Hydrogen/Hydride Ion Relay – A Mechanism for Early Electron Transfer in Cytochrome <u>c</u> Oxidases

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ABSTRACT

Cytochrome \underline{c} oxidase (COX) employs electrons obtained from cytochrome \underline{c} to bring about the reduction of oxygen to water. It is known that the electrons originate from the haem edge of cytochrome \underline{c} and enters bovine COX at Trp-104. It is also known that Tyr-105, Glu-198 and Asp-158 of COX subunit II play roles in the enzyme's catalysis but how these roles are linked to electron transfer remain unclear. Recently, we proposed that electrons travel from the haem edge of cytochrome \underline{c} to CuA, the first metal redox centre of COX, by a hydrogen/hydride ion relay using six residues. Now using a similar computer assisted approach, we investigate the extent to which this hydride/hydrogen ion mechanism is common amongst oxidases. The crystal structures of COX from P denitrificans, R sphaeroides and T thermophilus and quinol oxidase from E coli were downloaded and their binding domains analysed. As with bovine, all four oxidases had only nine amino acid residues in that region and both the sequences and three-dimensional structures were highly conserved. We propose that these residues function as a hydrogen/hydride ion relay, participating directly in electron transfer to CuA. We further suggest that this electron transfer mechanism might be a common feature in oxidases.

Keywords: Amino acid cable, bacterial oxidases, cytochrome <u>c</u>, cytochrome <u>c</u> oxidase, electron pathway, hydrogen/hydride ion relay

El Relé Iónico Hidrógeno-Hidruro: Un Mecanismo de Transferencia Temprana de Electrones en las Citocromo c Oxidasas

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RESUMEN

La citocromo c oxidasa (COX) emplea electrones obtenidos del citocromo c para producir la reducción del oxígeno a agua. Se sabe que los electrones originan a partir del hemo del citocromo c, y entran en la COX bovina en Trp-104. También se conoce que Tyr-105, Glu-198 y Asp-158 de la subunidad II de COX, desempeñan papeles en la catálisis de la enzima, pero no hay todavía claridad en cuanto a cómo estos papeles se hallan vinculados con la transferencia de electrones. Recientemente, sugerimos que los electrones viajan del borde del hemo del citocromo c al CuA, el primer centro metálico de reacción redox de la COX, por un relé iónico hidrógeno-hidruro, usando seis residuos. Ahora, usando un enfoque similar computarizado, investigamos hasta que punto este mecanismo de iones hidrógeno/hidruro es común entre las oxidasas. Se bajaron y analizaron los dominios de unión de las estructuras cristalinas de la COX de P denitrificans, R sphaeroides, y T thermophilus, y de la quinol oxidasa de la E coli. Como en el caso

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de la bovina, las cuatro oxidasas tenían sólo nueve residuos de aminoácido en esa región, y tanto las secuencias como las estructuras tridimensionales presentaban un alto grado de conservación. Proponemos que estos residuos funcionan como un relé iónico hidrógeno-hidruro, participando directamente en una transferencia de electrones al CuA. Asimismo, sugerimos que este mecanismo de transferencia de electrones podría ser un rasgo común de las oxidasas.

Palabras claves: Cable aminoácido, oxidasas bacterianas, citocromo c, citocromo c oxidasa, trayectoria del electrón, relé iónico hidrógeno-hidruro

West Indian Med J 2013; 62 (1): 4

INTRODUCTION

The general principles of the electron transport chain and its relationship to the production of ATP has been worked out more than two decades ago [1, 2). Electrons travel along the chain, the components of which are arranged in order of increasing redox potential (3). The ultimate acceptor of the electrons is molecular oxygen which is reduced to water (4). Cytochrome c oxidase (COX) is the final member of the electron transport chain and catalyses the transfer of electrons from cytochrome c, the penultimate member, to oxygen (4).

Cytochrome c oxidase is widely distributed throughout nature with the complexity of its structure showing a direct relationship with the complexity of the life form. Whereas in mammals the enzyme is dimeric with each monomer consisting of thirteen subunits (5), bacterial oxidases are much simpler structurally; they are monomeric and contain between two and five subunits (6, 7). A wide range of approaches, including NMR, EPR, kinetics, crystal structure analysis and optical spectroscopy, have established that for both classes of COX, the catalytic core of the enzyme comprises two redox active copper centres and two redox active haems located in subunits I and II (6–13).

