Electronic Supplementary Information

Orientation of the axial ligands and magnetic properties of the hemes in the cytochrome c_7 family from *Geobacter sulfurreducens* determined by paramagnetic NMR

Joana M. Dantas, Ivo H. Saraiva, Leonor Morgado, Marta A. Silva, Marianne Schiffer, Carlos A. Salgueiro*, Ricardo O. Louro,*

Assignment strategy of the heme α-substituents in the oxidized form

The assignment strategy of ^{13}C and ^{1}H heme α-substituents for paramagnetic multiheme cytochromes was firstly described for the tetraheme *Desulfovibrio vulgaris* (Hildenborough) cytochrome c_3 [20,24]. This strategy combines data from 2D-^{1}H-NOESY, 2D-^{1}H-TOCSY and 2D-^{1}H–^{13}C-HSQC NMR spectra and was also used to assign the heme α-substituents of triheme cytochromes PpcB, PpcC, PpcD and PpcE in the oxidized state. Briefly, in ^{1}H-^{13}C HSQC NMR spectrum, the heme α-substituents (heme methyls, α-propionates and thiother protons – see Fig. S1) appear in characteristic positions. This is illustrated in the 2D-^{1}H–^{13}C-HSQC NMR spectrum of PpcC (Fig S2). The signals of heme α-propionate CH_2 protons connected to the same carbon atom are easily identified in this spectrum (see straight lines in Fig. S2) and are the starting point of the assignment. In a first stage the scalar couplings between each pair of resonances are confirmed in the 2D-^{1}H-TOCSY spectra. Next, their NOE connectivities to heme methyls, observed in 2D-^{1}H-NOESY spectra, are used to identify the heme methyls $12^{1}CH_3$ or $18^{1}CH_3$ (see Fig. S1). One of these shows a NOE to heme methyl $2^{1}CH_3$ and allows distinguishing between the four heme methyls (Fig. S1). The NOE connectivities observed between the heme methyls $2^{1}CH_3$, $7^{1}CH_3$ and $12^{1}CH_3$ and thiother groups ($3^{1}CH$/ $8^{1}CH$ and $3^{2}CH_3$/$8^{2}CH_3$) are used to further confirm the assignment of the heme substituents. The connectivities observed for heme I of PpcC in 2D-^{1}H-TOCSY and 2D-^{1}H-NOESY are illustrated in Fig. S3. The final stage is the specific assignment of the heme groups in the structure and is achieved by examining the interheme NOE connectivities measured from the 2D-^{1}H-NOESY spectrum. These connectivites are illustrated in Fig. S4. The same type of connectivities is observed for PpcB, PpcC, PpcD and PpcE as the heme core is conserved in these proteins [6].

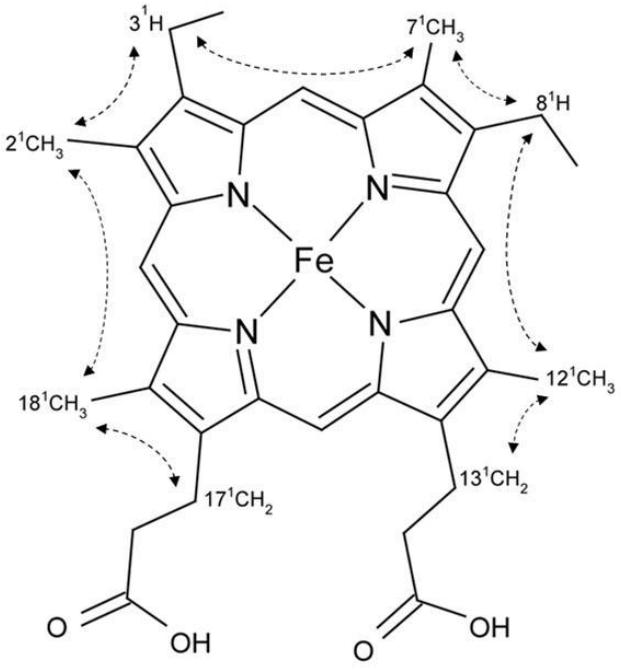

Fig. S1 Diagram of a heme *c* numbered according to the IUPAC-IUB nomenclature for the α-substituents. The dashed lines indicate the intraheme NOE connectivities between the heme α-substituents observed in the 2D-^{1}H-NOESY spectra of each cytochrome in the oxidized state

