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Pathways linked by hydrogen bonds with redox-dependent breaks implicated in electron transfer in human cytochrome c protein

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Pathways of hydrogen-bond-linked peptide units, polar side chains of the amino acid residues and buried water molecules have been traced in human cytochrome c protein. These connect heme-Fe to the surface through axially coordinated Met80-S and His18-N on the two sides of the heme plate. Oxygen atoms of the heme-propionate side chain and of the internal invariant water molecules form hydrogen bonds in connecting these pathways. With 28 out of the 37 amino acid residues being in the conserved list, these pathways are likely to be common in the highly conserved cytochrome proteins. Selective breaks appear in hydrogen bonds on the His18 side in the oxidized form and on the Met80 side in the reduced form consequent to the accompanying structural changes consistent with a regulatory role. These changes are defined by φ, ψ angles of the backbone and dihedral angles of the side chains, between the redox states. The pathways are identical in both the redox forms. They are suitable for intramolecular atom-to-atom electron transfer with hydrogen bond now experimentally found to transfer electrons better than covalent σ-bond, hitherto used for making the paths.

Keywords: Atom-to-atom pathways, Cytochrome c, Delocalized electron units, Electron transfer, Hydrogen bonds, Polar side chains.

Electron transfer occurs in protein complexes of cytochromes in the mitochondrial electron transport chain down the energy gradient reaching the ultimate electron sink, molecular oxygen. Pathways present in proteins, including enzymes, direct this by a process of "way in, a trap and way out" according to Moore and Williams[1]. Proteins in bulk, however, are insulators as the band gap (activation energy) is high at 3 eV and above[2]. Side chains of amino acid residues occur in proteins which have short delocalized electron systems such as O=C-N-H (Asn, Gln), O=C-O-H (Asp, Glu), -N=C-NH (His, Arg). These can facilitate transfer of electrons provided the π-clouds of these units are bridged by the connecting hydrogen bonds. Alternating peptide group with π-clouds and hydrogen bond [O=C-N-H– –O=C-N-H], named 'suprahelix'[3], occur commonly in the secondary structure of proteins. In a theoretical study Chandra *et al.*[4] stated that "delocalization of an extraneous electron is pronounced when it enters low-lying virtual orbital of the π-electronic structures of peptide-linked by hydrogen bonds", thus supporting electron transfer in such suprahelical structures, referred as π-H pathways[5].

Cytochrome c is a well-known example of electron transfer through heme-Fe occurring across the protein. Supporting evidence of connectivity between heme-Fe buried in the interior protein and the surface histidine residues non-perturbatively liganded to ruthenium[6,7] of cytochrome c was obtained by measuring electron transfer rates, using a flash-quench technique. The pathways proposed by Gray and coworkers[8] in 1990 included many covalent σ-bonds of the side chain amino acid residues. These studies revived interest in the pathway models of long range electron transfer. Balaban *et al.*[9] described a method to derive most probable electron transfer pathways in selective polypeptide structures "through covalent bonds and through space jumps". Using this method a pathway was proposed from heme-Fe, Cys14 and Lys13 of cytochrome c connecting with Tyr105, Met207 and CuA of subunit II of cytochrome oxidase passing

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through σ-bonds of carbon chains of the amino acid residues and through-space jumps[10]. Meaningful long range electron transfer can occur in a carbon chain with conjugated double bonds capable of resonance shifting to delocalize π-electrons over the structure, such as carioviologens, described by Lehn and coworkers[11]. Pathways built with σ-bonds of side chains of amino acid residues, but without electron delocalization, are likely to be poor for electron transfer.

Direct evaluation of electron coupling mediated by hydrogen bonds is now experimentally supported. Therien and coworkers[12] measuring photo-induced electron transfer rate constants emphatically statedin 1995 that "In contrast to generally accepted theory, electron coupling modulated by hydrogen-bond interface is greater than that provided by an analogous interface composed entirely of carbon-carbon σ-bonds". Nishino *et al.*[13] reiterated in 2013 that "a H-bond conducts electrons better than a covalent σ bond at short range" based on their experiments measuring the electron transfer with scanning tunneling microscopy. These experimental findings support hydrogen bonds, rather than covalent σ bonds, as bridging elements in electron transfer pathways in proteins.

