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*Minireview*

# Binuclear centre structure of terminal protonmotive oxidases

Simon Brown, A. John Moody, Roy Mitchell and Peter R. Rich

*Glynn Research Institute, Bodmin, Cornwall, PL30 4AU, UK*

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The recent proliferation of data obtained from mutant forms of cytochrome oxidase and analogous enzymes has necessitated a re-examination of existing structural models. A new model is proposed, consistent with these data, which brings several protonatable residues (Y244, D298, D300, T309, T316, K319, T326) into the vicinity of the binuclear centre, suggestive of a proton-transferring function. In addition, we also consider those residues which may participate in electron transport between $Cu_A$ and haem *a*. We suggest several potential lines of investigation.

Cytochrome *c* oxidase; Cytochrome *bo*; structure; Sequence conservation

## 1. INTRODUCTION

Cytochrome *c* oxidase has four redox centres which form a haem–copper binuclear centre (haem *a*3 and $Cu_B$), a low-spin haem (haem *a*) and $Cu_A$. These mediate the reduction of oxygen, which is linked to the translocation of protons. The two haems and $Cu_B$ are ligated to subunit I of the enzyme and $Cu_A$ is ligated to subunit II. Within subunit I there are seven highly conserved histidines (H61, H240, H290, H291, H368, H376, H378, in the bovine cytochrome oxidase sequence, corresponding to H106, H284, H333, H334, H411, H419 and H421, respectively, in the sequence of the structurally related cytochrome *bo* from *Escherichia coli*), six of which are necessary to provide ligands to the three redox centres (Fig. 1) [1–4].

Biophysical measurements suggest that the six-coordinate, low-spin haem (*a* or *b*) has two histidine ligands, that the five-coordinate, high-spin haem (*a*3 or *o*) has only one histidine ligand from the protein, and that $Cu_B$ has either two or three nitrogenous ligands. Some models of subunit I have haem *a*3 ligated to H368, which is in the sequence linking helices IX and X in Fig. 1 [5], but recent results from site-directed mutants of bacterial oxidases [3,4,6] have shown that this residue can be replaced (variously with Ala, Leu or Gly) without loss of the high-spin haem. These same authors have shown that haem *a* is ligated by H61 and H378, and that replacement of H290 or H291 with alanine leads to a loss of copper from subunit I [3]. These assignments leave only the third ligand to $Cu_B$ and the ligand to haem *a*3 undetermined. Of the two remaining conserved histidines, recent models have assigned H240 as the ligand to haem *a*3 and H376 as the third ligand to $Cu_B$ [3,4,6]. This arrangement is consistent with previous assignments of the $Cu_B$ ligands which were based on geometric constraints [1,2] and EXAFS data, showing that $Cu_B$ is coordinated by at least three nitrogenous ligands, at least one of which is an imidazole [7–10]. However, the data are also consistent with an alternative assignment where H240 is a ligand to $Cu_B$ and H376 is the ligand to haem *a*3. We favour this arrangement because it brings the highly conserved E242 into the vicinity of haem *a*3, where it may act as the 'slow' ligand [11,12]. We have gone on to consider those residues which might be involved in the catalytic cycle of the enzyme. A preliminary report of this work has appeared elsewhere [12].

*Correspondence address.* P.R. Rich, Glynn Research Institute, Bodmin, Cornwall, PL30 4AU, UK. Fax: (44) (20882) 575.

