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On Similarity of Cytochrome c Oxidases in Different Organisms

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Abstract

Most of biological oxygen reduction is catalyzed by the heme-copper oxygen reductases. These enzymes are redox-driven proton pumps that take part in generating the proton gradient in both prokaryotes and mitochondria that drives synthesis of ATP. The enzymes have been divided into three evolutionarily related groups: the A-, B- and C-families. Recent comparative studies suggest that all oxygen reductases perform the same chemistry for oxygen reduction and comprise the same essential elements of the proton pumping mechanism, such as the proton loading and kinetic gating sites, which, however, appear to be different in different families. All species of the A-family, however, demonstrate remarkable similarity of the central processing unit of the enzyme, as revealed by their recent crystal structures. Here we demonstrate that cytochrome c oxidases (CcO) of such diverse organisms as a mammal (bovine heart mitochondrial CcO), photosynthetic bacteria (*Rhodobacter sphaeroides* CcO), and soil bacteria (*Paracoccus denitrificans* CcO) are not only structurally similar, but almost identical in microscopic electrostatics and thermodynamics properties of their key amino-acids. By using pK_a calculations of some of the key residues of the catalytic site, D- and K- proton input, and putative proton output channels of these three different enzymes, we demonstrate that the microscopic properties of key residues are almost identical, which strongly suggests the same mechanism in these species. The quantitative precision with which the microscopic physical properties of these enzymes have remained constant despite different evolutionary routes undertaken is striking.

Keywords

heme-copper oxygen reductases; cytochrome c oxidase; electron transfer; proton transfer; pK_a calculations; redox potentials

1. Introduction

The solution of the structure of Cytochrome c Oxidase (CcO) from several organisms, and the followed rapid advances in understanding the molecular mechanism of its proton pumping have prompted a discussion of intriguing evolutionary issues of the super-family of heme-Cu oxidases to which Cytochrome oxidase belongs. These enzymes are redox-driven proton pumps that generate the proton gradient in both prokaryotes and mitochondria that drives synthesis of ATP. In light of non-trivial mechanistic principles that the enzyme appear to be using, and overall complexity of the mechanism (four electrons and eight

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protons are involved in one turnover) by which oxygen reduction is coupled to proton translocation (see refs. ¹⁻⁴), the evolutionary aspects of the proton pumping mechanism become particularly interesting.

The enzymes have been divided into three evolutionarily related groups: the A-, B- and C-families⁵. Recent comparative studies⁶ suggest that all oxygen reductases perform the same chemistry for oxygen reduction and comprise the essential elements of the proton pumping mechanism, such as the proton loading and kinetic gating sites, which, however, appear to be different in different families. For example, the A-type enzymes require two proton input pathways (D- and K-channels) to transfer protons used for oxygen reduction chemistry and for proton pumping, with the D-channel transporting all pumped protons. But as recently has been discovered⁶, the B-family, does not contain a D-channel. Rather it utilizes only one proton input channel, analogous to that of the A-family K-channel, and it delivers protons to the active site for both chemistry and pumping.

All species of A-family, however, demonstrate remarkable similarity of the central processing unit of the enzyme, as revealed by recent crystal structures. Here we demonstrate that the A-family cytochrome c oxidases of such diverse organisms as a mammal (bovine heart mitochondrial CcO), photosynthetic bacteria (*Rhodobacter sphaeroides* CcO), and soil bacteria (*Paracoccus denitrificans* CcO) are not only structurally similar, but almost identical in microscopic electrostatics and thermodynamics properties of their key amino-acids. We have performed pK_a calculations of some of the key residues of the catalytic site, D- and K- proton input, and putative proton output channels of these three different enzymes, and demonstrate that the microscopic properties of key residues are almost identical, which strongly suggest similar mechanism in these species. Many similar calculations have been reported in the past, including those by this group; however, a side-by-side comparison, using the same methods, has never been performed before. The quantitative precision with which the microscopic physical properties of these enzymes have remained constant in evolution despite differences in sequences and number of subunits is striking.

