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pH and Ionic Strength Effects on Electron Transfer Rate Constants and Reduction Potentials of the Bacterial Di-Heme Protein *Pseudomonas stutzeri* Cytochrome c_4

Jens J. Karlsson, Thomas E. Rostrup and Jens Ulstrup†

Chemistry Department A, Building 207, The Technical University of Denmark, DK-2800 Lyngby, Denmark

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We have investigated the ionic strength (0.1–0.5 M NaCl) and pH dependence (4.0–7.5) of the electron transfer (ET) rate constants for oxidation and reduction of the bacterial di-heme protein cytochrome c_4 (cyt c_4; *Pseudomonas stutzeri*, ATCC No. 11607) by $[Co(bipy)_3]^{3+/2+}$ (bipy = 2,2′-bipyridine). The kinetics is bi- or tri-phasic, and a mechanism based on cooperative ET at both hemes, slow intramolecular ET and electrostatically dominated inter-heme interaction is presently best in line with all the available data.

The ionic strength and pH dependence of the rate constants and reduction potentials is weak. The rate constants mostly decrease by 0–50% in the ionic strength range 0.1–0.5 M. The *macroscopic* potentials decrease by < 10 mV. Three of the *microscopic* potentials increase by 10–25 mV, while the fourth one decreases by 50 mV, but the accuracy of the microscopic reduction potential values is low. There is no pH dependence of the rate constants in the range 6.0–7.5, but most rate constants drop to half the 6.0–7.5 value in the range 4.0–6.0, leaving the reduction potentials almost unaffected.

The small effects are unexpected in view of the highly charged and strongly dipolar character, and the many hydrogen bond contacts of cyt c_4. These small effects must be related to the detailed rather the overall charge distribution of cyt c_4.

Multi-centre metalloprotein organization is one of the most important structural features in respiratory and photosynthetic electron transfer (ET).[1–4] This has two functional implications in particular. One is to ensure facile long-range ET by electronic coupling between individual metallic centres.[3,5–9] The other is that electrostatic and electronic–vibrational coupling associated with electron or hole insertion at a given centre induces physical changes in other centres, poising thermodynamically and electronically the protein for positive or negative multi-ET cooperativity.[10–15]

While long-range single-ET has been broadly investigated, cooperative multi-centre metalloprotein dynamics is characterized in much less detail. Cooperative dioxygen equilibria of hemoglobin,[16] and cytochrome c_3 ET dynamics and redox Bohr effects[10,12,17] are probably the systems where characterization incorporates most details. Reasons for these limitations are the prohibitively large number of microscopic interactions. Two-centre proteins would be attractive alternatives in this respect. Compared with the four-centre proteins the number of microscopic reduction potentials is reduced from 32 to 4, and the number of rate constants (oxidation *or* reduction) from 80 to 6. Kinetic resolution of two-centre proteins is thus within reach, whereas this prospective is far too remote for more composite proteins.

Bacterial, plant and mammalian two-centre proteins are encountered in photosynthetic and respiratory ET chains.[18–21] With a view on cooperative ET we have recently reported UV/VIS spectral and kinetic analysis of the ET reactions of the bacterial di-heme protein *Pseudomonas stutzeri* cyt c_4.[22] Both the sequence[23] and the 2.2 A X-ray crystallographic structure of this protein[24,25] have also become available. The structure and ET dynamics provide the following intriguing view:

(1) The kinetics is bi- or tri-phasic with different amplitudes. The most important mechanisms in line with these observations are discussed below.

(2) Tri-phasic ET is presently best in line with the data, and *electrostatically* dominated interaction potentials emerge from the rate constants.

(3) The sequence homology between cyt c_4 from *A. vinelandii*, *P. aeruginosa* and *P. stutzeri* is high (ca. 80%).[23]

(4) The structure has two domains, each holding a heme

† To whom correspondence should be addressed.

group.[24,25] The heme groups are in hydrogen-bond contact via a single propionate, and a hydrogen-bond network between the domains can be distinguished. The heme groups are organized in a non-coplanar fashion, with a tilt angle of 30°, not favourable for intramolecular ET.

(5) The molecule is dipolar, the *N*-terminus holding approximately four excess negative, and the *C*-terminus one excess positive charge.

(6) Both heme groups are axially coordinated to His and Met. Met-coordination is unusual, inducing low-spin/high-spin equilibrium in the oxidized state.[26] This could be a reason for slow intramolecular ET, as the local reorganization energy would be large.