In spite of the extensive research, the question of how exactly electrons are exchanged between cytochrome c and COX, and the mechanism by which COX move electrons from one of its redox centre to the next are still not well understood. A number of studies have suggested that the transfer of electrons from cytochrome c to COX occurs via the haem edge of the former (14). Witt et al (15), on the basis of site directed mutagenesis studies, have concluded that electrons enter bovine COX via Trp-104 located at the enzyme's binding domain. These workers had previously shown that Glu-246, Tyr-122 and Asp-206 of *P denitrificans* COX, which corresponds to Glu-198, Tyr-105 and Asp-158 of bovine COX are important for COX catalysis (16, 17). More recently, Alleyne and Sampson (18) proposed that a methyl group on the exposed haem edge of cytochrome \underline{c} catalyses the transfer of an electron to COX by formation of a positively charged Trp-104 intermediate.

Three methods for the movement of electrons between redox centres within biological systems are recognized. One method, quantum tunnelling, occurs through space and is independent of the properties of the structures that lie between the redox centres concerned (19, 20). A second method takes advantage of the loosely held π -electrons of stacked aromatic

residues (21). The third method is through bonds and depends critically on the structure and chemical properties of the groups that lie between the two redox active points (22). Recently, on the basis of a computer generated cytochrome <u>c</u>-COX enzymesubstrate adduct (23), we proposed that having entered bovine COX at Trp-104, the electrons travel by a hydrogen/hydride ion relay along a channel of aromatic and charged amino acid residues until they arrive at the enzyme's CuA centre (18). Now on the basis of sequence and structural homology, we present evidence for the existence of a similar pathway in the bacterial oxidases. We propose this mechanism of electron transfer as a general strategy for COX and related families like the quinol oxidases.

METHODS

The crystal structures of COX from the cow, *P denitrificans*, *R sphaeroides* and *T thermophilus* and quinol oxidase from *E coli* were downloaded from the Brookhaven Protein Data Bank. The Swiss-Pdb Viewer 4.0.1 developed at the Swiss Institute of Bioinformatics was used to analyse the structures. For the bovine enzyme, the electron transport pathway proposed previously (18) was located and the nine residues stretching from Trp-104 to the CuA centre identified. Next, for each bacterial oxidase, the nine residues corresponding to those of bovine origin were identified. Then, one at a time, the structure of the nine-residue sector of each bacterial oxidase was overlayed, using the computer, on to the corresponding nine-residue 'electron pathway' of bovine COX.

RESULTS

Structural analysis of bovine COX confirmed that, as previously reported (18), nine residues lie along the axis stretching from Trp-104 on the surface of subunit II to the CuA redox centre. The residues, (Trp-104, Tyr-105, Trp-106, His-102, Asp-158, Glu-198, Gly-101, Gln-103 and Asn-203) were mostly a mix of aromatics and charged amino acids (Table, Fig. 1A). Analysis of the structure of COX from the *P denitrificans* revealed an identical sequence of amino acid residues in the CuA region under investigation (Table). Moreover, computer manipulation of the three dimensional structures of the two proteins showed that the nine residues of *P denitrificans* COX could be perfectly aligned with those of the bovine enzyme (Fig. 1B). Of particular interest was the perfect alignment of the six (Trp-104, Tyr-105, Trp-106, His-102, Asp-158, Glu-198) thought to be involved in electron translocation.



Fig. 1: Proposed pathway for early electron transfer steps in cytochrome c oxidase (COX). It is widely agreed that electrons enter COX at Trp-104 for bovine (A) or at Trp-121 for *P denitrificans* (B). Figure 1B demonstrates total conservation of sequence and 3-D structure between bovine COX (blue) and *P denitrificans* COX (orange) in the enzymes' substrate binding domain. The CuA redox centre is shown as two green spheres. Structural alignment was performed with the Swiss Pdb Viewer 4.0.1.

 Table:
 Comparison of amino acids located between the putative electron entry point and CuA centre of cytochrome c oxidase (COX)

Bovine	P denitrificans	R sphaeroides	T thermophilus	Quinol oxidase
Trp 104	Trp 121	Trp 143	Phe 88	Trp 136
Tyr 105	Tyr 122	Tyr 144	Gly 89	Lys 137
His 102	His 119	Tyr 141	Phe 86	Met 134
Trp 106	Trp123	Trp 145	Tyr 90	Trp 138
Asp 158	Asp178	Asp 214	Asp 111	Ser 169
Glu 198	Glu 218	Glu 254	Gln 151	Glu 209
Gly 101	Gly 118	Gly 257	Ala 85	Ser 133
Gln 103	Gln 120	Gln 142	Ala 87	Asp 135
Asn 203	Asn 223	Ser 259	Gly 156	Gly 214

It is proposed that residues in **bold** form charge relays which facilitate electron transfer in the respective enzymes: Across the five species of COX, the residues are either totally or functionally conserved.