2. Proposed proton pumping mechanism

Computational studies⁷⁻⁹ point to a mechanism of pumping shown in Fig.1. Recent experiments^{10,11} support the mechanism in its general form; however, some details remain experimentally untested and may in fact be different (see e.g. discussion in refs^{10,11}). The calculations reported previously were done on the structure of mitochondrial CcO from bovine heart (pdb code, 2OCC¹²). Here we extend these calculations to new structures. According to the computational model, during the pumping cycle, the stable state of the catalytic center, before an additional electron is supplied to the system, is that in which one of the metal centers is formally oxidized, e.g., Fe³⁺-H₂O, or Cu²⁺-H₂O. This state is established in a previous step of the cycle, when a chemical proton is accepted by one of the hydroxyl ligands of the BNC. In this state, His291 (i.e., N81 position) is deprotonated and Glu242 is protonated.

As the cycle advances, an electron is supplied (by cyt c) to the heme a_3 -Cu_B binuclear center (BNC) via Cu_A and heme a redox centers of the enzyme. As a result, one of the metal centers of BNC is reduced, and the overall charge of the BNC becomes one charge unit more negative. The driving force of electron transfer is about 20 meV⁷. In response to the increased negative charge of the BNC, the proton from Glu242 has now a driving force to move closer to the BNC. There are two pathways leading from Glu242 to two possible sites: one is leading to the BNC itself, the second is leading to His291 N81 position¹³. The assumption (kinetic gating, or other type of gating¹⁴) is made that the rate of proton transfer to His291 is much higher than that of transfer to the BNC (the basis for this key assumption is discussed later in the paper). Therefore, the protonation of His291 occurs before that of the BNC. The fast proton transfer from Glu242 to His291 occurs by Grotthuss mechanism¹⁵ via Arg438 and PRDa3, details of the transfer are discussed below. The driving force for this transfer is about 100 meV.⁷ With this transition, His291 becomes protonated. Subsequently, the arrival of the chemical proton to BNC, either via D channel or K channel, expels the proton from His291 to the P-side of the membrane, resulting in a translocation of one proton across the membrane.

The two-step pumping mechanism, in which the pumped proton is first loaded to the Proton Loading Site (PLS) from the N-side of the membrane and then expelled by the chemical proton arriving to BNC to the P-side of the membrane, is part of all most recently discussed models. The main difference between the models is in the nature of the PLS site, whose identity is still not known; however, recent experimental and theoretical studies point to one of the residues in the group His291, Prop A or D of heme a_3 , or a group (including a water molecule) nearby^{10,11,16,17}. The proton translocation from Glu242 to His291 in response to the reduction of BNC (i.e. the assumption that PLS is His291, see however discussion in Refs. 18-20) is a distinguishing point of the mechanism discussed in this paper; otherwise the mechanism considered is quite generic.

In this work, we compare CcO enzymes taken from different organisms to check for similarities or differences in the properties of key residues of the described mechanism. For quantitative comparisons, we examine pK_a values of the bovine Glu242 and His291 analogs for so-called^{7,8,21,22} OORO and OORR states of CcO. In this notation of the redox state "O" or "R" letters mean formally oxidized (O) or reduced (R) state of each CcO redox center: Cu_A, heme a, heme a_3 and Cu_B, respectively. Thus, the two last letters specify redox state of BNC, e. g. BNC in OORR state is reduced in respect to the OORO state of CcO.

For a more complete picture of the proton pumping mechanism, we also included into consideration the protonatable sites of hemes, proton-entrance²³ and proton-exit pathways²². Since the proton-exit pathways are not known with certainty, we include the elements of the proposed²² exit pathways. The location of the key residues in the enzyme is shown in Fig.2.

*This means that in fact an electron is initially equilibrated between the two hemes, and the transfer is incomplete. However, this electron transfer is directly coupled with a proton transfer to the proton loading site, PLS, located near BNC, as explained later in the text. The proton transfer to PLS significantly increases the redox potential of BNC (about 0.5 mV) thereby stabilizing the electron at BNC, which in turn further increases the driving force for proton transfer to PLS. Therefore, one can say that the electron and the proton drive each other at this step to the more stable (intermediate) state of the enzyme where they occupy BNC and PLS, respectively.

