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Relationship between hydrogen-bonding network and reduction potential in *c*-type cytochromes

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c-Type cytochromes are biological electron shuttles involved in cyclic redox processes in many different organisms [1]. The midpoint redox potential of mitochondrial cytochromes *c* is about +260 mV, while it is usually about 100 mV higher in chloroplast cytochromes c_6, and it varies from +250 to +450 mV in bacterial cytochromes c_2. Determinations of local structural differences among the *c*-type cytochromes are of interest to investigate the factors controlling the reduction potentials of these soluble water proteins. Consequently, there has been considerable crystallographic work done in detecting structural changes between reduced and oxidised *c*-type cytochromes. The high-resolution determinations of several *c*-type cytochromes in both oxidation states have revealed that the conformational differences between the reduced and oxidised state are very small. The differences are manifested for the most part in terms of thermal parameter changes, small adjustments around the heme moiety and movement of internal water molecules, rather than in terms of explicit polypeptide chain shifts, which instead are found to be minimal. A buried water molecule located at the heme binding pocket near to the iron-bonded Met has been found in almost all the X-ray structures of eukaryotic and prokaryotic *c*-type cytochromes. The positional change of this water molecule is the most prominent structural difference observed with the change of the iron oxidation state in the eukaryotic cytochromes *c*.

In a recent contribution to this journal, S. Sogabe and K. Miki [2] reported a similar change for the analogous water molecule found in the bacterial *Blastochloris viridis* cytochrome c_2, which presents a reduction potential of +285 mV, close to that of eukaryotic cytochromes *c*. The authors suggested that this water molecule, found in various *c*-type cytochromes, plays an important role in adjusting the redox potential with alteration of the surrounding hydrogen-bond network. However, in order to confirm the role of the internal water molecule in the modulation of reduction potential, it is highly desirable to compare the structures of *c*-type cytochromes having a significantly different midpoint redox potential.

Recently, we have determined the X-ray structures of the oxidised and reduced forms of the cytochrome c_2 from *Rhodopseudomonas palustris* [3]. These are the first high-resolution crystal structures of a cytochrome c_2 exhibiting high redox potential (+365 mV), in both redox states. In all oxidised forms of *c*-type cytochromes with low reduction potential this water molecule is found in a position different from that found in the reduced forms. On the contrary, in the oxidised form of cytochrome c_2 from *R. palustris*, this water molecule is detected in a position close to that found in the reduced form. This is clearly shown in Fig. 1 where the structural comparison reported in fig. 4 of the Sogabe and Miki paper has been updated by adding the oxidised and reduced forms of cytochromes c_2 from *R. palustris*. Since thermodynamic studies suggest that the higher potential of bacterial cytochromes, such as *R. palustris* cytochrome c_2, appears to be determined mainly by enthalpy contributions [4], we have performed a rough evaluation of the electrostatic interactions (Table 1) between the buried water molecule and its H-bonded amino acids for the *c*-type cytochromes reported in Fig. 1. The shortening of the H-bond distances in the oxidised forms of *tuna* cytochrome *c* and *B. viridis* cytochrome c_2, with respect to those of the corresponding reduced forms (Table 1), leads to a stabilisation in energy of 4.1 and 2.5 kcal/mol, respectively. This gain in energy, mainly due to the positional change of the water molecule, corresponds to a decrease in the reduction potential of about 180 mV for the cytochrome *c*,

Table 1
H-bond distances (Å) of the buried water molecule detected in the reduced and oxidised forms of prokaryotic cytochromes c_2, from *R. palustris* and from *B. viridis*, and of eukaryotic cytochrome *c* from *tuna*

	PDB code	Resolution	B factor	Distance from the buried water molecule			
				Tyr OH	Asn ND2[a]	Thr OG1	E_{tot}
R. palustris (ox)[b]	1fj0	1.7	10.2 (2)	2.79 (4)	2.99 (2)	2.90 (2)	−16.6 (1)
R. palustris (red)[b]	1i8p	1.9	9.1 (11)	2.87 (6)	3.01 (5)	2.81 (2)	−17.2 (3)
B. viridis (ox)	1io3	2.0	16.4	2.70	2.72	2.72	−18.5
B. viridis (red)	1co6	1.6	11.2	2.85	3.03	2.90	−16.0
Tuna (ox)	3cyt	1.8	20.1	2.62	2.84	2.64	−21.4
Tuna (red)	5cyt	1.5	12.2	2.87	2.96	2.92	−17.3

