# Supplementary Material

# Protonation of the Cysteine axial ligand investigated in His/Cys c-type cytochrome by UV-Vis, mid- and Far-IR spectroscopy

Lidia Zuccarello, Catherine Berthomieu, Alain Boussac, Jean-Blaise Brubach, Irene Díaz-Moreno, Antonio J. Díaz Quintana, Rainer Hienerwadel

# **Experimental Section**

### Purification of c6wt and M58C

Cytochromes c6wt and M58C have been purified according to established protocol<sup>1</sup>.

Sample preparation

c6wt and M58C were concentrated to 0.2 to 3 mM in the final buffers Na-cacodylate 50 mM, NaCl 100 mM, pH 5.5; MES 50 mM, NaCl 100 mM, pH 6.5; Tris 50 mM, NaCl 100 mM, pH 7.5; or CAPS 50 mM, NaCl 100 mM, pH 10 and pH 11. Dilution/concentration steps on Amicon 10 kDa membranes were used to change the pH of samples or for the  $H_2O/^2H_2O$  exchange. For  $H_2O/^2H_2O$ exchange, the suitable buffer in  $H_2O$  was lyophilized using a speed-vacuum system and the dry pellet resuspended in  $^2H_2O$ .

## Spectro-Electrochemistry

Spectro-electrochemistry was performed using the three-electrode thin path-length cell <sup>2,3</sup> equipped with CaF<sub>2</sub> or CVD diamond windows. This cell is air-tight and allows recording spectra in the whole visible to Far-IR spectral region.

Ten  $\mu$ l of sample was deposited between the two CaF<sub>2</sub> or CVD diamond windows and the path-length of the closed electrochemical cell was adjusted to be less than 10  $\mu$ m by using the sample absorption at 1640 cm<sup>-1</sup>.

The following mediators were used to accelerate the redox reactions: methyl viologen ( $E_0$ : - 444 mV/NHE), benzyl viologen ( $E_0$ : - 358 mV), anthraquinone 2-sulfonate ( $E_0$ : - 223 mV), 2-hydroxy 1,4-naphtoquinone ( $E_0$ : - 123 mV), 2,5-dihydroxy p-benzoquinone ( $E_0$ : - 60 mV), duroquinone ( $E_0$ : + 10 mV), N-ethyldibenzopyrazine(ethyl)sulfonate ( $E_0$ : + 55 mV), methylphenazinium methyl salt ( $E_0$ : + 80 mV), 1,2-naphtoquinone 4-sulfonic acid ( $E_0$ : + 217 mV), diaminodurol ( $E_0$ : + 240 mV), tetramethylphenylene-diamine ( $E_0$ : + 270 mV), 2,5-dichloro 1,4-benzoquinone ( $E_0$ : + 300 mV) and ferricyanide ( $E_0$ : + 426 mV). The final concentration of each mediator was 40  $\mu$ M.

Redox titrations at equilibrium were performed by applying a series of external potentials with 20 mV intervals. Externally triggered potentiostats EG&G 262 or Tacussel Polarostat Type PGRE were used for the redox titrations. The applied potentials ranged from +550 mV to +110 mV *vs* NHE for c6wt at pH 7.5 and from -90 mV to -440 mV, from -190 mV to -540 mV and from -90 to -490 mV for M58C at pH 6.5, 7.5 and 10, respectively. Each potential was applied for 7 minutes before recording the UV-Vis spectra.

#### *UV-Vis spectrometry*

UV-Vis spectra were recorded using a Cary60 or Cary14 purged with dry air. The electrochemical cell was thermostated at  $15^{\circ}C \pm 0.1^{\circ}C$  with a water circulation system.

#### *FT-IR difference spectroscopy*

Mid-IR FTIR difference spectra were recorded using a Bruker Tensor 27 or a IFS88 spectrophotometer equipped with KBr beam splitters, MCT or DTGS detectors, and purged with dry air. Far-IR FTIR difference spectra were recorded using a vacuum-purged Bruker Vertex 70v spectrophotometer equipped with a helium-cooled Si-Bolometer and a 6  $\mu$ m Mylar beam splitter. The experiments were started after 30 minutes of equilibration at the fixed temperature and starting potential. Electrochemistry was performed at 10°C ± 0.1 °C. The sample was equilibrated for 15 minutes at oxidizing or reducing potentials before acquisition of the spectra. Each spectrum recorded during a single electrochemical cycle corresponds to the average of 300 scans at 4 cm<sup>-1</sup> resolution. The final reduced – *minus* - oxidized difference spectra correspond to the average of spectra obtained

with 10 to 20 electrochemical cycles in the Mid-IR and 20 to 30 electrochemical cycles in the Far-IR.

