The quest for a functional substrate access tunnel in FeFe hydrogenase
Supplementary information

Thomas Lautier[*] Pierre Ezanno[†] Carole Baffert[†]
Vincent Fourmond[†] Laurent Cournac[‡]
Juan C. Fontecilla-Camps[§] Philippe Soucaille[⋆]
Patrick Bertrand[†] Isabelle Meynial-Salles[⋆]
Christophe Léger[†¶]

[*]Université de Toulouse; INSA,UPS,INP; LISBP, 135 Avenue de Rangueil, F-31077 Toulouse, France.
INRA, UMR792 Ingénierie des Systèmes Biologiques et des Procédés, F-31400 Toulouse, France.
CNRS, UMR5504, F-31400 Toulouse, France

[†]CNRS. Laboratoire de Bioénergétique et Ingénierie des Protéines. UPR 9036. Institut de Microbiologie de la Méditerranée, 31 chemin Joseph Aiguier, 13402 Marseille Cedex 20. Aix-Marseille Université. http://bip.cnrs-mrs.fr/bip06

[‡]CEA, Institut de Biologie Environnementale et Biotechnologie, Laboratoire de Bioénergétique et Biotechnologie des Bactéries et Microalgues, F-13108 Saint-Paul-lez-Durance, France;
CNRS UMR Biologie Végétale et Microbiologie Environnementales, F-13108 Saint-Paul-lez-Durance, France;
Aix-Marseille Université.

[§]Laboratoire de Cristallographie et Cristallogenèse des Protéines, Institut de Biologie Structurale Jean-Pierre Ebel, CEA, CNRS, Université Joseph Fourier, 41 Rue Jules Horowitz, F-38027 Grenoble, France