Analysis of the structure of *R sphaeroides* found that for this enzyme too, there was considerable conservation of both sequence and structure. Again, nine residues were found along the axis stretching from the proposed electron entry point at Trp-143 to the CuA centre and again this sequence showed excellent alignment with the corresponding region of the bovine enzyme (Fig. 2A). Moreover, of the six residues in the pro-



Fig. 2: Early electron transfer steps in cytochrome c oxidase (COX) are compared. In (A) and (B), the proposed pathway for early electron transfer steps for bovine oxidase is shown in blue. In (A) the 3-D structure of *R sphaeroides* COX is overlayed in white, while in (B), the 3-D structure of *T thermophilus* COX is overlayed in turquoise. In both cases, the CuA redox centre is shown as two green spheres. The figure reveals significant conservation of 3-D structures. Structural alignment was performed with the Swiss Pdb Viewer 4.0.1.

posed electron relay network, five were exactly the same in bovine and *R sphaeroides* COX (Table); the single change involved replacement of His-102 of the bovine with Tyr-141 in *R sphaeroides*.

For COX of *T* thermophilus origin, again there were nine residues in the channel stretching from the electron entry point (Phe-88) to the CuA centre and again this sequence showed excellent alignment with the corresponding region of the bovine enzyme (Fig. 2B). Of the six residues linked to electron transfer, only one residue (Asp) was totally conserved but another three were conserved functionally. Functionally, Trp-104 and His-102 of the bovine were replaced by Phe-88 and Phe-86 in the *T* thermophilus; similarly, Trp-106 of the bovine was replaced by Tyr-90 (Table). For the remaining two of the critical six, Tyr-105 was replaced by Gly-89 and Glu-198 was replaced by Gln-151.

Examination of quinol oxidase revealed nine amino acid residues in the region between the exposed Trp-136 and CuA (Fig. 3, Table). As with COX from P denitrificans, R



Fig. 3: Early electron transfer steps in cytochrome c oxidase (COX) are compared. The proposed pathway for early electron transfer steps for bovine oxidase is shown in blue with the 3-D structure of quinol oxidase overlayed in red. The CuA redox centre is shown as two green spheres. The figure reveals significant conservation of 3-D structures. Structural alignment was performed with the Swiss Pdb Viewer 4.0.1.

sphaeroides and T thermophilus, the nine amino acids showed high structural conservation and could be aligned with the corresponding region for bovine oxidase. Of the six identified as the probable electron transfer route, three (Trp-136, Trp-138 and Glu-209) were totally conserved while the other three exhibited conservation of function. For example, Tyr-105 of the bovine was replaced by Lys-137, Met-134 replaced His-102 of bovine and Ser-169 replaced Asp-158. In all three cases, the substituted residue was capable of hydride/hydrogen ion relay, by making use of lone pairs of electrons, on oxygen, sulphur or nitrogen. Schemes 1–4 set out the mechanism of early electron transfer for COX from *P denitrificans, R sphaeroides* and *T thermophilus* and quinol oxidase from *E coli*.

DISCUSSION

Witt *et al* (15) have argued that electrons enter COX at Trp-104 of subunit II of the bovine enzyme and at equivalent positions in other oxidases. What remained unresolved is the mechanism by which electrons move from the entry point to CuA, which has been shown to be the first metal centre in COX to be reduced (9, 24). According to existing theories, there are three possibilities: the electrons can be moved from one centre to the other by quantum tunnelling (19, 20), by delocalization of electrons associated with aromatic rings or through bonds (21, 22). This work and our previous study (23) support the latter.

In our previous computer modelling studies (23), we showed that only nine residues (Trp-104, Tyr-105, Trp-106,

His-102, Asp-158, Glu-198, Gly-101, Gln-103 and Asn-203), all of subunit II, lie along a channel, 10 Å in diameter, that ran from Trp-104 on the surface to the CuA centre of bovine COX. In the conformational changes that accompany energy minimization of that cytochrome c-COX enzyme-substrate complex, His-102 and Trp-106 interchanged positions. In that work, we proposed that six of the nine residues located in the channel formed a hydride/hydrogen ion relay which moved electrons from Trp-104 to CuA. In a recent study in which the structures of oxidized and reduced COX were compared (25), we presented strong evidence that redox-linked conformational changes within bovine COX play vital roles in that enzyme's mode of action. In our present study, we have observed that COX isolated from P denitrificans, R sphaeroides and T thermophilus and the quinol oxidase from E coli each possessed a similar nine-residue sequence in its cytochrome \underline{c} binding domain to that seen in bovine COX, suggesting commonality in their mode of action.