# FIGURES AND TABLES

# Figure S1 Titration curves for A) c6wt and B) M58C at pH 6.5 (black) and pH 10 (red).

All the fitted midpoint potentials with either n free or set to 1 are listed in the supplementary Table 1. Pannel A shows the fits for c6wt at pH 6.5 (Em = 342 mV, n=0.91) and at pH 10 (Em = 288 mV, n=1). Pannel B shows the fits for M58C at pH 6.5 (Em = -313 mV, n=0.70) and at pH 10 (Em=-399 mV, n=1, black line). For M58C at pH 10, the experimental data are reproduced satisfactorily by considering a model in which  $\approx 62\%$  of the cytochromes are in a dimeric form with an electrostatic interaction equal to 140 mV. The fit involving the dimeric interaction was done as detailed thereafter. This is a qualitative illustration of the effect of biomolecular interactions that seem to occur for part of the proteins at alkaline pH.



The effect of electrostatic interactions on the redox properties of hemes has been recognized by the work of Moore concerning the effect of propionates<sup>4</sup> or that of Gunner and Honnig<sup>5</sup> or, for example Alric et al.<sup>6</sup>, concerning the effect of heme interactions. To analyse the titration curve obtained at pH 10, we considered dimers of M58C in which the two hemes are equivalent. Em denotes the midpoint potential of one heme when the other heme is reduced, and X the interaction energy, so that the midpoint potential becomes Em + X in the presence of the oxidised partner.

We denote by (0), (1), (2) the fractions of dimers with 0, 1, 2 reduced hemes, respectively. At equilibrium with the ambient potential E, one has the equilibriums:

 $(1)/(0) = 10^{(E-Em)/60}$  and  $(2)/(1) = 10^{(E-(Em+X))/60}$ 

Solving these equations and noting that (0)+(1)+(2)=1, one obtains:

 $(0) = [10^{((2E-2Em-X)/60)}]/[1+10^{(E-Em)/60} + 10^{(2E-2Em-X)/60}]$ (1) = [10^{((E-Em)/60)}]/[1+10^{(E-Em)/60} + 10^{(2E-2Em-X)/60}] (2) = 1/[1+10^{(E-Em)/60} + 10^{(2E-2Em-X)/60}]

And the fraction of reduced heme is obtained from these expressions as:  $0.5 \times (1) + (2)$ .

In cases where there is only a proportion  $\alpha$  of the molecules which make a dimer and where the remaining fraction (1- $\alpha$ ) titrates normally, the fraction of reduced heme becomes:  $\alpha[0.5\times(1) + (2)]+(1-\alpha)[(1/1+(10^{n(E-Em)/60}))]$ 

Importantly, in this latter equation the same Em is used with and without the interaction. Moreover, in the case of the dimer, the n value is considered to be 1 and we can also fix n=1 in the second member of the equation for the fitting of the data something that is been done here.

**Supplementary Figure S2:** Effect of  $H_2O/^2H_2O$  exchange at pH 7.5 on the reduced – *minus* – oxidized FTIR difference spectra. Spectra in a: Reduced – *minus* – oxidized spectrum recorded with c6wt in H<sub>2</sub>O (grey line) and in <sup>2</sup>H<sub>2</sub>O (black line). Spectra in b: Reduced – *minus* – oxidized spectra recorded with M58C in H<sub>2</sub>O (grey line) and in <sup>2</sup>H<sub>2</sub>O (red line). Insert: comparison of the 1875-1775 cm<sup>-1</sup> region of the spectra recorded in <sup>2</sup>H<sub>2</sub>O with c6wt (black line) and M58C (red line). Spectra in c: H<sub>2</sub>O – *minus* – <sup>2</sup>H<sub>2</sub>O double difference spectra recorded with c6wt (black) and M58C (red).



Two main types of signals can be distinguished in the difference spectra recorded in the Mid-IR region. The first type consists in signals related to structural changes involving amide modes and side-chain modes of amino acids. The second set of bands comprises signals from the heme and its propionate groups as well as side-chain modes of the axial ligands.

The reduced – *minus* – oxidized difference spectrum recorded with c6wt in the Mid-IR shows bands at 1748(-)/1733(+) cm<sup>-1</sup> (in H<sub>2</sub>O, spectrum a thin grey line, see also Figure 2) downshifted by 12-10 cm<sup>-1</sup> to 1736(-)/1723(+) in <sup>2</sup>H<sub>2</sub>O (spectrum a, black line). Equivalent bands at 1746(-)/1732(+) cm<sup>-1</sup> for M58C (in H<sub>2</sub>O) are downshifted by 14-11 cm<sup>-1</sup> to 1732(-)/1721(+) in <sup>2</sup>H<sub>2</sub>O (spectrum b, red line). The frequency of these bands and the shift in <sup>2</sup>H<sub>2</sub>O are characteristic for protonated carboxylic group. They can be assigned either to protonated Asp or Glu side chain <sup>7</sup> or to one of the two propionic groups of the heme by comparison with literature data <sup>8</sup>. Importantly, these carboxylic groups are protonated both for M58C<sub>RED</sub> and M58C<sub>OX</sub> and cannot account for the pH dependence of the M58C midpoint potential.