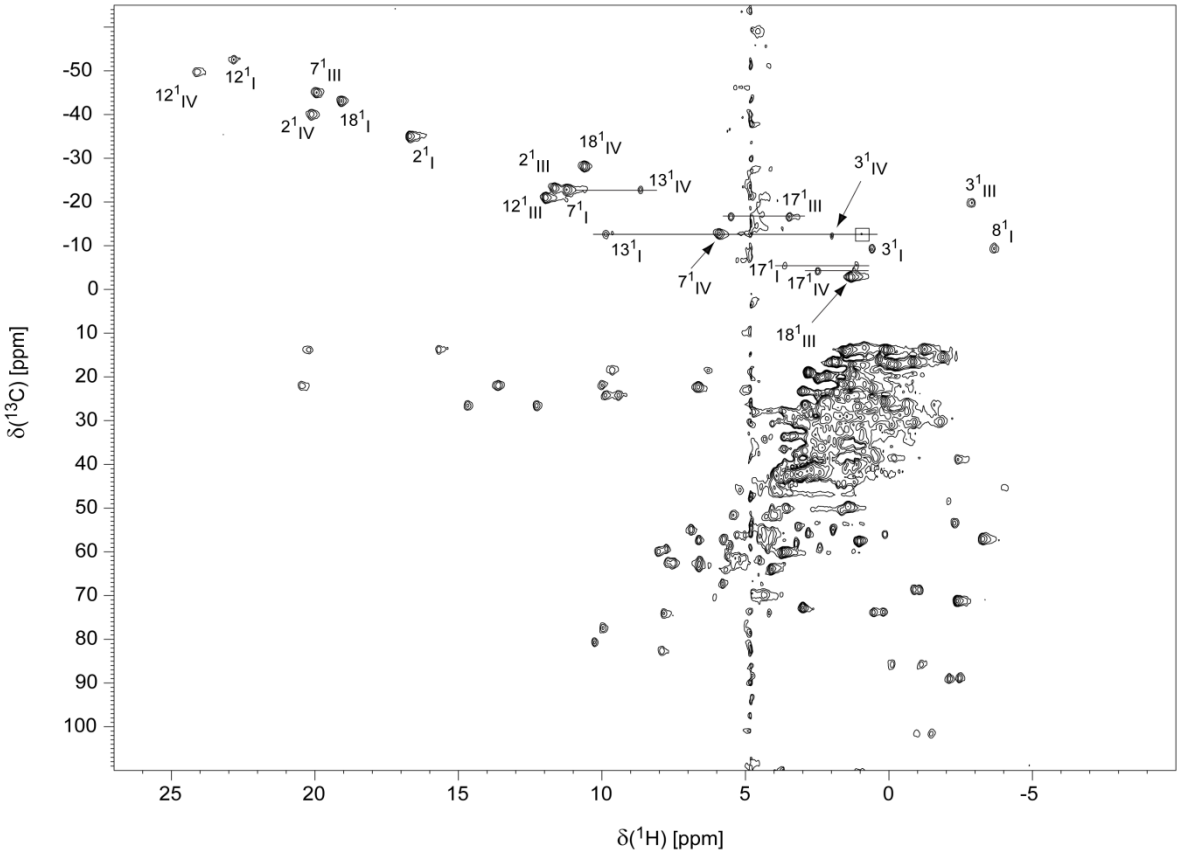

Fig. S2 ^{1}H-^{13}C HSQC spectrum of PpcC in the oxidized state at 298K and pH 7.1. Labels indicate the assigned heme α-substituents