CcO structural comparison for the species considered is given in the next section. Continuum electrostatic pK_a calculations and results are described in the section 3. Conclusions are summarized in section 4.

3. Systems studied

Four CcO structures from different organisms have been studied.

1. Bovine Heart Mitochondrion CcO

For mitochondrial enzyme calculations, we use **2OCC** pdb crystal structure resolved by Yoshikawa *et al.*¹² with 2.30 Å resolution, released 01-13-99. The catalytic core involves subunits I and II out of the total 13 subunits.

2. *Rhodobacter Sphaeroides* Bacterial CcO

Rhodobacter sphaeroides is a photosynthetic bacterium capable of aerobic and anaerobic respiration. For this organism, two structures (with and without Glu242 analog) have been considered. Namely, **2GSM** pdb crystal structure resolved by Qin *et al.*²⁴ with 2.00 Å resolution, released 10-10-06. The 2GSM structure has only 2 core subunits. The other, **1M57** pdb crystal structure resolved by Svensson-Ek *et al.*²⁵ with 3.00 Å resolution, released 08-28-02; it has 4 subunits. The Glu242 analog in this structure is mutated to non-titratable glutamine. The mutation of the critically important residue Glu242, which shuttles protons along the D-channel, to a residue that cannot function as a proton transfer group naturally renders the mutant **1M57** to be non-pumping. However, the other minor structural changes, in particular around the heme a3 propionates, that the mutation induces are considered to be also mechanistically significant; it is interesting to see what kind of pK_a changes these structural changes introduce. We therefore include this non-pumping mutant into comparison with the wild type enzymes.

3. *Paracoccus Denitrificans* Bacterial CcO

Paracoccus denitrificans is a soil bacterium capable of reducing nitrate to molecular nitrogen under anaerobic conditions but also capable of aerobic respiration. We examine **1AR1** pdb crystal structure resolved by Ostermeier *et al.*²⁶ with 2.70 Å resolution, released 02-11-98. The structure has 2 subunits.

To unify reference on residues across multiple structures we accept residue labeling corresponding to the bovine CcO structure, i.e. bovine residue number used for other structures means its functional analog. The few residues, analogs for which can not be determined in other structures, will be mentioned explicitly.

The residue statistics of CcO in different organisms is summarized in Table 1. Although the catalytic core of each enzyme is generally constituted by a unique sequence of amino acids, the cofactors and metal centers are essentially the same. Moreover, the mutual location of the cofactors, metal centers and some other functional groups responsible for the enzyme function is remarkably conserved across different structures (see Fig.3).

To quantify the similarity in the spatial arrangement of the putative proton pumping residues, we calculated their rmsd with respect to the bovine enzyme. The analysis has been performed by using the VMD program²⁷; the structure matching were achieved by best fitting of heme porphyrin rings. The list of key residues (shown in Fig.2), their analogs and corresponding rmsd's are given in the Table 2.

The location of the key titratable residues identified in the previous studies^{7,8,22} is seen to be mainly conserved (rmsd within 3.0Å); this may indicate that these residues and their location are indeed essential for the enzyme function, including its proton pumping mechanism. The exceptions are only for the residues of proton entrance and possible exit pathways which were not firmly established²². The absence of close analogs of some of the residues in different organisms most likely indicate that they are not essential for the CcO function.

To provide physically more reliable arguments, we calculated pKa values of all key residues listed in the Table 2.