The reduced - minus - oxidized difference spectrum recorded with c6wt shows also bands at 1691(- $\frac{1}{1668(+)}$  cm<sup>-1</sup>, 1656(-)/1646(+) cm<sup>-1</sup> and 1629(-)/1607(+) cm<sup>-1</sup> (spectrum a thin grey line, see also Figure 2). Equivalent bands are observed at slightly higher frequencies in the spectrum recorded with M58C, at 1696(-)/1676(+) cm<sup>-1</sup>, 1660(-)/1647(+) cm<sup>-1</sup> and 1632(-)/1612(+) cm<sup>-1</sup>, respectively (spectrum b thin grey line, Figure 2). The effect of  $H_2O/^2H_2O$  exchange on these bands consists in a downshift by 2 cm<sup>-1</sup> to 7 cm<sup>-1</sup> in the spectrum recorded with c6wt (spectrum a black line), except for the band at 1632 (-) which seems absent in  ${}^{2}H_{2}O$ . The effect of  $H_{2}O/{}^{2}H_{2}O$  exchange on these bands is slightly less important in M58C, with a downshift by a maximum of 5 cm<sup>-1</sup> in <sup>2</sup>H<sub>2</sub>O (spectrum b, red line). Shifts by less than 10 cm<sup>-1</sup> are expected upon  $H_2O/^2H_2O$  exchange for amide v(C=O) modes of the protein backbone, *i.e.* up to 10 cm<sup>-1</sup> for  $\alpha$ -helix and less than 5 cm<sup>-1</sup> for  $\beta$ -sheet secondary structures <sup>9-11</sup>. Therefore, the bands discussed above are assigned to peptide carbonyl stretching modes, indicating that contributions from the polypeptide backbone are dominating the spectra. Possible contributions from other functional groups of the protein, as amino acid side-chain modes from Arg or Lys, Asn or Gln, also expected in this spectra range <sup>7</sup>, cannot be distinguished directly. Therefore, we calculated a  $H_2O - minus - {}^2H_2O$  double-difference spectrum for c6wt (spectrum c black line) and for M58C (spectrum c, red line). In these spectra, only bands affected by  $H_2O/^2H_2O$ exchange contribute. In addition to the effects already discussed for the protonated carboxylic group at circa 1730 cm<sup>-1</sup> or for the polypeptide backbone, that gives rise to thin difference bands, the H<sub>2</sub>O  $-minus - {}^{2}H_{2}O$  spectra reveal a negative band at 1630 cm<sup>-1</sup> for c6wt and a positive band at 1689 cm<sup>-1</sup> <sup>1</sup> for M58C. These bands probably do not correspond to contributions from peptide carbonyl groups (note that the apparent intensity of these bands is due to their superimposition with contributions from the peptide carbonyl IR –shifts). The band at 1630 cm<sup>-1</sup> has no clear counterpart at lower frequency. Therefore, and according to literature, this band is either the IR mode of a lysine side chain in  $H_2O$ (the counterpart in  ${}^{2}H_{2}O$  is expected as a very small band at circa 1200 cm<sup>-1</sup>, which could not be detected) or the IR mode of a Gln or Asn side chain in <sup>2</sup>H<sub>2</sub>O <sup>7,12</sup>. In this case, the IR mode in H<sub>2</sub>O is expected in the 1687-1668 cm<sup>-1</sup> range, and could contribute at circa 1670 cm<sup>-1</sup> in spectrum c (black line). According to the structure of c6wt, the side chains of Lys29 and Asn56 are hydrogen bonded to propD, while the side chain of Gln50 is hydrogen bonded to the 6<sup>th</sup> heme axial ligand Met58 (Scheme 1, <sup>1</sup>). The IR bands could correspond to one of these amino acid side chains. At these frequencies, the spectrum recorded with c6wt and M58C are clearly different. A positive band is detected at 1689 cm<sup>-1</sup> for M58C<sub>RED</sub> in H<sub>2</sub>O and the counterpart in  ${}^{2}$ H<sub>2</sub>O is not clear. This band could be downshifted to 1641-1630 cm<sup>-1</sup> in <sup>2</sup>H<sub>2</sub>O. The spectral differences between the spectra recorded with c6wt and M58C strongly suggest that the IR bands correspond to the contribution of Gln50 in the reduced – minus – oxidized spectra. Indeed, while the interactions formed by the propionate group ProD seem highly conserved in the structures recorded with c6wt and M58C, those formed by Gln50 with the 6<sup>th</sup> ligand are expected to be different in c6wt and M58C, and affected by the redox state of the heme iron (Scheme 1). For M58C<sub>RED</sub>, a strong – and specific - interaction between Cys58 SH and the carbonyl group of Gln50 side chain is also inferred from the low frequency of the v(SH) mode of Cys58, identified at 2513 cm<sup>-1</sup> (Figure 2 insert, see below) as compared to literature (see below).

The H<sub>2</sub>O – *minus* – <sup>2</sup>H<sub>2</sub>O spectrum reveals small negative bands at 1556 cm<sup>-1</sup> (c6wt) or 1546 cm<sup>-1</sup> (M58C) and positive ones at 1436 cm<sup>-1</sup> (c6wt) and 1450 (M58C). They are interpreted as a downshift by 100-120 cm<sup>-1</sup> of the bands at 1556 or 1546 cm<sup>-1</sup> upon H<sub>2</sub>O/<sup>2</sup>H<sub>2</sub>O exchange, since this shift is typically that expected for the Amide II IR mode of the polypeptide backbone (<sup>12</sup> and references therein).