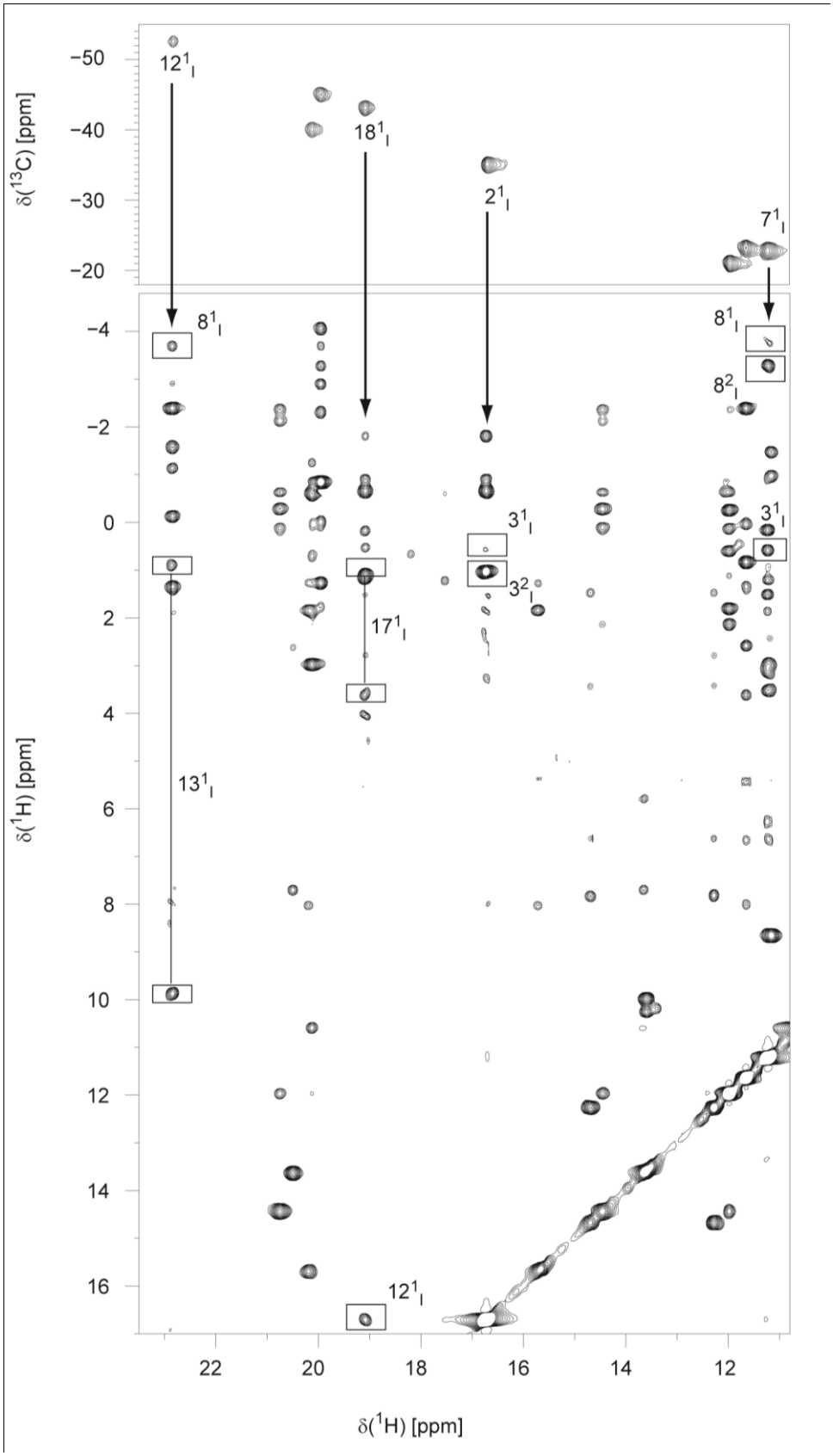

Fig. S3 Assignment of the PpcC heme I α-substituents in the oxidized state at 298K and pH 7.1. Expansion of the ^{1}H-^{13}C HSQC (top) and 2D-^{1}H NOESY (bottom) spectra. Arrows point

to proton frequency of heme I methyl groups in the 2D-^{1}H NOESY (see Fig. 2). Labeled cross-peaks indicate the intraheme NOE connectivities with each methyl group of heme I