[¶]E-mail: christophe.leger@ifr88.cnrs-mrs.fr

Figure 1

```
A, B, C, W = amino acids defining paths A, B, C, W.
a = amino acids close to the path A calculated with CAVER, footnote (a).
F or f = ligands of the FeS clusters
                                          f               f  f
Cp    --MKTIIINGVQFNTDEDTTILKFARDNNIDISALCFL----NNCNNDINKCEICTVEVE 54
Ca    --MKTIILNGNEVHTDKDITILELARENNVDIPTLCFL----KDCGN-FGKCGVCMVEVE 53
Dd    ------------------------------------------------------------
Cr    ------------------------------------------------------------
Df    MSMLTITIDGKTTSVPEGSTILDAAKTLDIDIPTLCYLNLEALSINNKAASCRVCVVEVE 60
Tm    --MKIYVDGREVIINDNERNLLEALKNVGIEIPNLCYLS-----EASIYGACRMCLVEIN 53

              f                                  f   f  f     f
Cp    GT-GLVTACDTLIEDGMIINTNSDAVNEKIKSRISQLLDIHEFKCGPCNRRENCEFLKLV 113
Ca    GK-GFRAACVAKVEDGMVINTESDEVKERIKKRVSMLLDKHEFKCGQCSRRENCEFLKLV 112
Dd    --------------------MSRTVMER------------------------------- 008L
Cr    ------------------------------------------------------------
Df    GRRNLAPSCATPVTDNMVVKTNSLRVLNARRTVLELLLSDHPKDCLVCAKSGECELQTLA 120
Tm    GQ--ITTSCTLKPYEGMKVKTNTPEIYEMRRNILELILATHNRDCTTCDRNGSCKLQKYA 111

                                      F  F  F   F
Cp    IKYKARASKPFLPKDKTEYVDERSKSLTVDRTKCLLCGRCVNACGKNTETYAMKFLNKNG 173
Ca    IKTKAKASKPFLPEDKDALVDNRSKAIVIDRSKCVLCGRCVAACKQHTSTCSIQFIKKDG 172
Dd    IEYEMHTPDPKADPDKLHFVQ-------IDEAKCIGCDTCSQYCPT-----AAIFGEMGE 056L
Cr    --MSALVLKPCAAVS-------------IRGSSCRARQVAPRAPLAASTVRVALATLEAP 045
Df    ERFGIRES-PYDGGEMSHYRKDISASIIRDMDKCIMCRRCETMCNTVQTCGVLSGVNRGF 179
Tm    EDFGIRKIRFEALKKEHVRDE--SAPVVRDTSKCILCGDCVRVCEEIQGVGVIEFAKRGF 169

                        F  F  F   F
Cp    KTIIGAEDEKCFDDTNCLLCGQCIIACPVAALSE-KSHMDRVKNALNAPEKHVIVAMAPS 232
Ca    QRAVGTVDDVCLDDSTCLLCGQCVIACPVAALKE-KSHIEKVQEALNDPKKHVIVAMAPS 231
Dd    PHSIPHIEA-------CINCGQCLTHCPENAIYEAQSWVPEVEKKLKDGKVKCIAMPAPA 109L
Cr    ARRLG-----------NVACAAAAPAAEAPLSHVQQALAELAKPKDDPTRKHVCVQVAPA 94
Df    TAVVAPAFEMNLADTVCTNCGQCVAVCPTGALVEHEYIWEVVEALAN-PDKVVIVQTAPA 238
Tm    ESVVTTAFDTPLIETECVLCGQCVAYCPTGALSIRNDIDKLIEALES--DKIVIGMIAPA 227

                                             C BCC BCA  AA  A
Cp    VRASIGELFNMGFGVDVTGKIYTALRQLGFDKIFDINFGADMTIMEEATELVQRIENNG- 291
Ca    VRTAMGELFKMGYGKDVTGKLYTALRMLGFDKVFDINFGADMTIMEEATELLGRVKNNG- 290
Dd    VRYALGDAFGMPVGSVTTGKMLAALQKLGFAHCWDTEFTADVTIWEEGSEFVERLTKKSD 169L
Cr    VRVAIAETLGLAPGATTPKQLAEGLRRLGFDEVFDTLFGADLTIMEEGSELLHRLTEHLE 154
Df    VRAALGEDLGVAPGTSVTGKMAAALRRLGFDHVFDTDFAADLTIMEEGSEFLDRLGKHLA 298
Tm    VRAAIQEEFGIDEDVAMAEKLVSFLKTIGFDKVFDVSFGADLVAYEEAHEFYERLKKGE- 286

              A AC WCF   W          W   WWB WC  B  BB  B
Cp    -------PFPMFTSCCPGWVRQAENYYPELLNNLSSAKSPQQIFGTASKTYYPSISGLDP 344
Ca    -------PFPMFTSCCPAWVRLAQNYHPELLDNLSSAKSPQQIFGTASKTYYPSISGIAP 343
Dd    M------PLPQFTSCCPGWQKYAETYYPELLPHFSTCKSPIGMNGALAKTYGAERMKYDP 223L
Cr    AHPHSDEPLPMFTSCCPGWIAMLEKSYPDLIPYVSSCKSPQMMLAAMVKSYLAEKKGIAP 214
Df    G--DTNVKLPILTSCCPGWVKFFEHQFPDMLDVPSTAKSPQQMFGAIAKTYYADLLGIPR 356