We report that π-H pathways that connect heme-Fe through axially coordinated Met80-S and His18-N to the surface of the protein are identical in both oxidized and reduced forms of human cytochrome c. They consist of the delocalized electron units in peptide bonds and in some side chains of amino acid residues, as well as some polar groups and the internal water molecules linked exclusively by hydrogen bonds with selective breaks in the redox forms.

Methods used in identifying the pathways

NMR structures of human recombinant cytochrome c proteins[14,15] of oxidized (conformer1; PDB: 2N9J) and reduced (conformer1; PDB: 2N9I) were analyzed using pymol software for 'Hydrogen Bonds'Starting from heme-Fe, all the polar atoms within 2.6 - 3.3 Å distance except for those described for proline[16] with multiple conformations[17] were manually marked. Unconventional hydrogen bonds described for proline[16] and methionine[18] and buried water molecules, known to be invariant in cytochrome c proteins across many sources and taken from the structures of mice (PDB: 5C0Z) and horse (PDB: 1HRC, 5IY5), were part of some connections in the pathways. The atom-to-atom pathways include the delocalized electron units of the peptide bonds and of the polar side chains, and O and N atoms bridged by hydrogen bonds.

Results

Hydrogen bond-linked pathways in human cytochrome c

Heme-Fe in cytochrome c, coordinated to the four pyrrole nitrogen atoms and embedded in the surrounding protein, is accessible through the axial connections on either side of the plate of His18-N and Met80-S, a conserved structural feature in all cytochromes c[19]. It can serve as a platform connected to tracks for assisted transfer of electrons across the protein with separate paths for entry and exit. The Met80 side of the pathway is for reduction of heme-Fe since modifying hydroxyl group of Tyr67 blocked reduction, but not oxidation[20]. The His18 side is considered the oxidation route in the pathway through the peptide bond (Lys86-Lys87) and a water molecule docked with the oxidant, ferricyanide (based on information from file PDB: 5C0Z).

We identified the π-H pathway, linked only by hydrogen-bonds[11,12] now accredited electron transfer bridges, passing through the invariant central heme structure (Met80-S— heme-Fe—His18-N) in cytochrome c proteins[21] (horse animal, tuna fish, rice plant and yeast microorganism). Another pathway connect sheme-Fe of cytochrome c with subunit II-CuA, the source of electrons to all other metal centers, in cytochrome c oxidase[22].

Identical pathways are found both in the oxidized and the reduced forms of cytochromes c (human) shown (Fig. 1) and on atom-to-atom basis (Fig. 2). Selective breaks of hydrogen bonds occur in the pathways shown by arrows on the His18 side in the oxidized form (with reduction path open) and on the Met80 side in the reduced form (with oxidation path open). The total pathway through the heme-Fe consists of 103 atoms (including H atoms) drawn from 36 amino acid residues (out of which 27 are conserved in animals), 14 peptide units, 4 delocalized electron units, 5 water molecules, 12 polar (O+N) groups and 34 hydrogen bonds. Covalent bonds present are part of the peptide and delocalized electron units.