We provide here ionic strength and pH profiles of the rate constants for oxidation and reduction of *P. stutzeri* cyt c_4 by $[Co(bipy)_3]^{3+/2+}$ (bipy = 2,2′-bipyridine) and the microscopic reduction potentials of the two heme groups. Notable effects might have been anticipated from the dipolar cyt c_4 structure. The unexpected result is, however, that these effects are in fact quite small.

Experimental

Reagents were of highest purity available. Millipore water (Milli-Q-Housing) was used throughout. $[Co(bipy)_3](ClO_4)_3$ was prepared and characterized by reported procedures.[27] $[Co(bipy)_3]^{2+}$ was prepared *in situ* by mixing $CoCl_2$ with slightly more than the equivalent amount of ligand. pH was adjusted by acetate (20 mM, $4.0 < pH < 5.0$), MES (2-[*N*-morpholino]ethanesulfonic acid, 20 mM, $5.5 < pH < 7.0$) and Tris (tris(hydroxymethyl)aminomethane, 20 mM, $pH > 7.0$) and the ionic strength, *I*, by NaCl.

Ps. stutzeri (ATCC No. 11607, DSM Braunschweig No. 50227) was grown and cyt c_4 isolated, purified and characterized as described in a previous report[22] (cf. Ref. 28). Yields were 20–27 mg cyt c_4/200 g wet cell paste. Absorption peaks of the α-, β- and Soret bands of reduced cyt c_4 were at 551, 522 and 415 nm, respectively. The α/β absorbance ratio was 1.2 and the 700 nm absorption coefficient was 1.700 M^{-1} cm^{-1}, referred to 44.400 M^{-1} cm^{-1} for reduced cyt c_4 at 550 nm.

Absorption spectra were recorded on a Milton Roy diode array spectrophotometer using quartz cells. Kinetics for cyt c_4(II,II) oxidation and cyt c_4(III,III) reduction was followed on a High-Tech SF53 stopped-flow instrument, using the OLIS (On-Line Instrument Systems, Jefferson, GA) software. The cyt c_4 concentration was in the range 0.1–2.0 μM and followed mostly at 420 nm, where the absorption change is largest. The inorganic reaction partners were always in a least 25-fold excess. Concentrations up to 400 μM were used. This is eight times higher than in the previous report, but with no indication of saturation kinetics. Completion of both cyt c_4(II,II) oxidation and cyt c_4(III,III) reduction could be achieved, and bi- or tri-exponential kinetics fitted to the data. The reduction potentials could be obtained from the rate constants for reduction and oxidation.

Results and discussion

The UV/VIS spectra followed the pattern previously reported.[22] This extends to the three-component α-band, indicative of specific heme group environments.

The reduction potential of $[Co(bipy)_3]^{3+/2+}$ is 310 mV (NHE)[29] and located between the midpoint potentials of the two heme groups (cf. below). The kinetics of both reactions can be fitted to either bi- or tri-exponential kinetics (cf. above), and numerical distinction is not presently feasible. All phases are proportional to the Co-complex concentration, and the amplitude of the slower phase in biexponential analysis about twice the amplitude of the faster phase. These observations are in line with one of the following *two* mechanisms[22] (cf. above):

(A) Parallel ET at each heme group and fast (on the stopped-flow timescale, i.e. $> 10^2$ s^{-1}) intramolecular redox equilibrium. This mechanism gives biexponential kinetics with different amplitudes. Fast intramolecular ET is not, however, observed by NMR.[26]

(B) Parallel, cooperative ET of each heme group. This implies that the intermolecular ET rate constants of a given heme group depends on the oxidation state of the other heme group, as in the following scheme (shown for oxidation):

$$h^1_{II}h^2_{II} + Co(III) \xrightarrow{k_{1a}} h^1_{III}h^2_{II} + Co(II)$$

$$h^1_{II}h^2_{II} + Co(III) \xrightarrow{k_{2a}} h^1_{II}h^2_{III} + Co(II)$$

$$h^1_{II}h^2_{III} + Co(III) \xrightarrow{k_{1b}} h^1_{III}h^2_{III} + Co(II)$$

$$h^1_{III}h^2_{II} + Co(III) \xrightarrow{k_{2b}} h^1_{III}h^2_{III} + Co(II)$$

where h^1 and h^2 are the two heme groups and the Roman numerals indicate the oxidation state. This scheme gives triexponential kinetics if $k_{1a} \neq k_{1b}$ and $k_{2a} \neq k_{2b}$ owing to electrostatic (and conformational) interactions when an electron or a hole is inserted on the second heme group.