These cytochromes, which have the same residues (Tyr, Asn, Thr) involved in the H-bond network with the buried water molecule, have been chosen in order to allow a homogeneous comparison. The resolution limit (Å) of the structures, the B factors (Å^2) of the water molecule, and the electrostatic contribution (kcal/mol) of the interactions between this water molecule and the three amino acids are also reported. The electrostatic contribution was calculated using the HyperChem programme. The H atoms were added to Tyr, Thr, and Asn residues and to the water molecule at calculated positions. The electrostatic interaction, modelled by a Coulombic interaction of atom-centred point charges, was calculated between the atoms of the water molecule and the atoms of a single amino acid residue with a dielectric constant $\varepsilon = 1$. The charges used were taken from Amber force field.
[a]OD1 in oxidised form of *tuna* cytochrome *c*.
[b]Mean values of four crystallographically independent molecules with standard error of the mean reported in brackets.

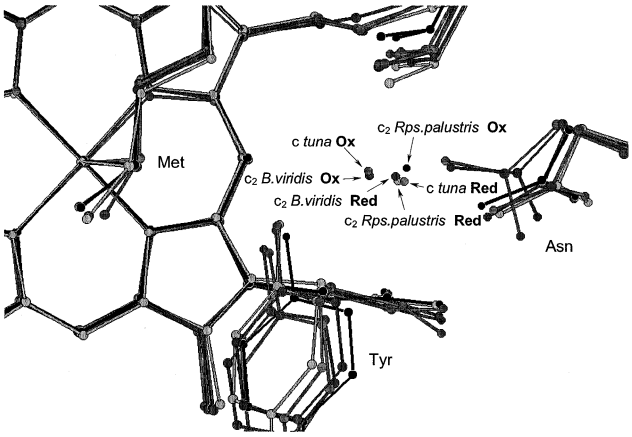

Fig. 1. View of the superimposition of the local environment around the buried water molecule located at the heme binding pocket near to the Met ligand, of the reduced and oxidised forms of eukaryotic cytochrome *c* from *tuna* and of prokaryotic cytochromes c_2 from *B. viridis* and *R. palustris*.

and 110 mV for the cytochrome c_2. However, the oxidised form of cytochrome c_2 from *R. palustris* shows H-bond distances and relative electrostatic interaction energies similar to those of the reduced forms. The weakness of these H-bonds with respect to the H-bonds found in the oxidised forms of cytochrome *c* and cytochrome c_2 from *B. viridis*, is consistent with the increase in the reduction potential value. It should be noted that no redox-dependent positional change of internal water molecules has been observed also in the X-ray structures of cytochrome c_6 from *Scenedesmus obliquus* [5]. These findings suggest that the movement of the buried solvent molecule lowers by about 100 mV the reduction potential through the stabilisation of the oxidised form.

Why the buried molecule found in *R. palustris* structures does not change its position with change of the redox state is the outstanding question. To answer this question we must look at the H-bond network that links the water molecule to the heme iron. The different behaviour of the water molecule in *R. palustris* may be associated to the hydrogen-bond distance between the hydroxyl group of the conservative Tyr residue and the sulphur atom of the Met axial ligand. In the cytochrome c_2 from *R. palustris* this distance is 3.34 Å in the oxidised form and 3.37 Å in the reduced one, significantly longer than those found in the oxidised and in the reduced forms of *B. viridis* cytochrome c_2 and of *tuna* cytochrome *c* (that ranges from 3.12 to 3.18 Å). The increase of this H-bond distance could be responsible for the low mobility of the buried water molecule with the change of the protein redox state in the *R. palustris* cytochrome c_2. In fact, the partial interruption of the H-bond network could disconnect the 'communication' between the metal centre and the water molecule. As a consequence, its position would not be influenced anymore by the iron oxidation state.

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