For c6wt, the negative band detected at 1556 cm<sup>-1</sup> in the H<sub>2</sub>O – *minus* – <sup>2</sup>H<sub>2</sub>O spectrum superimposes with positive ones in H<sub>2</sub>O, which are not sensitive to the H<sub>2</sub>O/<sup>2</sup>H<sub>2</sub>O exchange. Bands at 1576(-) and 1553-48(+) cm<sup>-1</sup> in H<sub>2</sub>O, which remain at 1571(-)/1555(+) cm<sup>-1</sup> in <sup>2</sup>H<sub>2</sub>O, are assigned to  $v(C_{\beta}C_{\beta})$  redox sensitive heme mode by comparison with previous experiments with the model compound microperoxidase and literature data <sup>13-19</sup>. A second difference band at 1259(-)/1239(+) cm<sup>-1</sup>, observed only in the c6wt spectrum and insensitive to H<sub>2</sub>O/<sup>2</sup>H<sub>2</sub>O exchange is assigned to the v42 ( $\delta C_m$ H) heme mode. The intensity of this mode was reported to be a marker for a six-coordinated LS Fe(II) heme <sup>18</sup>.

The Mid-IR region also contains contributions from side-chain modes of the heme axial ligands.

The v(SH) stretching mode of protonated Cys side-chains contributes in a spectral region, where no other protein contributions are expected, *i.e. at circa* 2550 cm<sup>-1</sup> and experiences a large downshift by

circa 700 cm<sup>-1</sup> upon H/<sup>2</sup>H exchange <sup>20</sup>. The positive band observed for M58C<sub>RED</sub> at 2513 cm<sup>-1</sup> is thus assigned to the v(SH) mode of protonated Cys58 in M58C<sub>RED</sub>, since it is the only Cys residue not involved in covalent bonding interaction with the heme. This band is downshifted by 680 cm<sup>-1</sup> to 1833 cm<sup>-1</sup> in <sup>2</sup>H<sub>2</sub>O (Spectrum a insert). The band frequency at 2513 cm<sup>-1</sup> is *circa* 40 cm<sup>-1</sup> below that reported for Cys in solution <sup>20</sup>. The frequency of the v(SH) mode is very sensitive to the interactions formed by the thiol group and its environment. Observed at circa 2585 cm<sup>-1</sup> in CCl<sub>4</sub>, this mode is identified at lower frequencies ranging from 2575-2580 cm<sup>-1</sup> to 2525-2560 cm<sup>-1</sup> for thiol groups acting as weak to strong hydrogen bond donors <sup>21,22</sup>. Hydrogen bonding interactions have been shown to shift the v(SH) mode by 20-40 cm<sup>-1</sup> in organic thiols placed in hydrogen bonding solvents or thiol hydrogen bonded to water molecule <sup>23-28</sup>. In Li and Thomas <sup>21</sup>, the lowest frequency value of v(SH) = 2528 cm<sup>-1</sup> was reported for a glutathione crystal, in which the thiol group was involved in four strong SH<sub>500</sub>O hydrogen bonding interactions. The Cys58 v(SH) frequency at 2519 cm<sup>-1</sup> is at the lower range of these frequency values. It probably indicates that besides a very strong interaction of Cys58 SH with Gln50 side chain, an electrostatic interaction still persists between the sulphur and the heme Fe(II).

The difference signal at 1113(+)/1103(-) cm<sup>-1</sup> in M58C difference spectrum [1118(+)/1103(-) cm<sup>-1</sup> for c6wt] is assigned with confidence to the v(C<sub>5</sub>N<sub>1</sub>) stretching mode of the imidazole side-chain of His18, the 5<sup>th</sup> axial ligand of the heme iron by comparison with literature data <sup>13,29-32</sup>. We and others have previously shown that i) this IR mode is enhanced when the histidine imidazole side-chain is a ligand of a metal (including heme iron), ii) the frequency of this mode is sensitive to the metal (heme iron) oxidation state, and iii) the frequency of this mode is also sensitive to the protonation state of the imidazole side-chain. These previous studies allow us to conclude that the 5<sup>th</sup> His ligand in c6wt and M58C is "neutral", i.e. with an imidazole (and not imidazolate) side-chain both for the reduced and oxidized forms.

**Supplementary Figure S3:** Effect of pH on the reduced – *minus* – oxidized difference spectra recorded with M58C in the 1800-1000 cm<sup>-1</sup> range. Spectrum in black is recorded in H<sub>2</sub>O at pH 5.5 (in cacodylate buffer), spectrum in green at pH 6.5 (in MES buffer), spectrum in red at pH 7.5 (in TRIS buffer), spectrum in blue at pH 10 (in CAPS buffer). Insert: 2650-2450 cm<sup>-1</sup> spectral range of the reduced – *minus* – oxidized FTIR difference spectra.



