain S	HIS-190 Chain S	HIS-241 Chain S	HIS-12 Chain L	HIS-53 Chain L	HIS-66 Chain L
HISH	HISB	HISH	HISH	HISB	HISH	HISA	HISH	HISB	HISB
HIS-100 Chain L	HIS-102 Chain L	HIS-106 Chain L	HIS-108 Chain L	HIS-110 Chain L	HIS-173 Chain L	HIS-189 Chain L	HIS-213 Chain L	HIS-316 Chain L	HIS-328 Chain L
HISA	HISB	HISB	HISB	HISB	HISB	HISH	HISB	HISB	HISB
HIS-329 Chain L	HIS-343 Chain L	HIS-377 Chain L	HIS-462 Chain L	HIS-519 Chain L					
HISB	HISH	HISB	HISB	HISH					

In Table S2 the protonation states of the ionizable histidine residues of the *Desulfovibrio vulgaris* [NiFeSe]-Hydrogenase are summarized.

Table S2 - Desulfovibrio vulgaris [NiFeSe] Hydrogenase Histidine residues protonation. The nomenclature of the protonation states is similar to Table 1.

HIS-24 Chain S	HIS-40 Chain S	HIS-50 Chain S	HIS-54 Chain S	HIS-90 Chain S	HIS-91 Chain S	HIS-156 Chain S	HIS-16 Chain L	HIS-68 Chain L	HIS-104 Chain L
HISB	HISB	HISH	HISB	HISH	HISH	HISB	HISH	HISB	HISB
HIS-107 Chain L	HIS-110 Chain L	HIS-159 Chain L	HIS-171 Chain L	HIS-246 Chain L	HIS-361 Chain L	HIS-363 Chain L			
HISB	HISB	HISB	HISB	HISH	HISB	HISB			

Glutamic acid residues were all selected as charged in both structures. There were some conflicting results when comparing data from different dielectric constants in the residue GLU-294 of the [NiFeSe] Hydrogenase. As it was not

possible to assert if the residue was protonated by the available data a qualitative structural analysis was performed. This consisted in aligning the [NiFeSe] and [NiFe] hydrogenases checking for homology between the residues of interest. There was homology between the GLU-294 and the [NiFeSe] hydrogenase's GLU-276. As GLU-276 has shown clear evidence of being charged we assumed that GLU-294 would likely be also in a charged state.

2. RMSD

To assess system stability, we performed RMSD analysis to both systems and both environments (with and without O2).

The following figure shows the average RMSD for all hydrogenase trajectories, over time, relative to the crystal structure's C-alphas.

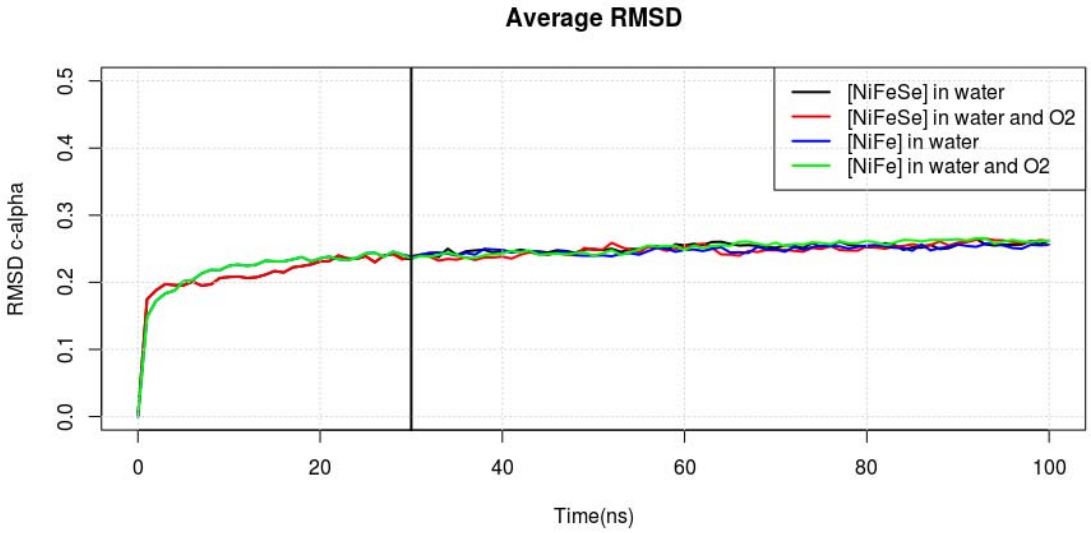

Figure S1 –C-alpha RMSD averages (over the five calculated trajectories for each condition) over time. Black line – O_2 introduction time

3. Non-conserved residues

The following table comprises the conserved and non-conserved residues (as compared with the [NiFeSe]-hydrogenase) near the [NiFe]-hydrogenase highest flux segments of the pathways. The corresponding residue in the [NiFeSe]-hydrogenase is also represented.