Tm    -------RLPQFTSCCPAWVKHAEHTYPQYLQNLSSVKSPQQALGTVIKKIYARKLGVPE 339
```

```
                F     W
Cp    KNVFTVTVMPCTSKKFEADRPQMEKDG---LRDIDAVITTRELAKMIKDAKIPFAKLEDS 401
Ca    EDVYTVTIMPCNDKKYEADIPFMETNS---LRDIDASLTTRELAKMIKDAKIKFADLEDG 400
Dd    KQVYTVSIMPCIAKKYEGLRPELKSSG---MRDIDATLTTRELAYMIKKAGIDFAKLPDG 280L
Cr    KDMVMVSIMPCTRKQSEADRDWFCVDADPTLRQLDHVITTVELGNIFKERGINLAELPEG 274
Df    EKLVVVSVMPCLAKKYECARPEFSVNG---NPDVDIVITTRELAKLVKRMNIDFAGLPDE 413
Tm    EKIFLVSFMPCTAKKFEAEREEHEG-----I--VDIVLTTRELAQLIKMSRIDINRVEPQ 392

                      C     CA  AA  A   a                         A A
Cp    EADPAMGEYSGAGAIFGATGGVMEAALRSAKDFAENAELEDIEYKQVRGLNGIKEAEVEI 461
Ca    EVDPAMGTYSGAGAIFGATGGVMEAAIRSAKDFAENKELENVDYTEVRGFKGIKEAEVEI 460
Dd    KRDSLMGESTGGATIFGVTGGVMEAALRFAYEAVTGKKPDSWDFKAVRGLDGIKEATVNV 340L
Cr    EWDNPMGVGSGAGVLFGTTGGVMEAALRTAYELFTGTPLPRLSLSEVRGMDGIKETNITM 334
Df    DFDAPLGASTGAAPIFGVTGGVIEAALRTAYELATGETLKKVDFEDVRGMDGVKKAKVKV 473
Tm    PFDRPYGVSSQAGLGFGKAGGVFSCVLSV---LNEEIGIEKVDVKSPE--DGIRVAEVTL 447

      a                                              a  AAA
Cp    N---------------------------------------------NNKYNVAVINGASN 476
Ca    A---------------------------------------------GNKLNVAVINGASN 475
Dd    G---------------------------------------------GTDVKVAVVHGAKR 355L
Cr    VPAPGSKFEELLKHRAAARAEAAAHGTPGPLAWDGGAGFTSEDGRGGITLRVAVANGLGN 394
Df    G---------------------------------------------DNELVIGVAHGLGN 488
Tm    K---------------------------------------------DGTSFKGAVIYGLGK 463

                     AA     F   F
Cp    LFKFMKSGMINEKQYHFIEVMACHGGCVNGGGQPHVNPKDLEKV-----DIKKVRASVLY 531
Ca    FFEFMKSGKMNEKQYHFIEVMACPGGCINGGGQPHVNALDRENV-----DYRKLRASVLY 530
Dd    FKQVCDDVKAGKSPYHFIEYMACPGGCVCGGGQP-VMPGVLEAAVKQIKDYMLDRINGVY 049S
Cr    AKKLITKMQAGEAKYDFVEIMACPAGCVGGGGQPRSTDKAITQK----------RQAALY 444
Df    ARELLKPCGAGET-FHAIEVMACPGGCIGGGGQPYHHG----DV-----ELLKKRTQVLY 537
Tm    VKKFLEE----RKDVEIIEVMACNYGCVGGGGQPYPNDS----------RIREHRAKVL- 508

                           BB  BB      BB  BB WWW
Cp    NQDE-HLSKRKSHENTALVKMYQNYFGKPGEGRAHEILHFKYKK---------------- 574
Ca    NQDKNVLSKRKSHDNPAIIKMYDSYFGKPGEGLAHKLLHVKYTKDKNVSKHE-------- 582
Dd    GADA-KFPVRASQDNTQVKALYKSYLEKPLGHKSHDLLHTHWFDKSKGVKELTTAGKLPN 111S
Cr    NLDE-KSTLRRSHENPSIRELYDTYLGEPLGHKAHELLHTHYVAGGVEEKDEKK------ 497
Df    AEDA-GKPLRKSHENPYIIELYEKFLGKPLSERSHQLLHTHYFKRQRL------------ 585
Tm    RDTMGIKSLLTPVENLFLMKLYEEDLKD--EHTRHEILHTTYRPRRRYPEKDVEILPVPN 571

Cp    ------------------------------------------------------------
Ca    ------------------------------------------------------------
Dd    PRASEFEGPYPYE------------------------------------------------
Cr    ------------------------------------------------------------
Df    ------------------------------------------------------------
Tm    GEKRTVKVCLGTSCYTKGSYEILKKLVDYVKENDMEGKIEVLGTFCVENCGASPNVIVDD 626

Cp    -------------------
Ca    -------------------
Dd    -------------------
Cr    -------------------
Df    -------------------
Tm    KIIGGATFEKVLEELSKNG 645
```