4. Methods and Results

The number of titratable sites in the CcO is variable across the multiple structures (see Table 1). For the analysis of the behavior of the titratable groups, the continuum electrostatic calculations were employed as described in ref. ⁷ In the calculations, the X-ray atomic coordinates and the corresponding partial atomic charges, which are placed in a low dielectric medium ($\epsilon = 4$), describe the protein. The external solvent is defined as a high dielectric continuum ($\epsilon = 80$). The effect from the internal solvent molecules is also described by the dielectric of 80 except for the water molecules ligated to the metal centers Mg^{2+} , Na^+ or Ca^{2+} . (For a discussion of dielectric properties of internal cavities and their possible dielectric constants, see Refs. ^{21,28}.) The ligands to heme a_3 and Cu_B are also treated implicitly by adding their charges to the corresponding metal centers. The calculations for all structures were performed on chains A and B. In addition, we included the membrane that shields the membrane-spanning helices of subunit A from the solvent. The membrane was modeled as a low dielectric slab ($\epsilon = 4$) of 40 Å.

The coordinates of hydrogen atoms were added and optimized by the AMBER7 package.²⁹ For this optimization, all titratable groups were in their standard protonation state (charged state for acidic and basic amino acids, neutral state for polar and hydrophobic amino acids, and reduced state for all redox-active centers).

The site-site interaction energies in the protein-membrane-solvent system were obtained by solving the Poisson equation as implemented in the MULTIFLEX program^{30,31} with a three-step grid focusing procedure.^{32,33} A solvent probe radius of 1.4 Å was used to define the dielectric boundaries. The external mobile ions and ionic strength were not included in the computation, because their influence on the pumping mechanism and catalytic function of CcO was found to be minor.⁷ The titration curves and bias energies of the protonatable groups of CcO were subsequently obtained by applying the Monte Carlo method as implemented in the KARSBERG program.³⁴

Most of the charges used in this application have been successfully used previously in calculations of redox potentials and protonation patterns of other proteins.^{7,32,33} Basically, we refer to the CHARMM22 partial atomic charges and radii,³⁵ when they are available, while charges of the residues in their nonstandard protonation states are taken from our previous publications.^{32,33} Several above water molecules included in the calculation explicitly were assigned the TIP3P charges.³⁶ The atomic partial charges for all four redox-active cofactors in their reduced state were computed as Mulliken charges using the ZINDO program.³⁷ In these quantum-chemical calculations, the redox centers were taken together with their ligated residues (bis-histidine ligated heme *a*, mono-histidine coordinated heme *a*₃, the Cu_A center consisting of the two Cu atoms, two His and two Cys, and the Cu_B center containing three ligated His and Tyr244 covalently bound to the His240). The atomic charges for the oxidized states of redox cofactors were obtained from the corresponding reduced states by evenly distributing a +1 charge between the metal ion and atoms directly coordinated to it.

The results of p*K*_a calculations at pH=7 for OORO and OORR redox state of CcO are summarized in Table 3. It is seen that in different organisms the p*K*_a values of His291 analogs are significantly lower than 7.0 when Cu_B cofactor is oxidized, while the values become dramatically higher than pH once Cu_B is reduced. Physically this means that His291 residue is rapidly protonated once the additional electron is supplied to BNC. The proton should be provided by one of closest sites which proton affinity in the OORR state is lower than one of His291, i.e. Glu242, Arg438 or Arg439 residue analogs. The most favorable candidate is Glu242 since in all structures it has lowest p*K*_a value. After donating a proton, Glu242 is quickly reprotonated, as its p*K*_a still high enough to pull a proton from the N-side of the membrane; thus Glu242 is acting as a proton intermediate. Therefore, one of the key elements of the mechanism is that upon BNC reduction, the proton translocation occurring from Glu242 to His291 is conserved in all considered organisms of CcO.

Another important point is that heme propionates remain deprotonated in both OORO and OORR states of the enzyme due to significant proton repulsion with salt-bridged positive residues Arg438 and Arg439. A possibility of protonation of both heme *a*₃ propionates has been often discussed in the literature, e.g. recently in Ref.¹⁴; however, the computational method used in our calculations does not predict such a possibility.

