Cytochrome $c_{6A}$: discovery, structure and properties responsible for its low haem redox potential

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Abstract

Cytochrome $c_{6A}$ is a unique dithio-cytochrome of green algae and plants. It has a very similar core structure to that of bacterial and algal cytochromes $c_6$, but is unable to fulful the same function of transferring electrons from cytochrome $f$ to Photosystem I. A key feature of cytochrome $c_{6A}$ is that its haem midpoint potential is more than 200 mV below that of cytochrome $c_6$ ($E_m \approx +340$ mV) despite both cytochromes having histidine and methionine residues as axial haem-iron ligands. One salient difference between the haem pockets is that a valine residue in cytochrome $c_{6A}$ replaces a highly conserved glutamine residue in cytochrome $c_6$. This difference has been probed using site-directed mutagenesis, X-ray crystallography and protein film voltammetry studies. It has been found that the stereochemistry of the glutamine residue within the haem pocket has a destabilizing effect and is responsible for tuning the haem’s midpoint potential by over 100 mV. This large effect may have contributed to the evolution of a new biological function for cytochrome $c_{6A}$.

Discovery of a cyt (cytochrome) $c_6$-like protein in plants

Cyt $c_6$ is found in the thylakoid lumen of cyanobacteria and eukaryotic algae. Under copper-deficient conditions, cyt $c_6$ functions as an electron carrier in oxygenic photosynthesis, transferring reducing equivalents from cyt $f$ of the cyt $b_{6f}$ complex to Photosystem I [1]. Under copper-replete conditions, many cyanobacteria and green alga replace cyt $c_6$ with the copper-containing cupredoxin plastocyanin [2–4]. The two proteins show considerable differences in their tertiary structures, but the midpoint redox potential of the haem in cyt $c_6$ and the mononuclear copper site of plastocyanin are tuned to similar values ($\sim 380$ mV compared with NHE (normal hydrogen electrode), pH 7.0). This important physical property reflects their similar function in the photosynthetic electron-transfer chain.

Plastocyanin is universally present in chloroplasts of higher plants. Until recently, it was thought that a cyt $c_6$ did not exist in land plants. This was reasoned under evolutionary terms where the reducing atmosphere of early Earth made iron more readily available, but, upon increasing oxygen in the atmosphere, copper became more accessible, making plastocyanin the dominant electron carrier in oxygenic photosynthesis of plants [4–6]. Two groups independently reported the existence of a cyt $c_6$-like protein in a range of land plants, including the model plant system Arabidopsis thaliana [7,8]. Wastl et al. [8] inferred the presence of a cyt $c_6$ through searching plant genomic and EST (expressed sequence tag) databases with a cyanobacterial cyt $c_6$ sequence. This identified homologues of cyt $c_6$-coding sequences that predicted that the amino acid sequences for plants contained a conserved insertion of 12 amino acids. Gupta et al. [7] reported the existence of a plant cyt $c_6$ homologue as a protein interacting with the chloroplast immunophilin FKBP13 (13 kDa FK506-binding protein) in a yeast two-hybrid assay. The same authors using RNAi (RNA interference) experiments presented evidence that the plant cyt $c_6$ was a functional substitute for plastocyanin [7]. However, this interpretation was subsequently challenged using two different approaches.

The first, by Weigel et al. [9], showed that inactivation of the two plastocyanin genes of Arabidopsis by stable frameshift mutations resulted in plants unable to grow photoautotrophically, even when the cyt $c_6$-like protein was overexpressed at the same time. The second approach was an in vitro study that examined the electron-transfer reaction between the heterologously overexpressed cyt $c_6$-like protein and Photosystem I particles prepared from Arabidopsis [10]. This study revealed that ‘cyt $c_6$’ was unable to donate electrons at a sufficient rate to Photosystem I. Furthermore, it highlighted a key physical property of the ‘cyt $c_6$’; the haem Fe(III)/(II) midpoint potential ($E_m$) was significantly lower than in bacterial or algal proteins (initial estimates $\approx +150$ mV) [10]. Consequently, the plant cyt $c_6$ is unsuitable to accept electrons from cyt $f$ ($E_m \approx +350$ mV). Together, these studies suggest that it is unlikely that the cyt $c_6$-like protein is involved in the ‘normal’ electron-transfer pathway of the chloroplast.