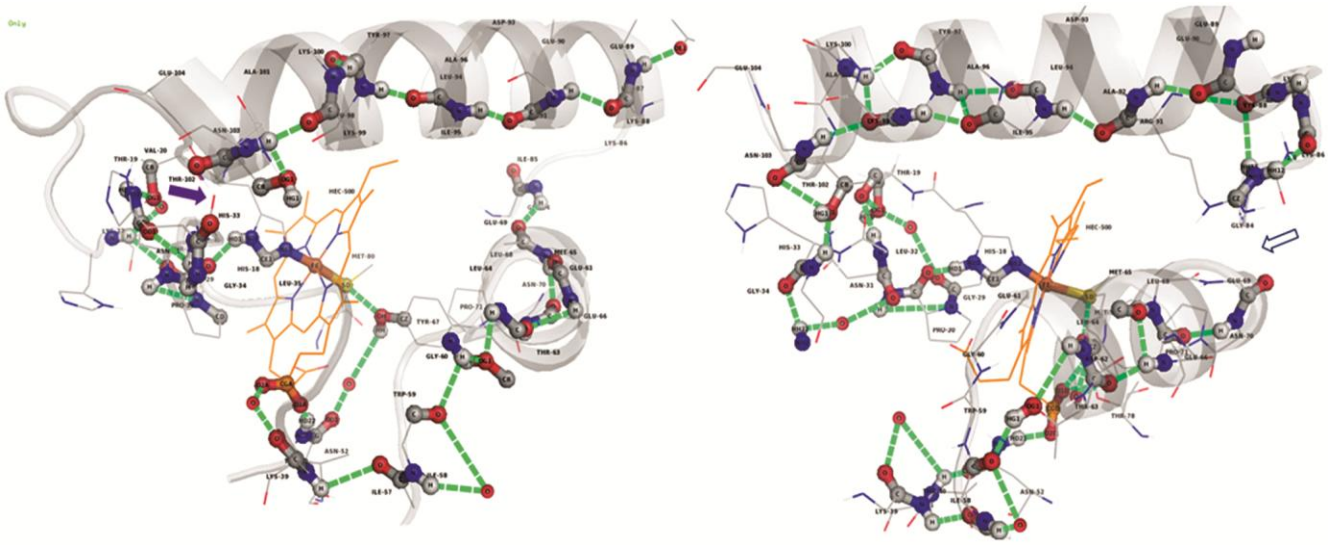

Fig. 1 — Pathways consisting of peptide bonds, polar groups of side chains of amino acid residues, and internal water molecules linked by hydrogen bonds in human cytochrome c protein. Oxidized form (left), reduced from (right). The helices (light gray) are represented as cartoon; the polypeptide backbone is represented as ball and sticks, and the heme as orange lines, respectively. The atoms in the pathways are identified as colored spheres - oxygen (red), nitrogen (blue), carbon (light gray), sulfur (yellow), iron (brown) - labeled with the respective name. Hydrogen bonds are shown as green broken lines