Tables 1 and 2 summarize the second-order rate constants at different ionic strengths in the range 0.1–0.5 M, pH 7.5 (20 mM Tris) for bi- and tri-exponential analysis. Table 3 shows the macroscopic reduction potentials determined from the ratio between the forward and reverse rate constants in the biexponential analysis, and the reduction potential of $[Co(bipy)_3]^{3+/2+}$. The ionic strength variation of the reduction potential of the latter couple was assumed to follow that of the analogous $[Co(phen)_3]^{3+/2+}$ (phen = 1,10-phenanthroline).[30,31]

Table 1. Second-order rate constants ($M^{-1}\,s^{-1}$) at different ionic strengths *I*(M, NaCl) for oxidation and reduction of cyt c_4 by $[Co(bipy)_3]^{3+/2+}$ [a]

	$[Co(bipy)_3]^{3+}$		$[Co(bipy)_3]^{2+}$	
I	k_1	k_2	k_{-1}	k_{-2}
0.1	$(7.8\pm0.7)\times10^4$	$(1.3\pm0.2)\times10^4$	$(9.3\pm0.5)\times10^3$	$(2.8\pm0.3)\times10^4$
0.2	$(6.2\pm0.6)\times10^4$	$(9.7\pm0.8)\times10^3$	$(8.2\pm0.7)\times10^3$	$(2.4\pm0.3)\times10^3$
0.3	$(4.1\pm0.5)\times10^4$	$(8.6\pm0.8)\times10^3$	$(9.3\pm0.7)\times10^3$	$(2.9\pm0.3)\times10^4$
0.5	$(5.6\pm0.5)\times10^4$	$(9.0\pm0.9)\times10^3$	$(1.0\pm0.1)\times10^4$	$(2.0\pm0.3)\times10^4$

[a] pH 7.5 (20 mM Tris). [cyt c_4]=0.1–1 μM. Co-complex concentrations in the range 60–400 μM. Biphasic kinetic analysis.

Table 2. Second-order rate constants ($M^{-1}\,s^{-1}$) at different ionic strengths *I*(M NaCl) for oxidation and reduction of cyt c_4 by $[Co(bipy)_3]^{3+/2+}$ [a]

	$[Co(bypy)_3]^{3+}$			
I	k_{1a}	k_{2a}	k_{1b}	k_{2b}
0.1	$(3.1\pm0.5)\times10^4$	$(1.3\pm0.3)\times10^4$	$(3.7\pm0.5)\times10^4$	$(4.3\pm0.5)\times10^4$
0.2	$(1.9\pm0.4)\times10^4$	$(0.9\pm0.2)\times10^4$	$(3.4\pm0.5)\times10^4$	$(6.0\pm0.8)\times10^4$
0.3	$(1.3\pm0.4)\times10^4$	$(0.7\pm0.2)\times10^4$	$(1.9\pm0.4)\times10^4$	$(6.4\pm0.8)\times10^4$
	$[Co(bipy)_3]^{2+}$			
I	k_{-1a}	k_{-2a}	k_{-1b}	k_{-2b}
0.1	$(0.6\pm0.2)\times10^4$	$(1.6\pm0.4)\times10^4$	$(0.9\pm0.2)\times10^4$	$(0.9\pm0.3)\times10^4$
0.2	$(1.3\pm0.3)\times10^4$	$(2.8\pm0.5)\times10^4$	$(1.0\pm0.3)\times10^4$	$(0.9\pm0.3)\times10^4$
0.3	$(1.0\pm0.2)\times10^4$	$(3.0\pm0.5)\times10^4$	$(1.0\pm0.3)\times10^4$	
0.5	$(1.8\pm0.4)\times10^4$	$(2.6\pm0.5)\times10^4$	$(1.0\pm0.3)\times10^4$	$(1.0\pm0.3)\times10^4$

[a] pH 7.5 (Tris). [cyt c_4]=0.1–1 μM. Co-complex concentrations in the range 60–400 μM. Triphasic fits and kinetic analysis as in Ref. 22.

Table 3. Midpoint potentials, E_1° and E_2° (mV, NHE) of the two heme groups of cyt c_4 at different ionic strengths calculated from the data in Table 1.