Further support for this comes from the discovery that the plant cyt $c_6$ homologue is not restricted to higher plants. In the eukaryotic green alga Chlamydomonas reinhardtii,
Figure 1 | X-ray crystal structure (PDB code 2CEO) of ferric Arabidopsis thaliana cyt c6A at 1.2 Å resolution
The four helices of the core structure are labelled 1–4, and the LIP is coloured pink. The disulphide bridge is shown in sticks along with the haem and the axial methionine and histidine ligands to the iron (red sphere). Figure prepared using PyMOL (DeLano Scientific, http://pymol.sourceforge.net/).

genes encoding a conventional cyt c and the higher plant homologue have been identified. This discovery led Wastl et al. [11] to name the plant homologue cyt c6A.

Cyt c6A structure
High-resolution X-ray structures of wild-type Arabidopsis cyt c6A and site-directed variants have been determined [12–14]. The structure in the ferric haem oxidation state reveals a highly conserved core cyt c fold consisting of four α-helices interconnected by loops (Figure 1) [12,13]. The single haem is covalently attached to the polypeptide through thioether linkages via the two vinyl groups of the porphyrin and the side-chain thiol groups of Cys16 and Cys19 that make up part of the conserved CXXCH motif. The arrangement of the helices and loops encapsulates the haem in a predominately hydrophobic crevice with the propionate-7 side chain of the porphyrin exposed to solvent. The axial haem-iron ligands were as predicted from the sequence and are His75, part of the conserved CXXCH motif, and the thioether of Met60. Thus the core structure of cyt c6A is very similar to bacterial and algal cyts c6. This was the presence of a 12 residue insertion predicted in silico to form an extension to a loop connecting helices 3 and 4 [8,15]. This LIP (loop insertion peptide) contains two cysteine residues (67 and 73) separated by five intervening residues and predicted to form a disulfide bond. The X-ray structure confirmed this and revealed a well-defined loop lying on the surface of the protein with average temperature factor for the α-carbon atoms of 19.3 Å² (Figure 1) [13]. Disulfides have been observed in other haemoproteins and cytochromes, but these are often confined to the hydrophobic interior and play a predominantly structural role [16–22]. The surface-lying LIP disulfide in cyt c6A is therefore unusual and has stimulated discussion as to whether it could be involved in a functional role [15,23].

A functional role for the disulfide-containing LIP in cyt c6A
One hypothesis put forward by Howe et al. [15] was that a haem oxidation state change could lead to a conformational rearrangement of the LIP. This would trigger the transmission of a signal to some other molecule in the chloroplast or enable cyt c6A to bind to immunophilin either by directly altering the immunophilin-binding site or through causing the release of cyt c6A from some other complex, freeing it to bind with an immunophilin [15]. To test for tertiary structural changes in the LIP or the core, Marcaida et al. [13] determined the structures of cyt c6A in the ferric and ferrous haem forms. The oxidation state of the protein in the crystal was determined through the characteristic spectroscopic signatures of ferric or ferrous haem in the visible region of the spectrum using microspectrophotometry, and the oxidation state was ascertained before and after X-ray diffraction measurements [13]. Structure determination of ferrous and ferric datasets, confirmed by microspectrophotometry, revealed no tertiary structural changes to the protein core or in the LIP region. These observations also extended to the porphyrin ring and to the geometry and bond lengths of the haem-iron axial ligands [13]. It was therefore concluded that the LIP in the dithiol form does not communicate with the haem by a conformational change [13].

A hypothesis put forward by Schlarb-Ridley et al. [23] argues a scenario for direct electron transfer between the disulphide and the haem. This is set against the proposed backdrop of cyt c6A catalysing disulfide bridge formation in proteins of the thylakoid lumen, with plastocyanin and the haem of cyt c6A acting as electron acceptors in the reoxidation of the resulting reduced LIP disulfide [23]. The structural stability of the LIP and the distance between the disulfide of the LIP and the haem iron (≈13 Å) make a conduit for electron transfer favourable, but such an exchange has yet to be proved experimentally.