Table 3 lists essentially all the residues that have been discussed in the past as possibly having some relevance to proton translocation in CcO. Just a casual inspection of the calculated p*K*_a's of these groups shows that mostly their proton transfer properties (reflected in their p*K*_a's) if not identical, are very similar quantitatively[†]. This is quite remarkable, because the enzymes outside the two central subunits are in fact rather different. Moreover, the sequence similarity between the structures of the different organisms is below 50%. Given the time of genetic separation of the considered species, i.e. length of their separate

[†]It should be noted too that for residues that do not *directly* participate in proton translocation, it is their charge (i.e. protonation state) but not the specific value of p*K*_a that may be important, as long as the variations of p*K*_a do not affect the protonation state of the residue.

evolution, the precision of preservation of the properties of some of the key residues of the enzyme in completely different cellular environments is remarkable.

5. Conclusions

Three different forms of cytochrome c oxidase (bovine, *Rhodobacter sphaeroides* and *Paracoccus denitrificans*), all belonging to the same A-family, have been studied by a comparative analysis of the putative proton pumping functional groups. Though a constitution of all structures is generally different, a remarkable conservation of the key residues in different organisms is observed. The spatial distribution of the proton pumping residues also is matching with high precision (rmsd < 3.0 Å, see Table 2).

To perform further analysis we calculated pK_a values of titratable residues which in our earlier studies^{7,8} have been identified as possible proton pumping elements (see list in the Table 2). The proton translocation from Glu242 to His291 upon BNC reduction proposed for mitochondrial bovine heart CcO also most likely occurs in the bacterial enzyme, because the energetic characteristics of proton translocation between these groups are almost identical in all enzymes studied. Of course, our present conclusions, as well as the assumption of the His291 role, are based only on calculations; the accuracy of these and similar calculations arguably is difficult to estimate. However, even if the computational scheme used here is not sufficiently accurate (for example to distinguish different candidates for the Proton Loading Site^{10,11}), the observed quantitative similarity of the energy characteristics of the key residues in enzymes of different organisms is rather remarkable, and points to a strongly preserved, unique mechanism of proton pumping.

It is interesting to notice similarity of the pK_a values of the key residues between the wild type enzymes and 1M57 non-pumping mutant (with the exception of Glu242, which is mutated in 1M57 structure.) There were suggestions that the structural changes in this mutant, in particular around heme propionates, are of mechanistic significance, and could mimic what occurs when Glu-242 is deprotonated²⁵. The current work suggests that there is no major difference in the pK values of key residues. Also, there were reports of spectroscopic changes due to this mutation³⁸; the data indicate that the pK values may not be so sensitive.

The similarity in pK_a values of key proton-translocation residues between the two bacterial and the bovine enzymes suggest the similarity in their mechanism; this may also be used to argue against the model of proton pump proposed by Yoshikawa and coworkers, who suggest that the mammalian oxidase does not pump protons in the same way as do the two bacterial enzymes³⁹.

The need to conserve the details of the mechanism may be due to a relative complexity and non-trivial physical principles of the mechanism which requires subtle balance of both energetic and kinetic properties of the system. As shown recently^{40,41}, even a mutation of a seemingly unimportant residue (i.e. not blocking proton transfer) in the D-channel far removed from the catalytic site can render the enzyme incapable of proton pumping (but still able to reduce oxygen). Thus in some sense, there is no much freedom in variation of the

parameters for the enzyme to be functional, so that once found, the mechanism is necessarily preserved in further evolutionary steps. The mechanism by which key residues of the enzyme retained their proton-translocation properties in evolution, and stay fine-tuned to perform the proton pumping function, despite the significant differences accumulated in their overall structure, remain unclear and will require further studies. It will be also interesting in this respect to compare oxidases from B- and C- families.

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Fig.1.

(A) The key structural elements of the proposed pumping mechanism of CcO and the sequence of transitions during one pumping cycle. Two protonation sites, PLS and one in BNC, are shown as H-circles. PT and ET steps are shown by blue and red arrows, respectively. (B) The schematics of the model. The key assumption of the model is that the proton transfer rate upon ET between the hemes (step 2) is much higher along the pumping channel 3 than along the chemical channel 5, in other words step 3 occurs before step 5.

Fig.2.