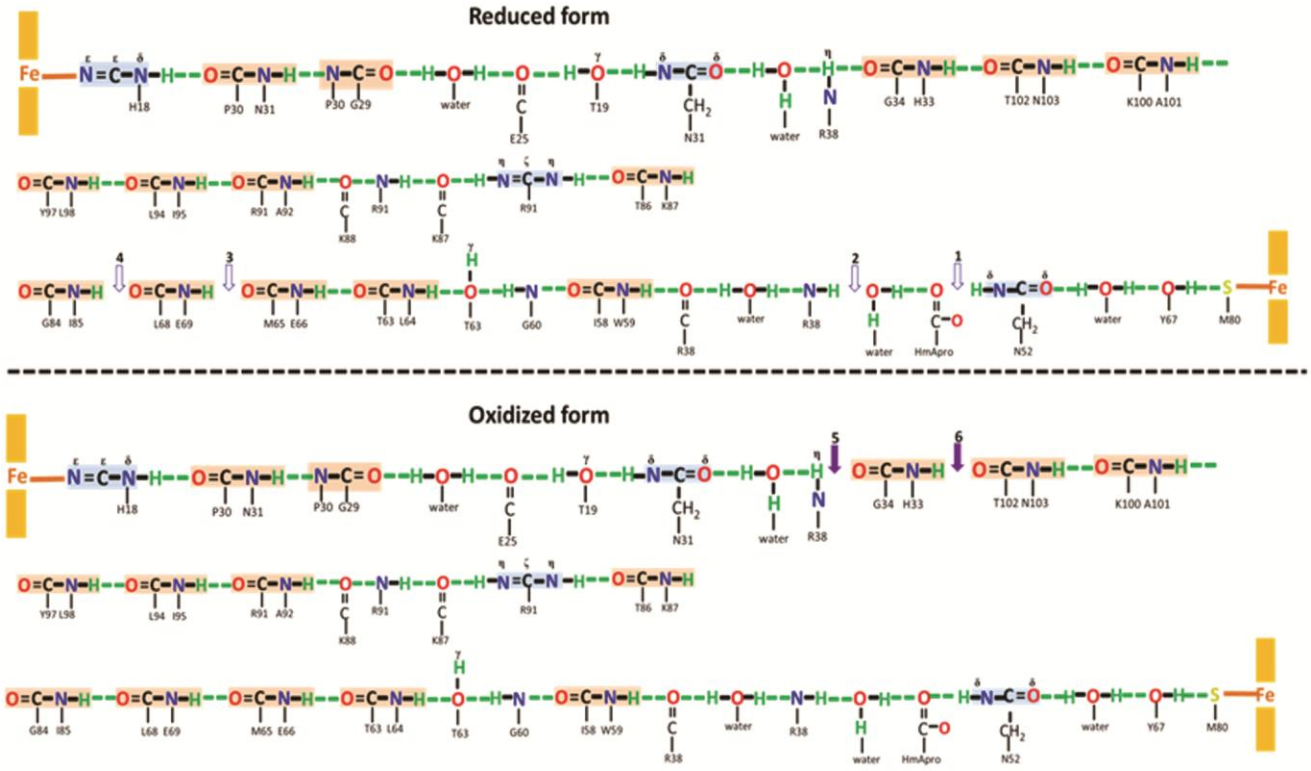

Fig. 2 — The hydrogen-bond-linked atom-to-atom pathways in oxidized and reduced forms of human cytochrome c. These pathways are built with hydrogen bonds linking the π-electron clouds of peptide bonds (shaded pink blocks) and of side chain amino acids (shaded blue blocks), other polar O and N atoms and bound water molecules extending from heme-Fe (long pathways from His18 are given in two lines). The breaks in the pathways, shown by vertical arrows, occur in both the redox forms, blocking the oxidation path in the oxidized form (2 breaks; marked by purple closed arrows) and the reduction path in the reduced form (4 breaks; marked by blue open arrows)

Peptide bonds in suprahelix are used in the pathway (3 on the Met80 side and 6 on His 18 side).

Changes in the backbone and the side chains in the two redox forms

Presence or absence of one electron on the heme-Fe atom of cytochrome c amazingly causes redox-controlled movements of the backbone and some of the side chains[23]. The loss of an electron from heme-Fe was found to strengthen the Fe–Met80 axial bond due to the higher affinity of the sulfur atom to the ferric iron[24]. This lone redox change is accompanied by several perceptible backbone movements, albeit how it is achieved is presently not comprehensible. Superposed reduced and oxidized structures show good overlap with low RMSD values[23]. Yet several changes, small and large, occur in the backbone and in side chains of amino acid residues. Some of these sites are marked by arrows in (Fig. 3). Movements of the backbone are observed in C-terminal helix one turn moving away from the heme, in the side chains of the heme and in unstructured coils at the surface in the regions of 21-28, 42-48, and 48-60. These are defined by φ, ψ angles of the backbone and dihedral angles of the side chains in the oxidized (PDB: 2N9J) and the reduced (PDB: 2N9I) forms of human cytochrome c given for record in (Table 1). The 19 amino acids listed in the table showed significant changes in the φ, ψ angles, particularly the ψ angles, on reduction of heme-Fe.

The breaks in the pathways

The structural modifications consequent to redox change in heme-Fe that cause the breaks of hydrogen bonds are shown in (Fig. 4). The changes in dihedral angles do confirm the movement of the side chain of propionate of heme ring A and of Asn52 which caused the breaks (#1 and #2) in hydrogen bonds of both the propionate-carboxyl oxygen