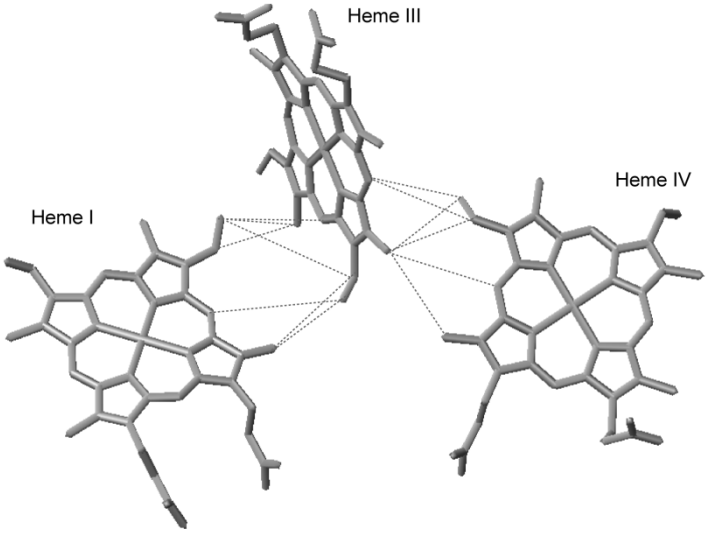

Fig. S4 Interheme NOE connectivities (dashed lines) used to specifically assign the heme groups in the structures. The heme core of PpcC is used in this figure as representative of the five cytochromes c_7 from *Geobacter sulfurreducens*

Table S1. Structural assignment of the ^{1}H and ^{13}C resonances (ppm) to the PpcB-E heme α-substituents at 289 and 298 K, pH 7.1. The unassigned resonances are indicated by '-'.

Protein	T (K)	Group	Heme I		Heme III		Heme IV	
			^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
PpcB	288K	2^1	-33.58	14.89	-26.31	13.18	-34.91	15.43
		3^1	-13.34	-0.45	-23.28	-2.41	-9.15	0.55
		7^1	-30.54	13.72	-37.27	15.91	-22.61	9.72
		8^1	-12.02	-3.89	-	-	-	-
		12^1	-45.27	17.82	-28.43	17.33	-44.29	19.58
		13^1	-17.81	1.22	-58.90	17.36	-19.44	5.13
				6.80		21.54		5.43
		17^1	-13.77	2.14	-16.86	3.96	-12.76	2.07
				-		4.28		3.14
		18^1	-40.84	17.21	-2.28	1.01	-37.01	14.15
	298K	2^1	-31.96	14.55	-25.22	13.22	-33.48	15.21
		3^1	-11.22	-0.19	-21.10	-1.99	-7.83	0.79
		7^1	-29.31	13.71	-35.58	15.81	-21.49	9.63
		8^1	-10.85	-3.43	-	-	-	-
		12^1	-43.27	17.38	-27.32	17.23	-42.71	19.30
		13^1	-16.11	1.31	-55.97	16.93	-18.16	5.26
				6.66		21.32		5.57
		17^1	-12.52	2.52	-14.95	4.14	-11.65	2.30
				5.12		4.42		3.32
		18^1	-40.06	17.40	-2.25	1.59	-35.76	14.08
PpcC	288K	2^1	-37.39	17.54	-24.03	11.71	-41.89	20.60
		3^1	-11.61	0.50	-21.45	-3.26	-13.41	1.79
		7^1	-22.65	10.62	-46.91	20.41	-13.14	5.69
		8^1	-9.84	-4.16	-	-	-	-
		12^1	-55.82	24.13	-21.57	11.99	-51.84	24.65
		13^1	-14.49	0.72	-	14.70	-24.43	8.85
				10.61		21.14		11.16
		17^1	-5.09	0.27	-17.81	3.37	-4.99	0.85
				3.21		5.51		2.24
		18^1	-43.58	18.63	-2.80	1.02	-29.04	10.48
	298K	2^1	-35.01	16.70	-23.11	11.65	-39.98	20.12
		3^1	-8.92	0.59	-19.74	-2.89	-11.76	1.99
		7^1	-22.73	11.23	-44.94	19.94	-12.60	5.91
		8^1	-9.26	-3.67	-	-	-	-
		12^1	-52.47	22.83	-20.91	11.97	-49.62	24.11
		13^1	-12.60	0.92	-	14.45	-22.70	8.66
				9.87		20.75		11.13
		17^1	-5.30	1.11	-16.48	3.47	-4.29	1.21
				3.63		5.50		2.48
		18^1	-43.02	19.08	-2.73	1.33	-28.07	10.61

