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Electronic supplementary information

New insights into the electron transfer mechanism within flavohemoglobins: tunnelling pathways, packing density, thermodynamic and kinetic analyses

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Protein expression and purification

FlavoHb of *R. eutropha* was expressed in *E. coli* BL21 strains and purified as described previously (El Hammi et al. 2011 Biochemistry). Static absorption spectrum was recorded at room temperature using an Uvikon dual-beam spectrophotometer. The sodium dithionite-reduced *minus* oxidized difference of absorption spectra were recorded between 350 and 650nm. Concentrated and purified FHP (80µM) was dialyzed overnight against 10 mM Formiate buffer. The anaerobic samples to be irradiated (typically 300 µl) contained 20 µM of FHP in formiate buffer (10 mM) N_2O-saturated. Experiments were made in triplicate.

Pulse radiolysis.

Pulse radiolysis measurements were performed as described elsewhere. Free radicals were generated by the application into an N_2O-saturated aqueous solution of a 0.2-0.8 µs pulses of high energy electrons (*ca.* 4 MeV) from the linear accelerator located at the Institut Curie, Orsay, France. Under such conditions, the radicals generated by water radiolysis are converted into carboxylate radicals.

The doses per pulse were calibrated from the absorption of the thiocyanate radical $(SCN)^{2\bullet-}$ obtained by radiolysis of the thiocyanate ion in N_2O-saturated solution ($[SCN^-] = 10^{-2}$ M, $G((SCN)^{2\bullet-}) = 0.55$ µmol. J^{-1}, $\varepsilon_{472\ nm} = 7580\ M^{-1}\ cm^{-1}$). The dose varies linearly with the pulse length, for instance a dose of ca. 5 Gy per pulse (0.2 µs long) resulted in ca. 2.8 µM of $O_2^{\bullet-}$.[1] Reactions were followed spectrophotometrically, using a Hamamatsu SuperQuiet xenon-mercury lamp (150 W) between 310

and 750 nm or a tungsten lamp between 450 and 750 nm, at 20 °C in a 2 cm path length fused silica cuvette. The Xenon lamp was not submitted to a surtension. In all the pulse radiolysis experiments, a cut-off filter cutting all wavelengths below 320 nm was positioned between the lamp and the cuvette. Kinetic traces were analyzed using a Levenberg-Marquardt algorithm from the Kaleidagraph® software package (Synergy Software).

$CO_2^{\bullet-}$ radicals was generated during the scavenging by formiate of the radiolytically produced hydroxyl radical, $HO^{\bullet}$.[2]

H_2O~ -> H, OH, e-aq, H2, H_2O_2

$H+HCOO^-$ -> H_2 + $COO^{\bullet-}$

$OH+HCOO^-$ -> H_2O + $COO^{\bullet-}$

e-aq + N_2O -> N_2 + O^-

O^-+ H^+ -> OH

The kinetic traces induced by a single pulse were obtained from measurements done at individual wavelengths between 400 and 650 nm.

Theoretical modeling

Various computational approaches are employed in this study: hybrid DFT/MM calculations for geometry optimizations of the heme and of the flavin cofactors as well as classical molecular dynamics simulations (MDS) for ET pathway analysis. Finally a combination of DFT and classical MDS for the evacuation of the driving force and reorganization energy is used.

Structure preparations: The starting structure of flavoHb is the crystallized form of the flavohemoglobin from *R. Eutropha* resolved at 1.75 Å (Protein Data Bank entry: 1CQX).[3] A phospholipid is present in the distal pocket of the heme group. The protein was hydrogenated with the HBUILD module of the CHARMM package (version 35)[4] and solvated in a box of 90x90x80 $Å^3$ of flexible SPC water molecules.[5] Sodium counter-ions were added to ensure electrical neutrality. The molecular systems were then relaxed by 1ns of molecular dynamics simulations and subsequent geometry optimizations to provide the starting geometries for DFT/MM optimizations and classical MDS-PM analysis.