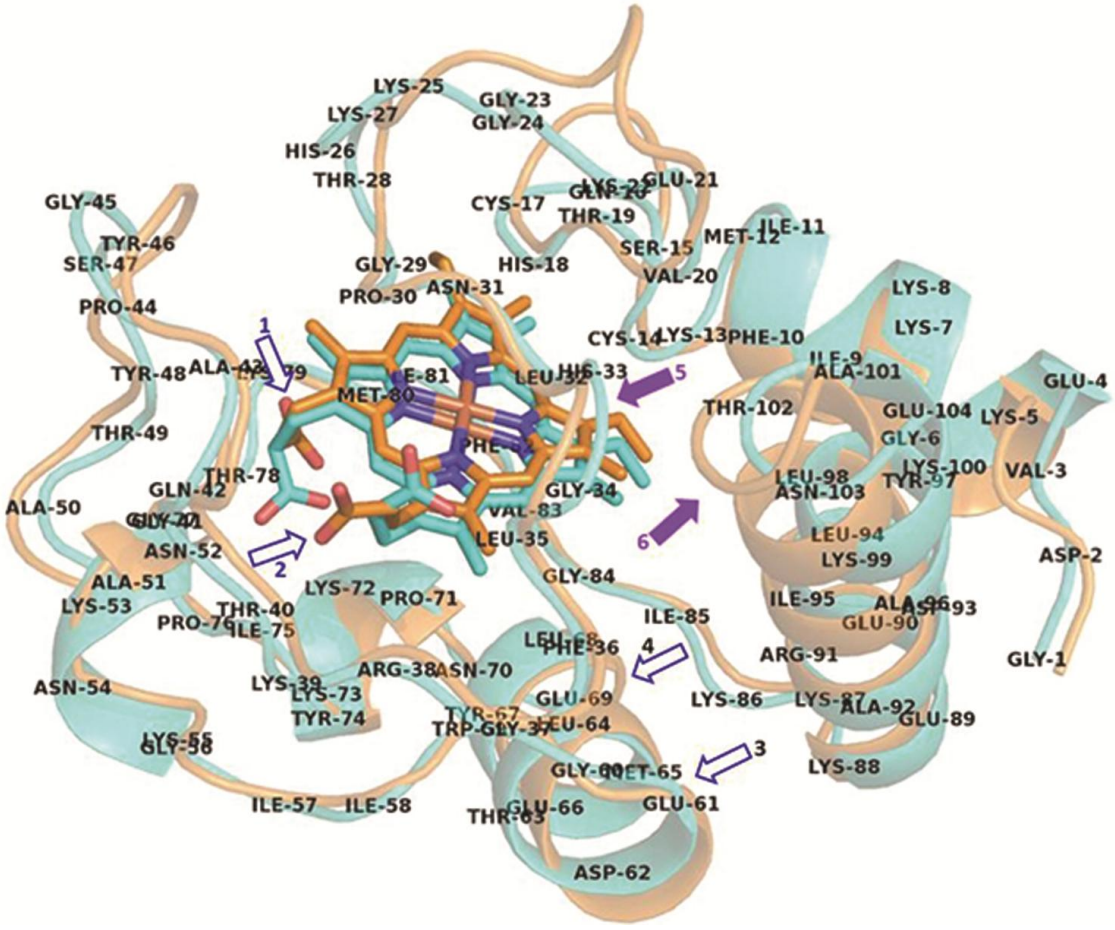

Fig. 3 — Superimposed structures of the oxidized form (brown) and the reduced form (cyan) of human cytochrome c. The arrows indicate the location of breaks in the pathways: Met80 side (blue open arrows, 1 to 4), His18 side (purple closed arrow, 5 and 6)

Table 1 — The φ, ψ angles of the backbone and dihedral angles of the side chains in oxidized (2n9j) and reduced (2n9i) forms of human cytochrome c. The significant conformational changes in φ, ψ angles with respect to backbone and dihedral angles with respect to side chains in the oxidized and the reduced forms of the cytochrome c protein are recorded. The changes in protein backbone and side chains and in the heme side chain propionate that lead to breaks in the π-H pathways are highlighted