## 2. BIOPHYSICAL CHARACTERISTICS OF THE REDOX CENTRES IN SUBUNIT I

It has been shown for both cytochrome *c* oxidase and cytochrome *bo* that both haem planes are approximately perpendicular to the plane of the membrane [13,14]. The haems are 12–16 Å; apart (Fe–Fe distance) [15,16] and the Fe-Fe vector is 30–60° from the membrane normal [15], which corresponds to a 'vertical' displacement of 8 ± 1.4 Å; [15]. In the model shown in Fig. 1B the 'vertical' Fe–Fe distance is 3–4 Å, corresponding to an Fe–Fe vector which is 10–20° from the membrane normal, and the lateral distance is 12–16 Å. The 'vertical' distance is less than that obtained experimentally, but no attempt has been made to consider the possibility of tilting either or both of helix II and helix X. The distance between $Cu_B$ and haem *a*3 has been

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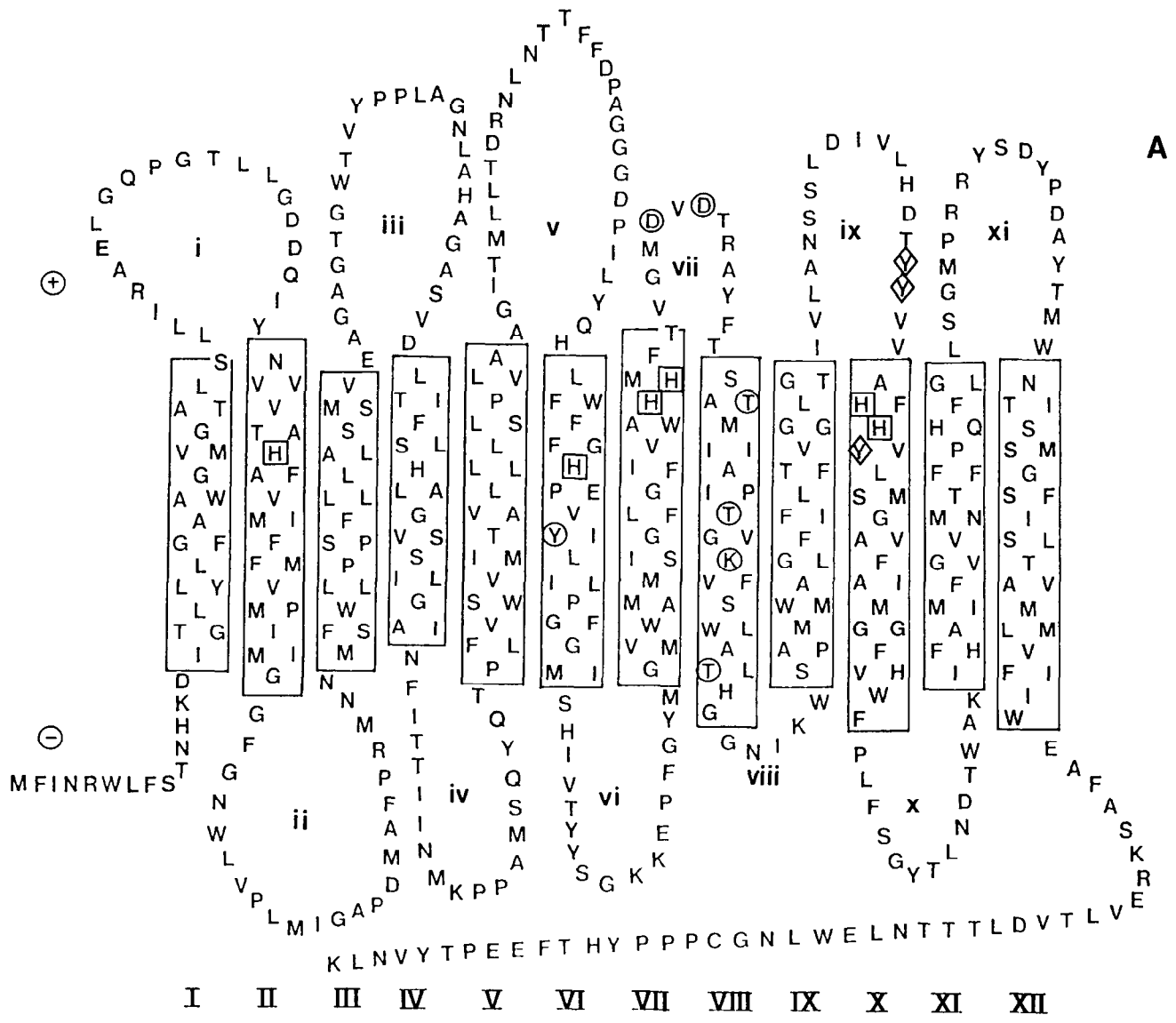
variously reported at up to about 5.25 Å [17,18] and $Cu_A$ is 8–13 Å from haem *a* [19].