Location of key residues in bovine cytochrome c oxidase. The residues are mainly distributed in subunit A, but those located in subunit B are marked with the letter "B" at the end of its label. D- and K-channel residues are shown in blue and brown color, respectively. Possible key residues of exit pathways are in purple color. Ligands of Cu_B co-factor are in red color and the hemes along with the salt-bridged Arginines are shown in cyan color.

Fig.3. Superimposed CcO Catalytic Centers: Bovine (red), *R. sphaeroides* (blue), *P. denitrificans* (cyan). The key residue labels correspond to the bovine structure. The structure matching has been done by VMD program and corresponds to the best fitting of heme porphyrin rings.

Table 1

Some comparative characteristics of CcO redox cofactors, and the number of titratable residues in the enzymes from different organisms.

life form		Bovine	R. Sphaeroides		P. Denitrificans
structure		2OCC	2GSM ^{*)}	1M57	1AR1
Redox cofactors	1	Cu_A	Cu_A	Cu_A	Cu_A
	2	<i>heme a</i>	<i>heme a</i>	<i>heme a</i>	<i>heme a</i>
	3	<i>heme a₃</i>	<i>heme a₃</i>	<i>heme a₃</i>	<i>heme a₃</i>
	4	Cu_B	Cu_B	Cu_B	Cu_B
Metal centers	1	Mg^{2+}	Mg^{2+}	Mg^{2+}	Mg^{2+}
	2	Na^+	Ca^{2+}	Ca^{2+}	Ca^{2+}
Titratable residues ^{**)}	Arg	14	21	24	18
	Asp ^{**))}	25	16	17	25
	Cys	1	-	-	4
	Glu ^{**))}	23	30	32	24
	His ^{**))}	15	16	15	15
	Lys	15	15	16	17
	Tyr	29	32	33	35

^{*)} 2GSM structure has also metal center Cd^{2+} added for technological reasons

^{**))} residues ligated to co-factor or metal center, are excluded from the statistics

Table 2

RMSD of the key residues with respect to bovine 2OCC structure

Organism		Bovine	R. Sphaeroides				P. Denitrificans	
structure		2OCC	2GSM		1M57		1AR1	
Redox cofactors	Cu _A	Cu228B	Cu3003	2.61	Cu1003B	2.54	Cu270B	2.53
		Cu229B	Cu3004	2.43	Cu1004B	2.61	Cu271B	2.47
	<i>heme a</i>	Hea515A	Hea2001A	1.01	Hea1001A	0.61	Hea562A	0.28
	<i>heme a₃</i>	Hea516A	Hea2002A	0.92	Hea1002A	0.36	Hea563A	0.62
	Cu _B	Cu517A	Cu3005	0.15	Cu1005A	0.24	Cu559A	0.72
		Tyr244A	Tyr288A	0.27	Tyr288A	0.53	Tyr280A	0.39
		His291A	His334A	0.21	His334A	0.33	His326A	0.56
Metal centers	Mg ²⁺	Mg518A	Mg3006	0.14	Mg2006	0.24	Mg560A	0.82
	Na ⁺	NA519A	Ca3007	2.96	Ca1007A	2.55	Ca561A	1.99
Protonatable key sites	D-channel entrance residues	His503A	His549A	0.63	His549A	1.17	His541A	1.15
		Asp91A	Asp132A	0.9	Asp132A	1.15	Asp124A	0.69
		His12A	His26A	1.09	His26A	1.74	His28A	1.41
		Tyr19A	Tyr33A	1.68	Tyr33A	1.72	Tyr35A	0.65
		Glu242A	Glu286A	0.91	Gln286A	1.23	Glu278A	0.73
	K-channel entrance residues	His395A ^{*)}						
		Glu493A	Glu539A	0.95	Glu539A	0.93	Glu531A	1.31
		His256A	His300A	0.36	His300A	0.44	His292A	0.57
		Lys319A	Lys362A	0.33	Lys362A	0.26	Lys354A	0.61
		Glu60B ^{*)}						
	Arginine salt bridge	Glu62B	Glu101B	2.54	Glu101B	1.8	Glu78B	1.31
		Arg438A	Arg481A	0.16	Arg481A	0.47	Arg473A	0.65
	Possible exit pathways	Arg439A	Arg482A	0.16	Arg482A	0.6	Arg474A	0.26
		His24B	His55B	6.83	His55B	7.03	His36B	5.76
		Asp25B	Asp58B	5.33	Asp58B	5.38	Asp35B	5.49
		Asp51A ^{**)}	Glu182A	0	Glu182A	0.61	Glu174A	1.42
			His93A	0	His93A	0.23	His85A	0.41
		Asp364A	Asp407A	0.52	Asp407A	0.82	Asp399A	0.68
		Ile365A ^{**)*)}	Arg408A	0	Arg408A	0.87	Arg400A	0.42
		Lys171B	Lys227B	0.66	Lys227B	1.27	Lys191B	0.7
		Asp173B	Asp229B	1.11	Asp229B	0.51	Asp193B	0.51