QM/MM computations: we use the CUBY framework developed by Řezáč *et al.* to perform all the hybrid DFT/MM computations. Cuby interfaces the MM software CHARMM with the DFT program deMon2k.[6] The DFT computations are realized with the OPBE[7] functional in the unrestricted formalism with empirical corrections to account for dispersion effects (except on iron for which no parameters are available). A double dzeta with polarization functions basis set adapted for GGA functionals (DZVP-GGA) has been used on all atoms. The Dunning auxiliary basis sets GEN-A2 (for C, H and O) and GEN-A2* (for Fe and N, the star denotes the inclusion of f and g auxiliary functions) have been employed to expand the auxiliary electronic densities as employed in the so-called auxiliary DFT framework.[8] An adaptive DFT grid of fine accuracy[9] has been used for the numerical evaluations of the XC energy and potential and a multipolar expansion scheme has been employed to evaluate the DFT long range Coulomb integrals.[10] Population analysis of the DFT electronic densities are realized according to the iterative Hirshfeld scheme[11] (HPA-I) recently implemented in deMon2k. The HPA-I cycles were repeated until the atomic charges variation were below 0.0005 between two cycles.

The QM/MM protocol follows a subtractive scheme with electrostatic embedding which is achieved through the inclusion in the DFT calculation of the environmental point charges of the MM atoms situated in less than 10 Å from the QM atoms. The QM/MM boundaries are treated with the link atom technique.[12] The DFT/MM geometry optimizations are performed on MM optimized structures keeping frozen the atoms situated beyond a distance of 10 Å from the QM atoms. When optimizing the iron center the quantum region includes the entire porphyrin (including the priopionate groups), the metal ion and the His85 residue, that was cut at the level of the C_α-C_β bond. On the other hand when optimizing the flavin, the QM partition includes the isoalloxazine portion and the entire ribitol tail.

Pathway model and Packing Density analysis: 5ns of classical MDS have been realized within the Langevin approach and a time step of 0.5fs. The temperature was set to 300K. Snapshots were extracted from the MDS trajectories every 100 fs and then pruned to conserve only the residues suspected to be directly involved in the ET pathways. These are the Ile82, Ala82, Asn83, Lys84, His85, Ala86, Ser87, Leu88, Tyr190, Gln207, Glu394, Val395, Phe396 residues as well as the FAD and heme prosthetic groups. The phospholipid was also included in the pruned geometries. Finally all the water molecules whose oxygen atoms were localized within 8Å of the heme propionate A group were included in the PM and PD analysis.

DFT+MM computations: The driving force and the reorganization energy FAD_{red} + Fe(III) → FAD_{ox} + Fe(III) reactions were calculated following the approach detailed by Blumberger and co-workers (see for example Ref) Both the $FADH^{\bullet}$ → $FADH^{+}$ and the FAD^{-} → FAD oxidations are investigated. The inner-

sphere contributions are obtained from single point computations performed on the different cofactors optimized at DFT/MM level (see above). The outer-sphere contributions are obtained using the Linear Response Approximation expressions. First the vertical energy gap between the initial (Fe(III)/FAD_{red}) and the final (Fe(II)/FAD_{ox}) redox states is sampled from 5.4 ns MDS from Brownian MDS (using the Langevin approach) realized on the initial and final. Only the last 4.4ns were used for data accumulation.

$\Delta E^{init} = E^{init}_{final}(t) - E^{init}_{init}(t)$ is computed along an MDS performed on the initial state

$\Delta E^{final} = E^{final}_{final}(t) - E^{final}_{init}(t)$ is computed along an MDS performed on the final state.