The close proximity of these redox centres causes electrochemical and magnetic interactions [20], although the CD spectrum does not reveal strong exciton coupling [21]. This contrasts with the situation in a variety of cytochrome $bc_1$ and $b_6f$ complexes in which there is a strong exciton coupling feature in the CD spectra [22] and it is difficult to detect redox interactions between the haems [23]. These differences between cytochrome oxidase and the $bc_1/b_6f$ complexes suggest that the structures are significantly different [21] in that the haems in the cytochrome oxidases are unlikely to be distributed 'vertically' across the membrane dielectric between the same pair of α-helices as they are in *b*-type cytochromes [24,25].

Data from MCD, EPR and Mössbauer measurements indicate that the low-spin haem is ligated by two histidines [18,26,27], while the high-spin haem is ligated by only one histidine [18,26,28], consistent with data from EXAFS [7,9] and ENDOR [8]. Mössbauer data, using yeast cytochrome *c* oxidase, indicated that about a third of haem *a*3 was low spin and presumably six coordinate [27]. Heterogeneity of haem *a*3 has also been observed in the cytochrome $c_1aa_3$ from *Thermus thermophilus* [29]. This may reflect a preparation artefact, such as variable amounts of 'slow' and 'fast' forms of the enzyme caused by heterogeneous haem *a*3–$Cu_B$ ligands [10], since MCD measurements indicate that there is only one low-spin haem in the bovine enzyme [30].

The number of nitrogenous ligands to $Cu_B$ remains an open question. Earlier EXAFS data indicate that there are only two [7], but more recent EXAFS and ENDOR data suggest that there are three [8,9]. Simulations of EPR measurements on cytochrome $ba_3$ from *T. thermophilus* were made on the basis of four, almost