As with the bovine, the nine residues identified in these other four oxidases fell into two groups. Six residues formed a 'cable' along one side while the other three were somewhat laterally displaced from this main group. Between the bovine and the *P denitrificans*, there was 100% conservation of the nine. The *R sphaeroides* oxidase showed only two substitutions. One was Tyr-141 for His-102 in the group of six and Ser-259 for Asn-203 in the group of three (Table). For the *T thermophilus* and the quinol oxidases, generally there was conservation of function in the group of six but not in the group of three (Table). We consider this conservation of primary sequence and three-dimensional structure across these five oxidases to be significant; it points towards the residues serving a specific purpose.

Further inspection of the structure of the residues in that group of six detects a mixture of aromatics, charged or polar amino acids that could facilitate the relay of electrons; we think that the other three residues probably have a role in maintaining structure. In the case of the earlier studies on bovine COX (18, 23), energy minimization of the cytochrome c-COX enzyme-substrate complex led to an exchange of positions of His-102 and Trp-106. We are proposing that since all of the oxidases examined in the present study had similar structures in that CuA region, similar conformational changes would accompany their catalytic turnover. The outcome would be a similar switch in the position of the residues equivalent to His-102 and Trp-106 of the bovine.

Schemes 1–4 summarize the proposed electron transfer mechanisms for the early stages of electron transfer in COX derived from *P denitrificans*, *R sphaeroides*, and *T thermophilus* and quinol oxidase from *E coli*. Scheme 1 for the *P denitrificans* is identical to that proposed for bovine COX (18). In stage 1, Trp-121 receives a hydride ion from a methyl group on cytochrome c forming a positively charged tryptophan intermediate. In stage 2, line B, partially charged Tyr, His and Trp develop as the hydride ion from the charged Trp-121 is passed



Scheme 1: Proposed mechanism for early electron transfer steps of cytochrome c oxidase (COX) from *P denitrificans*. The proposed mechanism is identical to that proposed for bovine (12). The residue numbering for the *P denitrificans* is shown above that for bovine which is in parentheses.

Step 1: A hydride ion from the haem of cytochrome <u>c</u> is transferred to Trp-121, either at the nitrogen (intermediate I) or the adjacent carbon (intermediate II): There is concomitant oxidation of the haem and reduction of the Trp-121 in the process.

Step 2: Hydride ions (coloured) are relayed from the activated Trp-121 intermediate to Glu-218, which is liganded to CuA. The latter centre, which is initially oxidized (A), becomes reduced in the process (C). Possible intermediates are shown in section B.

on. In the rate limiting step, Asp-178 passes a hydrogen ion to Glu-218, leading to reduction of CuA.

For *R sphaeroides* (Scheme 2, Fig 2A), the mechanism is the same as the bovine and *P denitrificans* except that Tyr-141 replaces His-102 of the bovine. In case of *T thermophilus*, Phe-88 replaced Trp-104 at the entry point, Phe-86 replaced His-102 and Tyr-90 replaced Trp-106. Finally Gln-151 re-

placed Glu-198 as the terminal residue of the group (Table, Scheme 3). Among these latter changes, two features stood out. First we could not assign a role for the translocation of a hydrogen/hydride ion to Gly-89, so the scheme for the *T thermophilus* is one residue shorter than the others with a bigger than normal gap between the first and second members of the relay. Given that the *T thermophilus* functions at elevated tem-



Scheme 2: Proposed mechanism for early electron transfer steps of cytochrome c oxidase (COX) from *R sphaeroides*.

Step 1: A hydride ion from the haem of cytochrome <u>c</u> is transferred to Trp-143, either at the nitrogen (intermediate I) or the adjacent carbon (intermediate II); there is concomitant oxidation of the haem and reduction of the Trp-143 in the process.

Step 2: Hydride ions (coloured) are relayed from the activated Trp-143 intermediate to Glu-254, which is liganded to CuA. The latter centre, which is initially oxidized (A), becomes reduced in the process (C). Possible intermediates are shown in section B. The mechanism is the same as outlined for bovine and *P denitrificans* (Scheme 1) except that Tyr-141 replaces His-102 and His-119, respectively.

peratures, we anticipate that the conformational changes occurring during turnover would be enhanced and would be sufficient to overcome this bigger than normal gap. The second area of interest was the substitution of Gln-151 for Glu-198; for all of the other oxidases, this CuA ligand was glutamic acid so that protonation-deprotonation was a well-understood process. Interestingly, X-ray diffraction studies (26) have demonstrated that the carbonyl group of amines can accept a proton. Again, the elevated temperatures in which the *T thermophilus* functions would facilitate this reaction. As such, Gln-151 can take part in the proposed ion relay as presented in Scheme 3.