^{*)} These residues are included because of their possible role in the bovine enzyme; however, there are no their counterparts in the bacterial enzymes.

^{**)*)} Asp51A and Ile365A residues of bovine enzyme have no direct analogues in the enzymes of other organisms; the closest possible candidates are shown, rmsdes for those residues have been calculated with respect to 2GSM structure.

Table 3
pKa values of CcO key residues in different organisms. The location of residues in bovine CcO is shown in Fig.2.

	Bovine			R. Sphaeroides						P. Denitrificans		
	20CC			2GSM			1M57			IAR1		
	residue	pK _a ^{a)}		residue	pK _a ^{a)}		residue	pK _a ^{a)}		residue	OORO	OORR
D-channel entrance residues	His-D 503A	7.9	7.9	His-D 549A	7.7	7.7	His-D 549A	7.3	7.3	His-D 541A	8.5	8.5
	His-E 503A	7.3	7.3	His-E 549A	6.9	7.0	His-E 549A	6.9	6.9	His-E 541A	8.6	8.7
	Asp 91A	3.7	3.7	Asp 132A	4.4	4.4	Asp 132A	6.2	6.2	Asp 124A	5.4	5.4
	His-D 12A	1.9	1.9	His-D 26A	4.9	4.8	His-D 26A	6.8	6.8	His-D 28A	5.9	6.0
	His-E 12A	12.1	12.2	His-E 26A	9.2	9.2	His-E 26A	7.3	7.3	His-E 28A	8.5	8.5
	Tyr 19A	17.4	17.5	Tyr 33A	16.4	16.5	Tyr 33A	16.4	16.5	Tyr 35A	19.4	19.5
	Glu 242A	14.7	15.0	Glu 286A	12.7	12.9	Glu 286A	N/A	N/A	Glu 278A	12.5	12.7
	His-D 395A	7.3	7.3									
K-channel entrance residues	His-E 395A	7.9	7.9									
	Glu 493A	6.6	6.6	Glu 539A	-1.1	-1.0	Glu 539A	1.4	1.5	Glu 531A	0.1	0.2
	His-D 256A	4.5	4.5	His-D 300A	4.2	4.2	His-D 300A	5.4	5.4	His-D 292A	3.9	3.9
	His-E 256A	9.6	9.5	His-E 300A	9.8	9.8	His-E 300A	8.6	8.6	His-E 292A	10.1	10.1
	Lys 319A	-2.5	-2.0	Lys 362A	-2.5	-2.1	Lys 362A	-0.9	-0.6	Lys 354A	0.4	0.8
	Glu 60B	14.4	14.5									
	Glu 62B	12.8	13.0	Glu 101B	-26.6 ^{b)}	-26.6 ^{b)}	Glu 101B	8.4	8.6	Glu 78B	4.1	4.3
	Tyr 244A	19.6	23.5	Tyr 288A	17.4	21.4	Tyr 288A	18.4	22.0	Tyr 280A	18.5	22.5
CuB ligands	His 291A	2.5	18.1	His 334A	-2.1	14.0	His 334A	2.3	16.5	His 326A	1.9	16.2
Heme propionate	Heme a: A	4.5	4.0	Heme a: A	-1.0	-1.1	Heme a: A	-2.7	-2.9	Heme a: A	-1.5	-1.8
	Heme a: D	-5.8	-5.6	Heme a: D	-7.0	-6.2	Heme a: D	-7.7	-7.1	Heme a: D	-6.3	-5.6
	Heme a ₃ : A	2.8	0.8	Heme a ₃ : A	2.3	0.5	Heme a ₃ : A	1.5	-0.2	Heme a ₃ : A	6.1	4.2
	Heme a ₃ : D	-1.5	-3.4	Heme a ₃ : D	-2.4	-3.5	Heme a ₃ : D	-0.1	-1.3	Heme a ₃ : D	-1.1	-2.4
Arginine salt bridge	Arg 438A	15.5	16.3	Arg 481A	15.1	13.0	Arg 481A	14.0	13.5	Arg 473A	14.8	14.4
	Arg 439A	18.0	17.1	Arg 482A	15.6	13.8	Arg 482A	15.5	14.0	Arg 474A	15.2	14.3