The idea that the disulfide bond of the LIP is part of an electron-transfer pathway in an as yet unassigned function of cyt c6A may turn out to be a red herring. Disulfide bonds are most commonly associated with enhancing protein stability [24]. Loop regions of proteins often display dynamics on a
wide range of time scales, which can influence the recognition and association of partners [25]. It may be that the disulfide bond present in cyt $c_{6A}$ contributes to stabilize the exposed structure of the LIP for presentation to a hypothetical partner protein. Recently, Schmidt et al. [26] classified protein disulfide bonds into 20 categories depending on the sign of the dihedral angle of the disulfide bond itself and of the two peptide bonds either side of it (Figure 2). Analysis of the LIP disulfide places it in the category $+/-$LHSpiral ($+,-,-,-,-$), which is of relatively low dihedral strain energy, characteristic of structural disulfides [26]. Most catalytic or allosteric disulfides fall into the higher-energy group $+/-$RHHook ($+,-,+,+,+,-$) [26]. Furthermore, a highly conserved arginine residue (Arg$^{70}$), which protrudes out into the solvent and may be a potential ‘hotspot’ residue for a partner protein. Figure prepared using PyMol (DeLano Scientific, http://pymol.sourceforge.net/)

**Molecular features determining the redox potential of cyt $c_{6A}$**

In all cyts $c$ involved in biological electron-transfer reactions, the most important functional characteristic is their $E_m$. In Class I cyts $c$, factors which determine the $E_m$ are the solvent exposure of the haem, the relative stabilities of the iron-porphyrin ring system in the reduced (neutral) and oxidized (positive) forms and the type of axial ligands. In cyts $c$, the nature of the sixth haem ligand divides the family into a low-potential (co-ordination with the imidazole side chain of a histidine residue) and a high-potential group (co-ordination with the thioether of a methionine residue). Within these two groups, the $E_m$ can be tuned further by varying the donating properties of the co-ordinating ligands through modulating metal–ligand bond strength [28–30] and/or through hydrogen-bonding interactions with the ligand [31].

Cysts $c_6$ have an $E_m$ of $+340$ mV compared with the NHE, which places them at the upper end of the high-potential group. Compared with mitochondrial cyts $c$ ($E_m + 260$ mV compared with the NHE), cyts $c_6$ possess a relatively simple hydrogen-bond network surrounding the haem cavity and the methionine ligand has no hydrogen-bond interactions with other protein residues. Before structural information for cyt $c_{6A}$, the surprisingly low $E_m$ was proposed to arise from the presence of the LIP [32]. However, site-directed mutagenesis to replace the two cysteine residues with serine or the construction of a deletion mutant with the complete removal of the LIP showed little significant effect on $E_m$ [32].

Using electrochemical methods, PFV (protein film voltammetry) gave an $E_m$ of $+71$ mV compared with the NHE at pH 7 for wild-type cyt $c_{6A}$ [14]. This is the lowest $E_m$ reported for a naturally occurring cyt $c$ with histidine/methionine as axial haem-iron ligands [14]. Initial inspection of the cyt $c_{6A}$ haem cavity with that of cyt $c_6$ structures revealed a comparable percentage of hydrophobic residues within 5 Å of the haem, and the haem solvent exposure varied little between the different structures [14]. A further common feature was the presence of a water molecule bridging the haem propionates via hydrogen bonds (Figure 3). This water appears to be conserved in all known cyt $c_6$ structures. There is no evidence to suggest that this water moves upon redox state change of the haem in either cyt $c_6$ or cyt $c_{6A}$ in analogy to water 166 in mitochondrial cyt $c$ [34,35].