The quinol oxidase was particularly interesting as it was the only one of the five examined that had a positively charged



Scheme 3: Proposed mechanism for early electron transfer steps in cytochrome c oxidase (COX) from *T thermophilus*. Step 1: A hydride ion from the haem of cytochrome <u>c</u> is transferred to Phe-88: There is concomitant oxidation of the haem and reduction of the Phe-88 in the process. Step 2: Hydride ions (coloured) are relayed from the activated Phe-88 intermediate to Gln-151, which is liganded to CuA. The latter centre, which is initially oxidized (A), becomes reduced in the process (B). Possible intermediates are shown in section A.

residue in the 'channel of nine'; also, compared to the others it had only two rather than three aromatic residues in this section. For this enzyme, Lys-157 replaced Tyr-105, Met-134 replaced His-102 and Ser-169 replaced Asp-158. In their work which proposed Trp-104 as the unique electron entry point for bovine COX, Witt *et al* (15) were unable to reconcile the presence of a positively charged lysine residue near to the electron entry point of the quinol oxidases. The lysine posed two problems. It could not facilitate the movement of electrons by loosely held π -electrons normally associated with aromatic rings. Secondly, it was felt that the lysine would increase the net positive charge on the molecule leading to repulsion of the substrate. Looking first at the charge, the introduction of a positive charge by lysine is neutralized by the loss of the adjacent histidine. Similarly, compared to the bovine, one acid residue is lost and one is gained in the group of nine (Table), so that overall there is little or no net change of charge in the substrate binding region. In terms of function, while lysine would not be able to contribute to the delocalization of electrons between aromatics, it is perfectly able to participate in the relay of a hydride/hydrogen ion as illustrated in Scheme 4. By making use of lone pairs of electrons on sulphur and oxygen, the same is also true for Met-134 and Ser-169, respectively, thus making Scheme 4 very plausible.



Scheme 4: Proposed mechanism for early electron transfer steps in quinol oxidase.

Step 1: A hydride ion from the haem of cytochrome \underline{c} is transferred to Trp-136, either at the nitrogen (intermediate I) or the adjacent carbon (intermediate II); there is concomitant oxidation of the haem and reduction of the Trp-136 in the process.

Step 2: Hydride ions (coloured) are relayed from the activated Trp-136 intermediate to Glu-209, which is liganded to CuA. The latter centre, which is initially oxidized (A) becomes reduced in the process (D). Possible intermediates are shown in sections B and C.

In all four cases considered, it was found that the region identified by Sampson *et al* for the bovine enzyme (23) was highly conserved and the folding was to all intent and purposes identical, allowing for almost perfect overlay of the five proteins. On top of that, in those instances where an amino acid was substituted, the replacement was, with one exception, always of the chemical type that could participate in the proposed charge relay *ie* there was conservation of function.

Overall, the high conservation of residues, the conservation of chemical function, combined with the alignment and location of the group of amino acids identified, all point to the residues serving a particular function: electron transfer. The charge relay would also account for the findings of Witt *et al* (15–17) who (i) identified Trp-104 of bovine COX and its equivalent in other oxidases as the electron entry points for COX and (ii) ascribed roles for bovine Tyr-105, Asp-158 and Glu-198 and their equivalents in COX catalysis.

In conclusion, it seems that the chymotrypsin-like charge relay mechanism previously proposed for bovine COX might be a general mechanism for early electron transfer steps in oxidases. Gennis (27) has proposed a similar mechanism, ie the use of a 'proton wire' as the method by which COX move protons from the matrix to the intermembrane space. The advantage of a relay system is that it is possible to have the net movement of a proton/hydride ion quickly, over a long distance, without any single charged species having to move very far. The fact that both the amino acids and the general structure in the region of the CuA centre have been conserved in the five oxidases examined is a very strong argument in support of redox reactions occurring via a proton/hydride ion relay mechanism. It would be interesting to see whether other electron transport chain components possess similar amino acid sequences and three-dimensional structures in the vicinity of their respective redox centres. It is possible that this discovery may open up the door for the synthesis of redox active peptides and proteins.

ACKNOWLEDGEMENTS

We thank Professor Peter Nicholls and Professor Mike Wilson for comments and discussions. We also thank Mr Michael Khan and Ms Heather Woodroffe for assistance with preparation of figures. We thank the Research and Publication Committee, The University of the West Indies, St Augustine for financial support.

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