Amino acid residue, number	Oxidized form	Reduced form
	(φ,ψ) angles	
Cys14	(−123.8, 77.1)	(−122.1, −49.7)
Ser15	(−109.4, 131.2)	(−41.4, −29.6)
Gln16	(38.9, 47.2)	(−39.2, −46.8)
Val20	(−136.5, −47.7)	(−86.8, 49.0)
Lys22	(−54.6, −39.1)	(−40.8, 121.1)
Gly23	(−63.8, −25.9)	(101.1, 10.7)
His26	(−67.8, 125.7)	(−91.7, 42.0)
His33	(−118.4, −50.4)	(−123.0, 89.2)
Gly34	(−101.4, 28.0)	(97.2, 20.6)
Lys39	(−77.3, −174.1)	(−80.8, 167.5)
Gln42	(−103.5, 27.6)	(−97.3, −45.0)
Pro44	(−75.0, 0.2)	(−75.0, 135.5)
Gly45	(−103.4, −14.3)	(83.5, 31.4)
Tyr48	(−93.8, −168.3)	(−41.1, 163.1)
Thr49	(−70.8, −176.0)	(−84.9, 153.5)
Lys55	(−77.0, 57.2)	(−40.8, −76.2)
Gly56	(−62.8, 85.9)	(65.9, 63.6)
Gly60	(−107.3, −176.9)	(178.9, −142.7)
Thr78	(−84.6, −179.7)	(0−73.5, 147.7)
Heme side chain changes	Oxidized form	Reduced form
	Dihedral angles	
Heme c ring D propionyls' carboxyl group $^{\gamma}$C	−31.8	85.9
Heme c ring D propionyls' carboxyl group o2	−58.4	108.3
Heme c ring D propionyls' carboxyl group o1	119.9	−73.2
Heme c ring A propionyls' carboxyl group $^{\gamma}$C	154.2	−30.2
Heme c ring A propionyls' carboxyl group o2	−163	139.9
Heme c ring A propionyls' carboxyl group o1	21.1	−35.8
Amino acid side chain changes	Oxidized	Reduced
	Dihedral angles	
Asn52$^{\gamma}$C from backbone C	86.6	61
Asn52 $^{\delta}$O	90.3	−112.9
Asn52 $^{\delta}$N	−89.5	67.1
Asn52$^{\gamma}$C from backbone N	−153.4	−179
Arg38 $^{\varepsilon}$N from $^{\beta}$C	89.8	78.8
Arg38 $^{\zeta}$C from $^{\gamma}$C	−167.6	154.5
Arg38/NH1 from $^{\delta}$C	0	0
Arg38/NH2 from $^{\delta}$C	−179.9	179.9

atoms. The breaks in hydrogen bonds between peptide bonds of M65-E66 and L68-E69 (break #3), and of L68-E69 and G84-I85 (break #4) in the reduced form occur as the hydrogen bonds fail to connect because of the backbone movement. Similarly, two breaks of hydrogen bonds between G34-T102 and H33-R38 (#5 and #6) in the oxidized form, the only two in the His18 side, occur as the distance between the residues increases. The residues loose hydrogen bond contact because of the