The reduced – *minus* – oxidized difference spectra recorded with M58C at pH 5.5, pH 6.5 and pH 7.5 are very similar, except for a slight upshift of the band at 1630 to1633 cm<sup>-1</sup>, small differences at circa 1550 cm<sup>-1</sup> and larger ones at 1067(-)/1043(+) cm<sup>-1</sup> at pH 7.5. These later bands in the spectrum recorded at pH 7.5 are contributions from the TRIS buffer, as shown in Figure S4. At pH 10 and pH 10.5, a general trend consists in a slight upshift of bands assigned to peptide carbonyl groups (Amide I) and NH+CN (Amide II) modes. These bands, ascribed to small redox-induced structural changes

of the protein backbone are not largely modified by pH. The difference signal from the histidine axial ligand at 1113(+)/1105(-) cm<sup>-1</sup> remains at the same frequency in the whole pH range, indicating that the His18 side-chain remains neutral (imidazole) in the whole pH range. The positive band at circa 1688 cm<sup>-1</sup>, tentatively assigned to the Gln50 side-chain, is absent at pH 10. The difference band at 1746(-)/1732(+) cm<sup>-1</sup> is also almost completely absent at pH 10, while a new band is observed at 1583 cm<sup>-1</sup>, in the range where deprotonated carboxylate groups are expected to contribute <sup>7</sup>. These data are consistent with the deprotonation of the redox-sensitive aspartic or glutamic acid or heme propionic group at pH 10. The pH has also an important influence on bands from the heme and its cysteine axial ligand. At alkaline pH, the positive band at 2519-2513 cm<sup>-1</sup> assigned to the Cys v(SH) mode is not detected. A positive band at 1241(+) cm<sup>-1</sup> is assigned to the v<sub>42</sub>( $\delta C_m H$ ) heme mode, characteristic of a 6c LS Fe(II) heme.

#### Supplementary Figure S4: Effect of M58C reduction on Tris buffer

Superimposition of the reduced – *minus* – oxidized FTIR difference spectrum recorded with M58C in TRIS buffer at pH 7.5 (black) with a difference spectrum recorded with the TRIS buffer only at pH 8.8 - minus - TRIS buffer at pH 7.3 (blue) in the 1800-1000 cm<sup>-1</sup> range.



The spectrum in blue corresponds to the deprotonation of a fraction of the TRIS molecules at pH 8.8 as compared to pH 7.3. The difference band at 1066(-)/1038(+) cm<sup>-1</sup> is due to the change in properties of the v(C-OH) IR mode of TRIS associated with the deprotonation of the amine group upon pH increase. IR bands of TRIS buffer also contribute at 1580-1500 cm<sup>-1</sup>. Superimposition of the spectra recorded with M58C at pH 7.5 and that recorded with the TRIS buffer (blue) clearly indicates that the reduction of M58C is associated with a deprotonation of TRIS molecules. This most probably corresponds to a proton transfer from TRIS to reduced M58C. Hence, the following chemical reaction can be written:

 $Fe^{3+} + TRIS - H \rightarrow Fe^{2+} + TRIS$ 

This chemical equation is coupled with the proton uptake upon reduction that occurs in M58C in the pH range, where a pH dependence of its midpoint potential exists.

$$Fe^{3+} + e^{-} + R^{-} + H^{+} \rightarrow Fe^{2+} + RH$$
$$Fe^{3+} + TRIS - H \rightarrow Fe^{2+} + TRIS + H^{+}$$

**Supplementary Table S1:** Midpoint potentials of c6wt and M58C. Potentials are expressed in mV *versus* NHE

	(	e6wt	M58C		
	n=1	n=1 n variable n=1		n variable	
Em (pH 6.5)	<b>.5)</b> 342 mV 342 mV, n=0.91		-312 mV	-313 mV, n=0.70	
Em (pH 7.5)	339 mV	339 mV, n=1.05	-375 mV	-375 mV, n=1.0	
Em (pH 10.0)	<b>10.0)</b> 288 mV 288 mV, n=1		-399 mV*	-413 mV, n=0.33	

Fits of the experimental data have been done from the amplitudes of the Soret band, by using solver or Excel. The fits have been done using a Nernst curve either by fixing the n value to 1 (n=1) or by letting the value free (n variable). No significant differences have been found except for M58C at pH 10.

\*For M58C at pH 10, the data could not be fitted by a Nernst curve with n=1. This likely originates from an electrostatic interaction between hemes of different cytochromes at this high pH value. This problem has been theoretically treated in the case of a dimer as detailed in supplementary Figure S1.

**Supplementary Table S2:** Proposed assignments of M58C bands in the Mid-IR difference spectra; shifts in <sup>2</sup>H<sub>2</sub>O are given under brackets.