I	E_1°	E_2°
0.1	256	330
0.2	251	326
0.3	263	332
0.5	255	319

Table 4 summarizes the microscopic reduction potentials determined from triexponential analysis. Interaction potentials calculated from the data in Table 4[22] are electrostatically dominated but the values, ranging from 0 to 80 mV, show considerable scatter and cannot be associated with a particular ionic strength dependence. The following other observations are appropriate:

(1) The rate constants and reduction potentials at pH 7.5, $I=0.1$ M follow closely the reported values.[22] The absence of saturation kinetics up to 400 μM (cf. above) is notable in view of the negative charge accumulation in the *C*-terminal domain of cyt c_4.

(2) The rate constants for cyt c_4 oxidation decrease slightly overall, i.e. by 20–50% with increasing ionic strength. Those for cyt c_4 reduction are mostly unaffected, but k_{-1a} in the triphasic pattern seems to increase notably (a factor of two or three) in the ionic strength range 0.1–0.5 M. The midpoint potential variations are insignificant. The overall effects are much smaller than anticipated from the high negative protein charge or the strongly dipolar charge distribution. The overall ionic strength effects on the biphasic rate constants have also been noted to be small in a previous report.[32]

(3) The rate constants for biphasic and triphasic kinetic analysis in the pH range 4.0–7.5 ($I=0.1$ M, NaCl) are summarized in Tables 5 and 6. The pH dependence is notable but small in view of the large number of negatively charged side groups and hydrogen bond contacts between the two cyt c_4 domains. The faster biphasic rate constant for cyt c_4 oxidation and reduction is independent of pH in the range 6.0–7.5 but drops to about half

Table 4. Microscopic reduction potentials (mV, NHE) of the two heme groups of cyt c_4 at different ionic strengths *I*, calculated from the data in Table 2.

I	e_1^{ox}	e_2^{ox}	e_1^{red}	e_2^{red}
0.1	269	316	273	269
0.2	292	332	271	254
0.3	294	339	284	219

[a] The subscripts refer to a given heme group and the superscript to the oxidized ('ox') or reduced ('red') state of the other heme group.

Table 5. Second-order rate constants ($M^{-1}\ s^{-1}$) at different pH for oxidation and reduction of cyt c_4 by $[Co(bipy)_3]^{3+/2+}$ (I=0.1 M, NaCl)[a].

	$[Co(bipy)_3]^{3+}$		$[Co(bipy)_3]^{2+}$	
pH	k_1	k_2	k_{-1}	k_{-2}
7.5	(7.8±0.7) ×10⁴	(1.3±0.2) ×10⁴	(0.9±0.1) ×10⁴	2.8±0.3) ×10⁴
7.0	(6.9±0.7)×10⁴	(1.2±0.2) ×10⁴	(0.8±0.1) ×10⁴	(1.7±0.2) ×10⁴
6.5	(7.1±0.7) ×10⁴	(1.2±0.2) ×10⁴	(0.8±0.1) ×10⁴	(2.0±0.2)×10⁴
5.5	(6.0±0.6) ×10⁴	(1.1±0.2) ×10⁴	(0.7±0.1) ×10⁴	(1.4±0.2) ×10⁴
5.0	(5.2±0.6) ×10⁴	(1.0±0.1) ×10⁴	(0.7±0.1)×10⁴	(1.4±0.2)×10⁴
4.5	(2.8±0.3)×10⁴	(0.7±0.1)×10⁴	(0.4±0.1)×10⁴	(1.2±0.2)×10⁴
4.0	(2.9±0.3)×10⁴	(0.6±0.1)×10⁴	(0.5±0.1)×10⁴	(1.0±0.1)×10⁴

[a][cyt c_4]=0.1–1 μM. Co-complex concentrations in the range 60–600 μM. Biphasic kinetic analysis.

Table 6. Second-order rate constants ($M^{-1}\ s^{-1}$) at different pH for oxidation and reduction of cyt c_4 by $[Co(bipy)_3]^{3+/2+}$ (I=0.1 M, NaCl).[a]