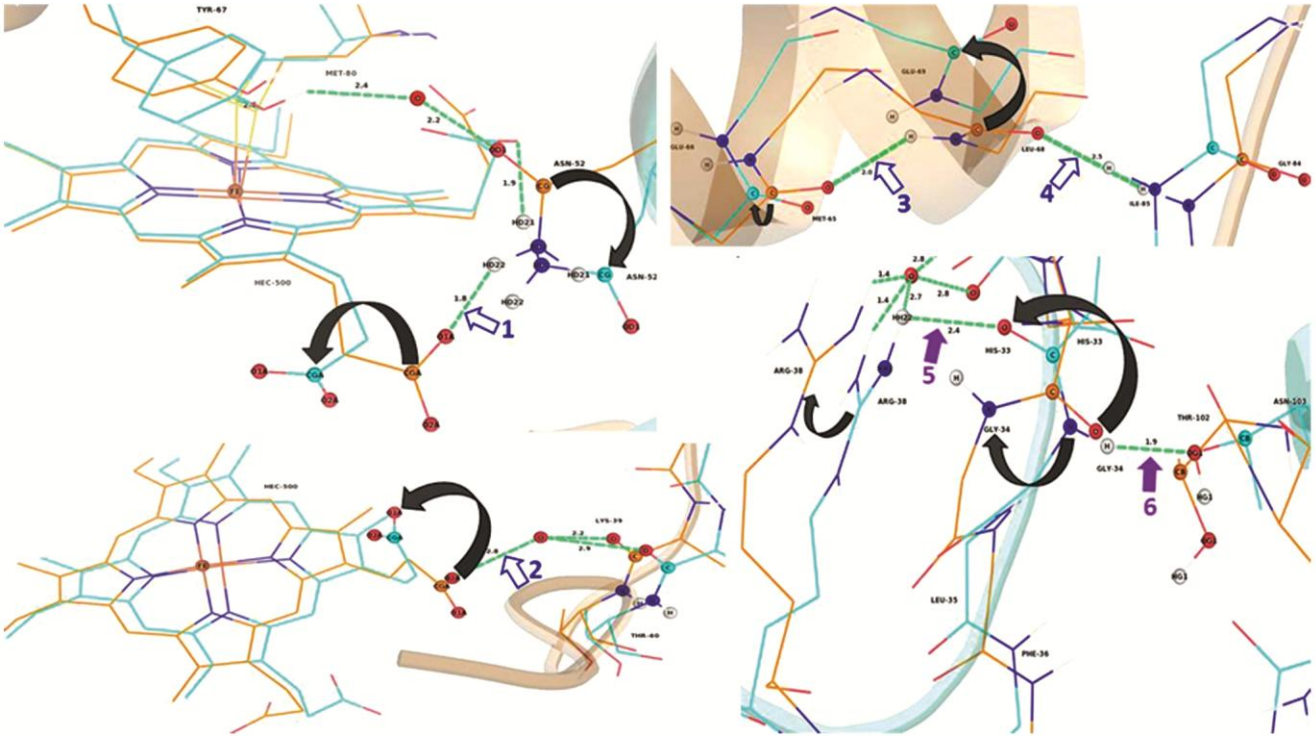

Fig. 4 — Conformational changes at the sites of the breaks between the oxidized form (brown) and the reduced form (cyan). All notations are the same as in Fig. 1. The change in side chain of amino acid Asn52 (top left) confers break in the reduction path (blue open arrow #1). The change in heme propionate side chain (bottom left) confers another break in the reduction path (blue open arrow #2). A break occurs at the beginning of the reduction path because of the changes in the backbone at Met65-Glu69 and at Ile85 (top right) (blue open arrows #3 and #4). The breaks at Arg38 side chain and flip of Gly34 and His33 backbone in the oxidation path in the oxidized form are shown as purple closed arrows #5 and #6, respectively (bottom right). The changes in the positions of atoms moved are shown by black curved arrows

relative movement of the top turn in the helix beyond Lys100 and of the unstructured coil at 30-40.

Discussion

Delocalized electron systems occur as O=C-O-H (Asp, Glu), O=C-N-H (Asn, Gln), -N=C-NH (His, Arg) in addition to peptide groups. These and other reactive groups such as C=O, N-H, O-H and S-H present in amino acid residues are interconnected by hydrogen bonds over which electron transfer is possible[12,13]. These are collectively referred as π-H pathways[5] and their importance in proteins is underscored as the four basic structural features of the protein - peptide bonds, side chain polar groups, hydrogen bonds and folding - are utilized in their formation.

The propionate side chains of the heme also show changes in the angles indicating their considerable flexibility. One of these is involved and is the site of a break in reduction pathway on the Met80 side. Heme is known to hold the Fe atom by the tetrapyrrole-nitrogen atoms of its porphyrin group, and the short side chain side is hydrophobic and is surmised to transfer electron through the cysteine chain of covalent bonds[10]. The current observation gives a functional role for the longer propionate side chain in connecting the hydrogen-bonded pathway.

The presence of independent pathways for oxidation and reduction of cytochrome c molecule implies its minimal movement in the inner membrane of mitochondria.

The two redox states of cytochrome c protein structurally overlap with some significant changes in the backbone and of the loop regions. In an otherwise identical pathway, these movements of the backbones offer a plausible explanation of the conspicuous breaks in the connecting segments around heme-Fe with a possible regulatory role.

Current models[6-10] use covalent σ-bonds and through space jumps but these need to be validated. In the pathways identified here, atom-to-atom connectivity is provided by delocalized electron units and by hydrogen bonds, both reliable elements for electron transfer[11-13].

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