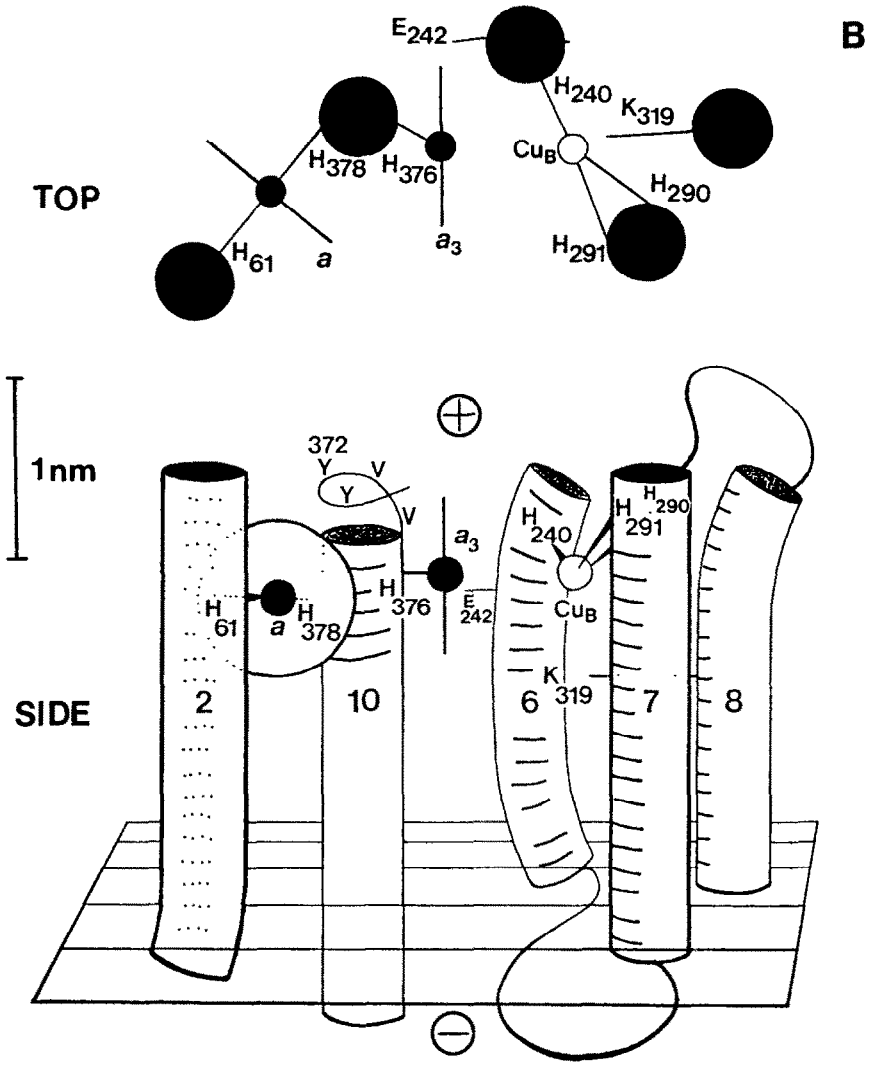



Fig. 1. Folding of bovine cytochrome *c* oxidase (A) and the model of subunit I of the enzyme (B) described in the text. Large Roman numerals denote the different helices and small Roman numerals correspond to the loops connecting helices. □, histidines taken to be ligands to the redox centres; ○, residues identified as potentially involved in protonation/deprotonation reactions; ◇, residues identified as potentially important in electron transport. The arrangement of the helices in B is only one of several possibilities.

equivalent, nitrogenous ligands to $Cu(II)_B$ which are arranged in a square planar configuration [31]. The ENDOR spectra, which are similar to those obtained with the bovine heart cytochrome *c* oxidase, were interpreted as indicating three or more nitrogenous ligands [31]. These authors favour the hypothesis that there are four histidine ligands to $Cu_B$. If this is the case it would suggest that the enzyme is quite different from other known oxidases, in spite of the similarity of its redox centres to other oxidases [32]. The most likely conclusion is that $Cu_B$ is ligated by three histidines in cytochrome *c* oxidase and in most bacterial quinol oxidases.

Haem A and haem O differ merely in that the haem A formyl group at pyrrole ring D is replaced with a methyl group in haem O [33], so it is likely that the amino acids which charge compensate the propionate groups are conserved (possibilities include Y54, T369, Y371 and Y372). Both haems possess a farnesyl side chain [33], but neither the role, if any, nor the location in the protein of this group, are known. It has clear similarities with the phytyl chain of (bacterio)chlorophyll, and some strains of *Chlorobium* and *Chloropseudomonas* have a chlorophyll in which the phytyl chain is replaced with a farnesyl group [34]. Unfortunately, the role of the phytyl chain in bacterial photosynthesis, if there is one, is not known, and suggestions that it might play a role in electron transport [35] have received only indirect support [36].

## 3. A NEW STRUCTURAL MODEL OF CYTOCHROME *c* OXIDASE AND CYTOCHROME *bo*

A topological folding model of subunit I of the bovine enzyme is shown in Fig. 1A. This has been deduced