	$[Co(bipy)_3]^{3+}$			
pH	k_{1a}	k_{2a}	k_{1b}	k_{2b}
7.5	(3.1±0.5)×10⁴	(1.3±0.3)×10⁴	(3.7±0.5)×10⁴	(4.3±0.5)×10⁴
7.0	(1.8±0.4)×10⁴	(1.0±0.2)×10⁴	(4.5±0.5)×10⁴	(6.9±0.8)×10⁴
5.5	(2.0±0.4)×10⁴	(1.0±0.2)×10⁴	(4.1±0.5)×10⁴	(4.2±0.5)×10⁴
5.0	(1.7±0.3)×10⁴	(0.9±0.2)×10⁴	(2.7±0.3)×10⁴	(3.4±0.4)×10⁴
4.5	(1.3±0.3)×10⁴	(0.6±0.1)×10⁴	(2.0±0.3)×10⁴	(2.9±0.4)×10⁴
4.0	(0.8±0.2)×10⁴	(0.5±0.1)×10⁴	(1.2±0.3)×10⁴	(3.2±0.4)×10⁴
	$[Co(bipy)_3]^{2+}$			
pH	k_{-1a}	k_{-2a}	k_{-1b}	k_{-2b}
7.5	(0.6±0.1)×10⁴	(1.6±0.2)×10⁴	(0.9±0.2)×10⁴	(0.9±0.2)×10⁴
7.0	(0.6±0.1)×10⁴	(2.2±0.3)×10⁴	(0.9±0.2)×10⁴	(0.8±0.1)×10⁴
6.5	(0.6±0.1)×10⁴	(0.9±0.2)×10⁴	(0.8±0.1)×10⁴	(0.8±0.1)×10⁴
5.5	(1.1±0.2)×10⁴	(1.6±0.2)×10⁴	(0.8±0.1)×10⁴	(0.9±0.2)×10⁴
5.0	(0.6±0.1)×10⁴	(1.8±0.3)×10⁴	0.7±0.1)×10⁴	(0.5±0.1)×10⁴
4.5	(1.0±0.2)×10⁴	(1.1±0.2)×10⁴	(0.6±0.1)×10⁴	(0.7±0.1)×10⁴
4.0	(0.5±0.1)×10⁴	(0.6±0.1)×10⁴	(0.5±0.1)×10⁴	(0.5±0.1)×10⁴

[a][cyt c_4]=0.1–1 μM. Co-complex concentrations in the range 60–400 μM. Triphasic fits and kinetic analysis as in Ref. 22.

the pH value in the range 4.0–6.0. In addition to protonation equilibria in the side groups and the hydrogen-bond network this could be caused by ligand substitution of axially coordinated Met. The reactions could not be followed at higher pH owing to hydrolysis of $[Co(bipy)_3]^{2+}$. Neither the slower biphasic rate constants nor the macroscopic midpoint potentials showed any pH dependence in the whole range. A somewhat similar pattern emerged from triphasic kinetic analysis where, however, all the rate constants decreased smoothly by 30–50% when pH was lowered from 7.5 to 4.0. Except for e_1^{ox} the microscopic reduction potentials, summarized in Table 7, are largely independent of pH. The interaction potentials are electrostatically dominated, in the range 0–60 mV, and with no systematic pH variation.

The following concluding observations are appropriate. Slow intramolecular ET in spite of close heme group proximity is unexpected. This could be rooted in unfavourable heme group orientation, hydrogen-bond reorganization, and particularly in the high-spin/low-spin equilibrium[26] which would induce notable axial ligand group reorganization. ET kinetics using more slowly electron exchanging reaction partners, and cyclic voltammetry using immobilized cyt c_4 could hold clues to the intramolecular rate constants: such investigations are in progress.

The weak pH dependence indicates that the hydrogen-bond network and propionate contact are robust in the pH range 5.0–7.5. Collective protonation of the negatively charged residues appears to occur only at lower pH

Table 7. Microscopic reduction potentials (mV, NHE) of the two heme groups of cyt c_4 at different pH, calculated from the data in Table 6, with the notation as in Table 4.

pH	e_1^{ox}	e_2^{ox}	e_1^{red}	e_2^{red}
7.5	269	316	273	269
7.0	283	332	269	254
5.5	294	321	269	269
5.0	281	329	276	262
4.5	302	325	279	271
4.0	296	318	289	265

where the protein is unstable. In this respect the protein resembles the plant plastocyanins, for which the ET profiles are dominated by single protonation,[31,33] presumably at copper-coordinated His in spite of the substantial negative charge accumulation on the remote side of the protein.

The unexpectedly small ionic strength effects must be viewed in relation to details of the charge distribution. Individual charge interactions on protein surfaces are, moreover, always strongly screened owing to dielectric image forces which can increase the effective dielectric constant to values well above that of bulk water.[34–36] In addition, although the protein is overall negatively charged and highly dipolar, inspection of the structure shows that the effects of the many local negative and positive charges are likely to cancel to a considerable extent. It thus appears that the small ionic strength effects can be traced to the moderate overall charge of the protein and the fact that all the positive and negative charges are close enough to the ET sites to cause a substantial congestion of their individual effects.

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