Experimental

Construction of the pPHhydA1-LL-Csteptag: optimisation of the C-terminal Strep tag II exposition was achieved by addition in the pPHhydA1-Cstrep-tag vector of a long amino acid linker between the 3' part of the *hydA* gene and the Strep tag II sequence, as previously described in the study of Von Abendroth et al., 2008.[1] The linker sequence was inserted in the pPHhydA1-Cstrep-tag[2] by site-directed mutagenesis and contained 36 bp (5'-CCCGGGGGTAGTGGTGGTGGTAGTGGTGGTGGTAGT-3'), adding a 12-amino acid linker in the synthesized protein.

Site-Directed Mutagenesis: The high fidelity Phusion DNA polymerase (Finnzymes (R), Espoo, Finland) was used to generate point mutations on hydA gene cloned into the pPHhydA1-LL-Csteptag vector. Plasmids containing the *hydA* mutated genes were transformed into *E. coli* strain TOP10 (Invitrogen (R), Carlsbad, California, USA). After plasmid extraction using the GenElute (TM) HP plasmid Midiprep kit (Sigma-Aldrich (R), Saint-Louis, Missouri, USA) the *hydA* mutated genes were fully sequenced. The recombinant plasmids expressing mutated *hydA* gene were introduced into *Clostridium acetobutylicum* ΔCAC1502 strain by electrotransformation under anaerobic conditions. The presence of the *hydA* mutated gene in the transformed cells was validated by sequencing the specific PCR product.

The purification procedure is described in ref 3. The H_2 / methyl viologen solution assays, previously described in ref 4, were carried out in an anaerobic glove box filled with N_2. All buffers and solutions were prepared in distilled water (previously boiled and degassed with oxygen-free nitrogen) and transferred under an hydrogen atmosphere just before using. Specific activities were determined in a range where linearity with protein concentration was established. Each enzyme assay was carried out in triplicate. Hydrogenase activity in the direction of MV reduction was assayed as described in ref 5, modified as follows: 100 mM potassium phosphate buffer (pH 7.2) under hydrogen atmosphere; 20 mM MV; 20 mM DTT; 1 atm of hydrogen gas in the headspace.

The isotope exchange assays, described in ref 6, were performed using the setup described in ref. 7. A 1.5mL solution of 20 mM K-phosphate buffer, pH 7.2, and saturated with D_2 was kept at 30°C in a thermostated vessel in a glove box filled with N_2. The solution was stirred continuously with a magnetic stirrer. The bottom of the vessel was sealed by a polypropylene membrane, allowing dissolved gases to be directly introduced through a vacuum line into the ion source of the mass spectrometer (model MM 880; VG Instruments, Cheshire, United Kingdom). The spectrometer sequentially scans the abundance of the different gases (H_2, D_2, HD, and O_2) by automatically adjusting the magnet current to the corresponding mass peaks ($m/e = 2$, 4, 3 and 32, respectively). Measuring one mass peak typically takes 0.5s. After the enzyme was added, the hydrogenase activity resulted in D^+/H^+ scrambling which is detected by continuously monitoring the consumption of D_2 and the formation of HD and H_2. The spectrometer sensitivity was calibrated, and the 1st order rate constants for gas consumption by the spectrometer were determined in control experiments where the solution was initially saturated under an atmosphere of known composition, and the exponential decrease of the concentration over time was monitored. This gas consumption by the mass spectrometer introduces a contribution that must be corrected before the data are analysed (see supplementary information of ref 6).

We used the electrochemical setup and equipment described in ref. 8. All PFV experiments were carried out in a glove box under a N_2 atmosphere ($O_2 < 3$ ppm). The electrochemical cell was thermostated at the desired T using a water circulation

system. A pyrolytic graphite "edge" (PGE)[9] rotating disk working electrode (area $A \approx 3$ mm^2) was used in conjunction with an electrode rotator, a platinum wire was used as a counter electrode, and a saturated calomel electrode (SCE), located in a side arm containing 0.1 M NaCl and maintained at room temperature, was used as a reference. All potentials are quoted versus the standard hydrogen electrode (SHE), $E_{SHE} = E_{SCE} + 240$ mV. The "mixed buffer" consisted of MES, HEPES, sodium acetate, TAPS, and CHES (5mM of each component), 1mM EDTA, and 0.1M NaCl as supporting electrolyte, titrated to desired pH using concentrated HCl or NaOH.

Before preparing an enzyme film, the PGE electrode was polished with an aqueous alumina slurry (Buehler, 1 μm) and sonicated thoroughly. Protein films were prepared by painting the electrode with about half a microliter of a stock solution of enzyme (≈ 0.1 mg/mL in the purification phosphate buffer at pH 7.2). The electrode could then be transferred to a fresh solution with very little loss in electroactive coverage over time.