Table S3 – Residues lining the highest flux zones from [NiFe] and their counterpart in [NiFeSe] for each pathway. The Pathways nomenclature is similar to Table 1.

NF-A		Conservation status
[NiFe]	[NiFeSe]	
ALA 52 S	GLU 53 S	
VAL 11 L	ILE 21 L	
SER 39 L	GLY 49 L	
MET 357 L	VAL 316 L	
SER 486 L	SER 445 L	Conserved
LEU 490 L	CYS 449 L	
VAL 502 L	VAL 461 L	Conserved
PRO 517 L	PRO 476 L	Conserved
ILE 520 L	VAL 479 L	
LEU 521 L	ALA 480 L	
VAL 524 L	ILE 483 L	
PRO 529 L	PRO 488 L	Conserved
CYS 530 L	CYS 489 L	Conserved
GLY 534 L	ALA 493 L	
VAL 535 L	VAL 494 L	Conserved
NF-B		
VAL 67 L	VAL 77 L	Conserved
THR 69 L	PRO 79 L	
VAL 71 L	ALA 81 L	
HIS 72 L	HIS 82 L	Conserved
LEU 74 L	THR 84 L	
MET 100 L	PHE 110 L	
TYR 104 L	TYR 114 L	
ASP 107 L	SER 117 L	
LEU 115 L	LEU 125 L	Conserved
ASN 460 L	GLU 419 L	
ALA 461 L	ALA 420 L	Conserved
PRO 462 L	PRO 421 L	Conserved
ARG 463 L	ARG 422 L	Conserved
LEU 466 L	LEU 425 L	Conserved
NF-C		
THR 61 L	VAL 61 L	
GLN 62 L	GLN 72 L	Conserved
ARG 63 L	ARG 73 L	Conserved
ALA 64 L	ILE 74 L	
CYS 65 L	CYS 75 L	Conserved
CYS 68 L	CYS 78 L	Conserved
THR 69 L	PRO 79 L	
TYR 70 L	THR 80 L	Conserved
HIS 72 L	HIS 82 L	Conserved

HIS 468 L	HIS 427 L	Conserved
LEU 484 L	ILE 495 L	

The following table comprises the conserved and non-conserved residues (as compared with the [NiFe]-hydrogenase) near the [NiFeSe]-hydrogenase highest flux segments of the pathways. The corresponding residue in the [NiFe]-hydrogenase is also represented.

Table S4 - Residues lining the highest flux zones from [NiFeSe] and their counterpart in [NiFe] for each pathway. The Pathways nomenclature is similar to Table 1.

NFS-A		Conservation status
[NiFeSe]	[NiFe]	
LEU 13 S	LEU 12 S	Conserved
GLY 17 S	GLU 16 S	
CYS 18 S	CYS 17 S	Conserved
PRO 184 S	PRO 218 S	Conserved
GLU 28 L	GLU 18 L	Conserved
ARG 53 L	ARG 43 L	Conserved
GLN 72 L	GLN 62 L	Conserved
ILE 74 L	PRO 64 L	
CYS 75 L	CYS 65 L	Conserved
GLY 76 L	GLY 66 L	Conserved
PRO 79 L	THR 69 L	
HIS 185 L	HIS 219 L	Conserved
GLY 491 L	ALA 532 L	
NFS-B		
VAL 77 L	VAL 67 L	Conserved
ASN 113 L	GLN 103 L	
GLN 116 L	HIS 106 L	
SER 117 L	ASP 107 L	
LEU 120 L	VAL 110 L	
HIS 121 L	HIS 111 L	Conserved
HIS 124 L	HIS 114 L	Conserved
TYR 162 L	TYR 196 L	Conserved
ALA 165 L	ALA 199 L	Conserved
LEU 166 L	LEU 200 L	Conserved
ARG 169 L	GLN 203 L	
PRO 421 L	PRO 462 L	Conserved
ARG 422 L	ARG 463 L	Conserved
NFS-C		
PHE 45 S	TYR 44 S	
ILE 58 L	ILE 48 L	Conserved
ARG 62 L	ARG 52 L	Conserved

ILE 70 L	PHE 60 L	
VAL 71 L	THR 61 L	
ILE 74 L	ALA 64 L	
CYS 492 L	CYS 533 L	Conserved
HIS 495 L	HIS 536 L	Conserved