рН 5.5		рН 6.5		рН 7.5		pH 10		Proposed
Ox	Red	Ox	Red	Ox	Red	Ox	Red	assignments
	2519		2519		2513 (-680)			ν(Cys-SH)
1746	1732	1746	1732	1746 (-14)	1732 (-11)	1746	1733	COOH Asp/Glu or propionic acid
1696	1676	1696	1677	1696 (-5)	1677 (-3)	1698	1679	Amide I, turn
	1688		1688		1688			Gln v(C=O)
1660	1647	1660	1647	1660 (-3)	1648 (-4)	1661	1649	Amide I
1632	1611	1630	1612	1633 (-4)	1612 (-1)	1636	1616	Amide I
							1583	COO <sup>-</sup> Asp/Glu or propionate
1546	1556	1545	1557	1545 (-95)	1557 (-87)	1549	1563	Amide II
							1534	
							1241	v <sub>42</sub> (δC <sub>m</sub> H) 6c-LS
1104	1114	1104	1113	1104 (0)	1113 (0)	1104	1113	His $\nu(C_5N_1)$
				1038	1066			v(C-O) of TRIS

**Supplementary data S3:** Detailed assignment of the redox-sensitive Far-IR modes of c6wt and M58C.

The difference band at 604(+)/581(-) cm<sup>-1</sup> in the spectrum recorded with c6wt (Figure 2) appears upshifted by 14-12 cm<sup>-1</sup> in the spectrum recorded with M58C. This difference band is not present in the spectrum recorded at alkaline pH (Figure 2). The origin of this band is not clear since literature data are scarce in this spectral range. However, correlation with changes in the Mid-IR allows to propose a contribution of the  $\delta$ (OCO) mode of the protonated carboxylic groups from Asp or Glu or from an heme propionate. Far-IR contributions from carboxylate modes were reported between 480 and 650 cm<sup>-1</sup> <sup>33</sup> and strong IR contributions have been reported for protonated carboxylic acids <sup>34</sup>. Since difference bands are observed, the IR mode is present in the oxidized and reduced forms, which shows that there isn't any change in protonation state of the chemical group upon change of the iron redox state of c6wt or M58C. This group does not explain the pH dependence of the heme iron midpoint potential.

The frequency of IR bands of  $c6wt_{OX}$  at 380 cm<sup>-1</sup> (-2), 361 cm<sup>-1</sup> (+2), 317 cm<sup>-1</sup> (-2) and 269 cm<sup>-1</sup> (-3) and the counterpart for  $c6wt_{RED}$  at 388 cm<sup>-1</sup> (-2), 371 cm<sup>-1</sup> (0) and 244 cm<sup>-1</sup> (+2) are almost unchanged in M58C (shift under brackets). Bands at similar frequencies were observed in the electrochemically-induced FTIR difference spectra recorded with cyt *c* <sup>35</sup>. These bands are thus in common between cyt *c*, c6wt and M58C and characteristic of *c*-type hemes with at least one histidine axial ligand of the iron. According to IR spectra of metalloporphyrins, intense bands contribute in the 420-350 cm<sup>-1</sup> region. These bands were assigned to in plane Fe-pyrrole stretching modes (v(Fe-N4<sub>Pyr</sub>), modes v8 and v50 and oop tilting/folding), likely coupled to Fe-ligand vibrations <sup>15,36-38</sup>. The v8 and/or v50 modes could contribute at 388-360 cm<sup>-1</sup>. IR active heme oop modes associated to pyrrole tilting, v6 reported at circa 363 cm<sup>-1</sup>, and methine wagging (C<sub>a</sub>C<sub>m</sub>)  $\gamma$ 7 reported at 315 cm<sup>-1</sup> or the pyrrole tilting v51, v(C<sub>β</sub>C<sub>1</sub>)<sub>as</sub> B2u mode reported at 318 cm<sup>-1</sup> could contribute in the FTIR spectra of both c6wt and M58C. Finally, the bands at 266(-) cm<sup>-1</sup> and 244-246(+) cm<sup>-1</sup> are tentatively assigned to the corresponding v52(vC<sub>β</sub>C<sub>1</sub>)<sub>s</sub> mode, i.e.  $\delta$ (C<sub>β</sub>Y) moiety vibration involving propionate groups by comparison with RR data <sup>37,38</sup>.

#### References

(1) Diaz-Moreno, I.; Hulsker, R.; Skubak, P.; Foerster, J. M.; Cavazzini, D.; Finiguerra, M. G.; Diaz-Quintana, A.; Moreno-Beltran, B.; Rossi, G. L.; Ullmann, G. M.; Pannu, N. S.; De la Rosa, M. A.; Ubbink, M. The Dynamic Complex of Cytochrome c(6) and Cytochrome f Studied with Paramagnetic NMR Spectroscopy. *Biochim. Biophys. Acta -Bioenergetics* **2014**, *1837*, 1305-1315.

Bernardina, S. D.; Alabarse, F.; Kalinko, A.; Roy, P.; Chapuis, M.; Vita, N.; Hienerwadel, R.;
Berthomieu, C.; Judeinstein, P.; Zanotti, J. M.; Bantignies, J. L.; Haines, J.; Catafesta, J.; Creff, G.; Manceron,
L.; Brubach, J. B. New Experimental Set-Ups for Studying Nanoconfined Water on the AILES Beamline at
SOLEIL. *Vib. Spectrosc.* 2014, *75*, 154-161.

(3) Moss, D.; Nabedryk, E.; Breton, J.; Mantele, W. Redox-Linked Conformational-Changes in Proteins Detected by a Combination of Infrared-Spectroscopy and Protein Electrochemistry - Evaluation of the Technique with Cytochrome-C. *Eur J Biochem* **1990**, *187*, 565-572.