For measuring the Michaelis constant for H_2, we used the method exposed in ref 6. The electrochemical cell was flushed with H_2 using a cannular to bubble the gas directly into the cell solution. While the activity was measured at -160mV, pH 7, ω =3krpm, H_2 initially dissolved in concentration $[H_2]_0$ was flushed away by bubbling argon in the cell. The change in activity vs time is a portion of a sigmoid and the value of $K_m/[H_2]_0$ can be determined by fitting the data. Hence the K_m value is obtained as a fraction of the concentration of H_2 that is initially dissolved.

For determining the kinetics of CO binding and release, the electrochemical cell was continuously flushed with H_2. The buffer used in the electrochemical cell, but saturated with CO at room temperature (with the safety precautions relevant to the high toxicity of carbon monoxide), was kept in a capped serum bottle. Small aliquots of this solution were injected into the electrochemical cell using gas-tight syringes. The volume injected divided by total volume of solution in the cell after the injection is also the ratio of the concentration of dissolved gas at time of injection over the concentration under saturating conditions. Hence "one atm of CO" refers to the concentration of CO in a solution that is equilibrated with 1 atm. of CO at 25°C.

For studying the kinetics of inhibition by O_2, the method is the same as that for CO except that we inject aliquots of solution saturated with either oxygen or air at 25°C, as described previously.[8,10]

We analyzed the electrochemical data using "SOAS," an in-house program described in ref 11 and available on our web site at `http://bip.cnrs-mrs.fr/bip06/software.html`.

The bimolecular rates of inhibition in units of s^{-1} per atm. of CO or O_2 can be converted in units of s^{-1} per mM using the Henry constants of 0.96 and 1.25 mM/atm, respectively.

References

[1] G. Vonabendroth, S. Stripp, A. Silakov, C. Croux, P. Soucaille, L. Girbal and T. Happe, *Int. J. Hydrog. Energ.*, 2008, **33**, 6076–6081.

[2] L. Girbal, G. von Abendroth, M. Winkler, P. M. C. Benton, I. Meynial-Salles, C. Croux, J. W. Peters, T. Happe and P. Soucaille, *Appl. Environ. Microbiol.*, 2005, **71**, 2777–2781.

[3] V. Fourmond, T. Lautier, C. Baffert, F. Leroux, P.-P. Liebgott, S. Dementin, M. Rousset, P. Arnoux, D. Pignol, I. Meynial-Salles, P. Soucaille, P. Bertrand and C. Léger, *Anal. Chem.*, 2009, **81**, 2962–2968.

[4] P.-P. Liebgott, F. Leroux, B. A. A. Burlat, S. A. Dementin, C. Baffert, T. Lautier, V. Fourmond, P. Ceccaldi, C. Cavazza, I. Meynial-Salles, P. Soucaille, J. C. Fontecilla-Camps, B. Guigliarelli, P. Bertrand, M. Rousset and C. Léger, *Nat. Chem. Biol.*, 2010, **6**, 63–70.

[5] A. M. Junelles, R. Janati-Idrissi, H. Petitdemange and R. Gay, *Curr. Microbiol.*, 1988, **17**, 299–303.

[6] F. Leroux, S. Dementin, B. Burlat, L. Cournac, A. Volbeda, S. Champ, L. Martin, B. Guigliarelli, P. Bertrand, J. Fontecilla-Camps, M. Rousset and C. Léger, *Proc. Nat. Acad. Sc. USA*, 2008, **105**, 11188–11193.

[7] P. Lindberg, P. Lindblad and L. Cournac, *Appl. Environ. Microbiol.*, 2004, **70**, 2137–2145.

[8] C. Léger, S. Dementin, P. Bertrand, M. Rousset and B. Guigliarelli, *J. Am. Chem. Soc.*, 2004, **126**, 12162–12172.

[9] C. Blanford and F. Armstrong, *Journal of Solid State Electrochemistry*, 2006, **10**, 826–832.

[10] C. Baffert, M. Demuez, L. Cournac, B. Burlat, B. Guigliarelli, P. Soucaille, P. Bertrand, L. Girbal and C. Léger, *Angew. Chem. Int. Edit.*, 2008, **47**, 2052–2055.

[11] V. Fourmond, K. Hoke, H. A. Heering, C. Baffert, F. Leroux, P. Bertrand and C. Léger, *Bioelectrochem.*, 2009, **76**, 141–147.