Two amino acid differences between the haem cavities were identified and subsequently proposed to modulate $E_m$ [14]. The first difference noted was the absence of an electrostatic interaction between an electropositive amino acid side chain and the haem propionate-7 in cyt $c_{6A}$ (Figure 3) [14]. In cyts $c_6$, this interaction is conserved and has been reported to destabilize the oxidized form of the haem [36]. In cyt $c_{6A}$ proteins, the residue at position 31 is uncharged, but positioning a lysine here increased the $E_m$ (measured by PFV), although the change of $+34$ mV is too small to account for the much greater difference between the cyt $c_{6A}$ and cyt $c_6$ [14]. The second difference was a conserved polar glutamine residue in cyts $c_6$, which in cyt $c_{6A}$ is replaced by the smaller valine residue (Figure 3). A priori, it is less intuitively apparent why this residue difference may contribute to lowering the $E_m$ in cyts $c_{6A}$, because a hydrophobic valine residue would be expected to increase $E_m$ through an increased destabilization of the positively charged ferric haem [14]. Replacing the valine in cyt $c_{6A}$ with a glutamine increased the $E_m$ by $+109$ mV and making the converse switch of the glutamine residue for a valine residue in the cyanobacterial Phormidium laminosum cyt $c_6$ resulted in a complementary decrease in $E_m$ of 100 mV. A possible explanation for this pronounced effect is that the polar glutamine side chain is perturbing the electronic
structure of the haem through a dipolar effect or by formation of an aromatic hydrogen bond with the closely contacted pyrrole \[14\].

Together, residues 31 and 52 (in the cyt \(c_{6A}\) numbering scheme) contribute \(\sim 150 \text{ mV}\) of haem redox potential in the cyt \(c_6\) family. This is substantial considering that this type of change is normally associated with substitution of an axial ligand. Other studies with cyt \(c\) have illustrated that orchestrated substitutions intended to increase the \(E_m\) almost always led to a decrease \[37\]. This highlights the fact that cyt \(c\) have evolved to stabilize the reduced state. For cyt \(c_{6A}\), it may be argued that its evolution has followed a trajectory of favouring the oxidized haem state. Assuming that the redox properties of the haem are an essential feature to the function of cyt \(c_{6A}\), then the lowering of \(E_m\) by \(>100 \text{ mV}\) with a glutamine-to-valine substitution is an example of how a single amino acid change could, in principle, alter the properties of the protein to abolish its original function and opens avenues to perform a new function more effectively.

The A to C of cyts \(c_6\)

Recent phylogenetic analysis of plant, algal, bacterial and cyanobacterial genomes has revealed two novel and widely distributed clusters of cyanobacterial cyt \(c_6\)-like proteins \[38\]. These are distinct from both the conventional cyts \(c_6\) and cyt \(c_{6A}\) (as they do not contain a disulfide LIP), but have the conventional histidine/methionine axial haem ligation, and modelling predicts a core structure typical of a cyt \(c_6\) \[38\]. In accordance with the nomenclature used for the plant cyt \(c_6\) homologue \[11\], these new cyt \(c_6\) groups have been named cyt \(c_{6B}\) and \(c_{6C}\) \[38\]. Sequence alignments reveal a valine/isoleucine/leucine residue at position 52 (cyt \(c_{6A}\) numbering). On the basis of work with cyt \(c_{6A}\), the \(E_m\) of the haem in cyts \(c_{6B}\) and \(c_{6C}\) would be predicted to be of a low potential \[14\]. The heterologously overexpressed cyt \(c_{6C}\) from \textit{Synechococcus} sp. PCC7002 confirms this, with an \(E_m\) of \(+148 \text{ mV}\) at pH 7 \[38\]. This value is slightly higher than for cyt \(c_{6A}\), but we note that position 31 in \(c_{6B}\) and \(c_{6C}\) (cyt \(c_{6A}\) numbering) is a positively charged residue (lysine or arginine) and will influence the \(E_m\) by making it more positive \[14\]. Therefore functional equivalence with plastocyanin appears to be unlikely, and the roles that cyts \(c_{6A}\) to \(c_{6C}\) play in their respected organisms remain yet to be revealed.

References

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The Biochemical Society is acknowledged for the award of a travel grant to J.A.R.W. The Wellcome Trust, BBSRC (Biotechnology and Biological Sciences Research Council) and the Newton Trust have sponsored parts of this work. We are grateful to the ESRF (European Synchrotron Radiation Facility), Grenoble, for access to their facilities.