E^a_b denotes the MM potential energy in state *b* computed on a molecular geometry corresponding to belonging to a NVT statistical ensemble corresponding to state *a*. To avoid any double counting with the inner-sphere contributions, the intra-molecular MM energy of the redox cofactors are retrieved from E^a_b . The outer-sphere contributions of driving force and of the reorganization energies are then computed according to:

$$\Delta G_o = \frac{1}{2}\left(\langle \Delta E^{init} \rangle + \langle \Delta E^{final} \rangle\right)$$

$$\lambda_o = \frac{1}{2}\left(\langle \Delta E^{init} \rangle - \langle \Delta E^{final} \rangle\right)$$

Table 1: Force field charges for the Fe(II) (triplet) and Fe(III) (quadruplet) complexes derived from the iterative Hirshfeld charges obtained on the OPB/MM optimized structure. The atom labeling corresponds to the heme atom definitions of the CHARMM force field.

	Fe(II)	Fe(III)		Fe(II)	Fe(III)		Fe(II)	Fe(III)
FE	1.204	1.5343	CAA	-0.1861	-0.2217	CMD	-0.5177	-0.5452
NA	-0.543	-0.6118	HAA1	0.1308	0.1426	HMD1	0.1473	0.168
NB	-0.5543	-0.6222	HAA2	0.1135	0.1331	HMD2	0.1665	0.1818
NC	-0.544	-0.6102	CBA	-0.5963	-0.5804	HMD3	0.158	0.1865
ND	-0.5748	-0.6439	HBA1	0.1564	0.1577	CAD	-0.1817	-0.223
C1A	0.2972	0.3117	HBA2	0.1677	0.1629	HAD1	0.138	0.1494
C2A	-0.0342	0.0167	CGA	1.0229	1.0144	HAD2	0.121	0.1438
C3A	0.0203	0.0453	O1A	-0.8999	-0.8774	CBD	-0.5889	-0.5663
C4A	0.2861	0.3123	O2A	-0.9019	-0.8863	HBD1	0.1533	0.1564
C1B	0.2888	0.3177	CMB	-0.5218	-0.5338	HBD2	0.1608	0.1563
C2B	0.0795	0.0873	HMB1	0.1461	0.1547	CGD	1.014	1.0023
C3B	-0.1253	-0.0818	HMB2	0.15	0.1566	O1D	-0.895	-0.8865
C4B	0.3259	0.3396	HMB3	0.1584	0.1678	O2D	-0.917	-0.8675
C1C	0.2672	0.2882	CAB	-0.0153	-0.0445	*His85:*		
C2C	0.0341	0.0598	HAB	0.1042	0.1209	CA	0.2245	0.2366
C3C	-0.1386	-0.097	CBB	-0.3938	-0.3478	CB	-0.6114	-0.6247
C4C	0.3065	0.3347	HBB1	0.1499	0.1494	HB1	0.2078	0.2154
C1D	0.2745	0.3104	HBB2	0.1314	0.1419	HB2	0.1743	0.1864
C2D	0.0366	0.0649	CMC	-0.503	-0.5262	ND1	-0.4959	-0.4871
C3D	-0.038	0.0238	HMC1	0.1289	0.1441	HD1	0.4952	0.5111
C4D	0.3103	0.3265	HMC2	0.1307	0.1522	CG	0.3414	0.3618
CHA	-0.4011	-0.3737	HMC3	0.1616	0.1817	CE1	0.2205	0.2507
HA	0.1787	0.1992	CAC	-0.0238	-0.058	HE1	0.0676	0.0751
CHB	-0.4038	-0.3992	HAC	0.0785	0.0964	NE2	-0.4321	-0.5231
HB	0.1562	0.1707	HD	0.1607	0.1778	CD2	-0.1338	-0.1123
CHC	-0.4436	-0.4319	CMA	-0.5243	-0.5439	HD2	0.1316	0.1367
HC	0.1521	0.1713	HMA1	0.1569	0.1702			
CHD	-0.4186	-0.4086	CBC	-0.3574	-0.296			
HMA2	0.1673	0.1848	HBC1	0.1574	0.1584			
HMA3	0.1419	0.1555	HBC2	0.1314	0.1462			

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