	Bovine			R. Sphaeroides					P. Denitrificans			
	2OCC			2GSM			1M57			1AR1		
	residue	pK _a ^{a)}		residue	pK _a ^{a)}		residue	pK _a ^{a)}		residue	pK _a ^{a)}	
		OORO	OORR		OORO	OORR		OORO	OORR		OORO	OORR
Possible exit pathway	His-D 24B	7.0	7.0	His-D 55B	8.6	8.6	His-D 55B	8.3	8.3	His-D 36B	7.5	7.5
	His-E 24B	7.0	7.0	His-E 55B	5.5	5.5	His-E 55B	6.0	5.9	His-E 36B	6.5	6.5
	Asp 25B ^{c)}	8.2	8.2	Asp 58B ^{c)}	3.7	3.7	Asp 58B ^{c)}	8.3	8.5	Asp 35B ^{c)}	3.5	3.7
	Asp 51A	4.8	4.6	Glu 182A	5.2	5.0	Glu 182A	6.3	6.3	Glu 174A	4.9	4.8
				His-D 93A	12.7	12.7	His-D 93A	13.7	13.7	His-D 85A	13.6	13.5
				His-E 93A	6.1	6.0	His-E 93A	7.7	7.7	His-E 85A	8.4	8.3
	Asp 364A	11.4	13.2	Asp 407A	11.7	13.5	Asp 407A	12.5	14.0	Asp 399A	7.9	9.8
	Ile 365A	N/A	N/A	Arg 408A	13.5	13.3	Arg 408A	11.6	11.9	Arg 400A	11.9	11.6
	Lys 171B	14.1	13.7	Lys 227B ^{d)}	10.8	10.3	Lys 227B ^{d)}	6.7	6.1	Lys 191B	8.9	8.3
	Asp 173B	-2.9	-3.7	Asp 229B	-4.7	-5.3	Asp 229B	-0.6	-1.4	Asp 193B	0.5	0.2

a) In the notation of states, O and R mean formally oxidized (O) or reduced (R) state of each CcO redox center: Cu_A, heme a, heme a₃ and Cu_B, respectively.

b) The low pKa of Glu101B is an artifact due to the presence of Cd²⁺ ion located just 2 Å apart from this residue; ions Cd²⁺ are not natural, and were introduced to 2GSM structure in preparation procedure.

c) The pKa scattering for Asp25B analogs is a result of the presence of 2 more protonatable residues (Arg and Tyr) at a distance of a hydrogen bond in these structures. In this case, the pKa of the residue describes only the partial proton population of the residue in the group Asp-Arg-Tyr; the protonation of the whole group would be more correctly to consider.

d) The difference between 2GSM and 1M57 results here is due to the difference in the distances between the protonatable site of Lys227B and Mg²⁺ ion; it is 6 Å in 1M57 structure, while it is 7.5 Å in 2GSM structure.