(4) Moore, G. R. Control of Redox Properties of Cytochrome c by Special Electrostatic Interactions. *Febs Lett* **1983**, *161*, 171-175.

(5) Gunner, M. R.; Honig, B. Electrostatic Control of Midpoint Potentials in the Cytochrome Subunit of the *Rhodopseudomonas viridis* Reaction Center. *Proc. Natl. Acad. Sci. U S A* **1991**, *88*, 9151-9155.

(6) Alric, J.; Tsukatani, Y.; Yoshida, M.; Matsuura, K.; Shimada, K.; Hienerwadel, R.; Schoepp-Cothenet, B.; Nitschke, W.; Nagashima, K. V. P.; Vermeglio, A. Structural and Functional Characterization of the Unusual Triheme Cytochrome Bound to the Reaction Center of *Rhodovulum sulfidophilum*. *J. Biol. Chem*. **2004**, *279*, 26090-26097.

(7) Venyaminov, S. Y.; Kalnin, N. N. Quantitative Ir Spectrophotometry of Peptide Compounds in
Water (H2o) Solutions .1. Spectral Parameters of Amino-Acid Residue Absorption-Bands. *Biopolymers* 1990, 30, 1243-1257.

(8) Mileni, M.; Haas, A. H.; Mantele, W.; Simon, J.; Lancaster, C. R. D. Probing Heme Propionate Involvement in Transmembrane Proton Transfer Coupled to Electron Transfer in Dihemic Quinol : Fumarate Reductase by C-13-Labeling and FTIR Difference Spectroscopy. *Biochemistry* **2005**, *44*, 16718-16728.

(9) Chirgadze, Y. N.; Brazhnikov, E. V. Intensities and Other Spectral Parameters of Infrared Amide Bands of Polypeptides in Alpha-Helical Form. *Biopolymers* **1974**, *13*, 1701-1712.

(10) Chirgadze, Y. N.; Shestopalov, B. V.; Venyaminov, S. Y. Intensities and Other Spectral Parameters of Infrared Amide Bands of Polypeptides in Beta- and Random Forms. *Biopolymers* **1973**, *12*, 1337-1351.

(11) Venyaminov, S. Y.; Kalnin, N. N. Quantitative Ir Spectrophotometry of Peptide Compounds in Water (H2o) Solutions .2. Amide Absorption-Bands of Polypeptides and Fibrous Proteins in Alpha-Coil, Beta-Coil, and Random Coil Conformations. *Biopolymers* **1990**, *30*, 1259-1271.

(12) Barth, A. Infrared Spectroscopy of Proteins. *Biochim. Biophys. Acta Bioenergetics* 2007, 1767, 1073-1101.

(13) Berthomieu, C.; Boussac, A.; Mantele, W.; Breton, J.; Nabedryk, E. Molecular-Changes Following Oxidoreduction of Cytochrome-B559 Characterized by Fourier-Transform Infrared Difference Spectroscopy and Electron-Paramagnetic Resonance - Photooxidation in Photosystem-II and Electrochemistry of Isolated Cytochrome-B559 and Iron Protoporphyrin-Ix-Bisimidazole Model Compounds. *Biochemistry* **1992**, *31*, 11460-11471.

(14) Boucher, L. J.; Katz, J. J. Infared Spectra of Metalloporphyrins (4000-160 Cm-1). J. Am. Chem. Soc. **1967**, *89*, 1340-1345.

(15) Desbois, A. Resonance Raman-Spectroscopy of C-Type Cytochromes. *Biochimie* **1994**, *76*, 693-707.

(16) Hu, S. Z.; Mukherjee, A.; Piffat, C.; Mak, R. S. W.; Li, X. Y.; Spiro, T. G. Modeling the Heme Vibrational Spectrum: Normal-Mode Analysis of Nickel(II) Etioporphyrin-I from Resonance Raman, FT-Raman, and Infrared Spectra of Multiple Isotopomers. *Biospectroscopy* **1995**, *1*, 395-412.

(17) Kitagawa, T., Osaki Y.: *Infrared and Raman spectra of metalloporphyrins.*, 1987; Vol. 64. pp.71.

(18) Marboutin, L.; Boussac, A.; Berthomieu, C. Redox Infrared Markers of the Heme and Axial Ligands in Microperoxidase: Bases for the Analysis of c-type Cytochromes. *J Biol Inorg Chem* **2006**, *11*, 811-823.

(19) Oshio, H.; Ama, T.; Watanabe, T.; Kincaid, J.; Nakamoto, K. Structure Sensitive Bands in the Vibrational-Spectra of Metal-Complexes of Tetraphenylporphine. *Spectrochim Acta A* **1984**, *40*, 863-870.

(20) Susi, H.; Byler, D. M.; Gerasimowicz, W. V. Vibrational Analysis of Amino-Acids - Cysteine, Serine, Beta-Chloroalanine. *J Mol Struct* **1983**, *102*, 63-79.