Table 1 (continued)

Protein	T (K)	Group	Heme I		Heme III		Heme IV	
			^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
PpcD	288K	2^1	-56.79	26.78	-21.45	10.22	-31.22	12.79
		3^1	-	-	-27.95	-2.39	-5.90	-0.60
		7^1	-2.05	3.77	-38.61	15.94	-30.85	14.64
		8^1	-34.74	-0.79	-	-	-13.63	0.07
		12^1	-54.77	21.91	-28.44	16.43	-32.70	13.32
		13^1	8.29	-2.63	-62.07	15.68	-18.73	2.33
				3.69		22.45		5.82
		17^1	5.39	0.29	-16.99	0.03	-20.76	5.19
				3.86		7.64		5.85
		18^1	-54.43	25.64	0.94	-1.20	-40.63	17.15
	298K	2^1	-54.16	26.00	-20.22	10.13	-29.73	12.71
		3^1	-	-	-25.64	-2.07	-4.63	-0.31
		7^1	-1.83	3.97	-36.85	15.70	-29.24	14.37
		8^1	-32.25	-0.65	-	-	-11.72	0.36
		12^1	-52.51	21.40	-27.07	16.10	-31.44	13.37
		13^1	8.59	-2.40	-59.14	15.58	-17.32	2.70
				3.83		21.77		5.87
		17^1	5.86	0.61	-15.61	0.36	-19.13	5.32
				3.81		7.50		5.86
		18^1	-52.18	25.05	0.94	-0.78	-38.94	16.92
PpcE	288K	2^1	-32.15	13.51	-27.06	13.65	-34.40	15.49
		3^1	-9.58	-0.90	-26.29	-0.46	-10.55	0.34
		7^1	-31.64	14.19	-30.91	12.47	-22.12	9.38
		8^1	-16.72	-2.87	-	-	-	-
		12^1	-38.05	14.51	-37.23	20.22	-43.17	19.06
		13^1	-10.75	-0.97	-60.31	18.16	-20.47	3.81
				5.07		19.81		6.72
		17^1	-18.08	4.09	-14.74	2.18	-14.14	2.84
				7.32		4.28		-
		18^1	-45.35	20.28	-0.83	-0.81	-36.73	14.73
	298K	2^1	-30.67	13.26	-25.72	13.40	-32.79	15.15
		3^1	-7.88	-0.62	-24.14	-0.16	-9.01	0.56
		7^1	-30.35	14.07	-29.71	12.39	-21.37	9.48
		8^1	-15.25	-2.51	-	-	-	-
		12^1	-36.58	14.27	-35.94	19.86	-41.40	18.65
		13^1	-9.56	-0.77	-57.57	17.99	-19.19	3.96
				5.12		19.40		6.69
		17^1	-16.89	4.32	-13.64	2.34	-13.14	3.15
				7.36		4.37		5.04
		18^1	-43.97	20.13	-1.07	-0.26	-35.51	14.68