(21) Li, H. M.; Thomas, G. J. Cysteine Conformation and Sulfhydryl Interactions in Proteins and Viruses .1. Correlation of the Raman S-H Band with Hydrogen-Bonding and Intramolecular Geometry in Model Compounds. *J Am Chem Soc* **1991**, *113*, 456-462.

(22) Raso, S. W.; Clark, P. L.; Haase-Pettingell, C.; King, J.; Thomas, G. J. Distinct cysteine sulfhydryl environments detected by analysis of Raman S-H markers of Cys -> Ser mutant proteins. *J Mol Biol* **2001**, *307*, 899-911.

(23) Becker, B., Felcyn, E.W., Herman, A., Pikies, J., Wojnowski, W. Schwingungsspektroskopische Untersuchungen der Acidität von Silanthiolen; quantitative Zusammenhänge zwischen der Struktur und den Dv(SH)-, HNP- und pK-Werten. *Z. anorg. allg. Chem.* **1982**, *488*, 229-234.

(24) Mielcarek, A.; Dolega, A. Weak Hydrogen Bonding Interaction S-H Center Dot center dot O=C studied by FT-IR Spectroscopy and DFT calculations. *J Mol Struct* **2016**, *1103*, 217-223.

(25) Bhattacherjee, A.; Matsuda, Y.; Fujii, A.; Wategaonkar, S. Acid-Base Formalism in Dispersion-Stabilized S-H Center dot Center dot center dot Y (Y=O,S) Hydrogen-Bonding Interactions. *J. Phys. Chem. A* **2015**, *119*, 1117-1126.

(26) David, J. G.; Hallam, H. E. Infra-Red Solvent Shifts + Molecular Interactions .8. Acidity of Thiophenols. *T Faraday Soc.* **1964**, *60*, 2013-&.

(27) David, J. G.; Hallam, H. E. Hydrogen-Bonding Studies of Thiophenols. *Spectrochim. Acta* **1965**, *21*, 841-&.

(28) Kieninger, M.; Ventura, O. N. Calculations of the Infrared and Raman Spectra of Simple Thiols and Thiol-Water Complexes. *Int. J. Quantum Chem.* **2011**, *111*, 1843-1857.

(29) Dupeyrat, F.; Vidaud, C.; Lorphelin, A.; Berthomieu, C. Long Distance Charge Redistribution upon Cu,Zn-Superoxide Dismutase Reduction - Significance for Dismutase Function. *J. Biol. Chem.* **2004**, *279*, 48091-48101.

(30) Hasegawa, K.; Ono, T.; Noguchi, T. Ab Initio Density Functional Theory Calculations and Vibrational Analysis of Zinc-bound 4-Methylimidazole as a Model of a Histidine Ligand in Metalloenzymes. *J Phys. Chem. A* **2002**, *106*, 3377-3390.

Hienerwadel, R.; Berthomieu, C. Bicarbonate Binding to the Non-Heme Iron of Photosystem
II Investigated by Fourier Transform Infrared Difference Spectroscopy and C-13-Labeled Bicarbonate.
*Biochemistry* 1995, *34*, 16288-16297.

(32) Noguchi, T.; Inoue, Y.; Tang, X. S. Structure of a Histidine Ligand in the Photosynthetic Oxygen-Evolving Complex as Studied by Light-Induced Fourier Transform Infrared Difference Spectroscopy. *Biochemistry-Us* **1999**, *38*, 10187-10195.

(33) Matei, A.; Drichko, N.; Gompf, B.; Dressel, M. Far-Infrared Spectra of Amino Acids. *Chem. Phys.* **2005**, *316*, 61-71.

(34) Umemura, J. Vibrational-Spectra, Normal Coordinate Analyses, and Molecular Configurations of Crystalline Propionic-Acid. *J. Mol. Struct.* **1977**, *36*, 35-54.

(35) Berthomieu, C.; Marboutin, L.; Dupeyrat, F.; Bouyer, P. Electrochemically induced FTIR difference spectroscopy in the mid- to far infrared (200 mu m) domain: A New Setup for the Analysis of Metal-Ligand Interactions in Redox Proteins. *Biopolymers* **2006**, *82*, 363-367.

(36) Othman, S.; Lelirzin, A.; Desbois, A. A Heme C-Peptide Model System for the Resonance Raman-Study of C-Type Cytochromes - Characterization of the Solvent-Dependence of Peptide-Histidine-Heme Interactions. *Biochemistry* **1993**, *32*, 9781-9791.

(37) Hu, S. Z.; Smith, K. M.; Spiro, T. G. Assignment of Protoheme Resonance Raman Spectrum by Heme Labeling in Myoglobin. *J. Am. Chem. Soc.* **1996**, *118*, 12638-12646.

(38) Li, X. Y.; Czernuszewicz, R. S.; Kincaid, J. R.; Su, Y. O.; Spiro, T. G. Consistent Porphyrin Force-Field .1. Normal-Mode Analysis for Nickel Porphine and Nickel Tetraphenylporphine from Resonance Raman and Infrared-Spectra and Isotope Shifts. *Journal of Physical Chemistry* **1990**, *94*, 31-47.