| | Helix II ▽ | | | | ▲ | | Helix VI ▽ | | | | ▲ | | Helix VII ▽ | | | | ▲ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *B. taurus* | NVVVT | AHAFV | MIFFM | VMPIM | IG | --- | LFWFF | GHPEV | YILIL | PGFGM | I | --- | GMVWA | MMSIG | FLGFI | VWAHH | MFT |
| *S. cerevisiae* | ..L.V | G..VL | ....L | ...AL | .. | --- | ..... | ..... | ....I | ....I | . | --- | S..Y. | .A... | L...L | ..S.. | .YI |
| *P. denitrificans* | ..MI. | Y.GVL | .M..V | .I.AL | F. | --- | IL... | ..... | ..I.. | ....I | . | --- | P..L. | .AA.. | I...V | ..... | .Y. |
| *B. subtilis* $aa_3$-605 | .E.M. | M.GTT | ...LA | A..LL | FA | --- | ...I. | ..... | ..... | .A..I | F | --- | S..F. | IV-L. | ....M | ..V.. | ... |
| *H. halobium* | .GLL. | S.GIT | .L.LF | GT.MI | AA | --- | ..... | ..... | .V.V. | .PM.I | V | --- | FV.YS | TLA.. | V.S.G | ..... | ... |
| *T. thermophilus* | .QIL. | L.GAT | .L..F | IIQAG | LT | --- | F...Y | S..T. | .VML. | .YL.I | L | --- | Q.... | Q.G.V | V..TM | ..... | ... |
| *S. acidocaldarius* | YSAL. | I.GWA | AMIAF | .PMAA | AA | --- | ....Y | ...V. | .YVPF | .L..A | L | --- | WAR.N | IYLLA | IGTMG | ..V.. | LQ. |
| *E. coli* | DQIF. | ..GVI | ....V | A..FV | .. | --- | .I.AW | ..... | ..... | .V..V | F | --- | SL... | TVC.T | V.S.. | ..L.. | F.. |
| *B. subtilis* $aa_3$-600 | .EIF. | T.GTM | I..MA | MPFLI | GL | --- | ...I. | ..... | ..V.. | .A..I | F | --- | A..GS | IIA.S | V.S.L | ..T.. | F.. |

| | Helix VIII ▽ | | | | | | ▲ | | Helix X ▽ | | | | | ▲ | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *B. taurus* | VGMDV | DTRAY | FTSAT | MIIAI | PTGVK | VFSWL | ATLHG | --- | HDTYY | VVAHF | HYVLS | MGAVF | AIMGG | FVHWF | --- |
| *S. cerevisiae* | ..L.A | ..... | ..... | ..... | ...I. | I.... | ..IY. | --- | ..... | ..G.. | ..... | ...I. | SLFA. | YYY.S | --- |
| *P. denitrificans* | A..SL | TQQ.. | .ML.. | .T..V | ...I. | ....I | ..MW. | --- | ..... | ..... | ..... | ...M. | L.... | G.FA. | VYY.I | --- |
| *B. subtilis* $aa_3$-605 | T.LGP | IAN.I | .AV.. | .A... | ...I. | I.N.. | L.IW. | --- | ....F | ..... | ..... | ...II | G.V.. | GLLA. | VHF.W | --- |
| *H. halobium* | T.I.P | RI.SS | .MAVS | LA.S. | .SA.. | ..N.I | T.MWN | --- | ..... | ..G.. | .FIVY | GAIG. | .LFAA | SYY.. | --- |
| *T. thermophilus* | ..EST | LFQIA | .AFF. | AL..V | ..... | L.NII | G.LW. | --- | ..S.F | ..... | .N..M | A.SG. | GAFA. | LYY.W | --- |
| *S. acidocaldarius* | WPLPI | VL.EW | VNLS. | L.L.T | GS.LT | .LNLG | L.IFT | --- | .NS.. | ..G.. | .LMIW | TLIIM | GYTTV | .LDML | --- |
| *E. coli* | M.AGA | NVN.F | .GIT. | ..... | ..... | I.N.. | F.MYQ | --- | .NSLF | LI... | .N.II | G.V.. | GCFA. | MTY.W | --- |
| *B. subtilis* $aa_3$-600 | M.NSA | SVNSF | .SIT. | .A.S. | ..... | I.N.. | F.MYK | --- | .N..F | L.S.. | ....I | A.T.. | .CFA. | .IF.Y | --- |

Fig. 2. Alignment of some of the putative helical regions of several sequences of subunit I. Sequences are of $aa_3$-type oxidases except for *E. coli* (cytochrome *bo*), and *T. thermophilus* (cytochrome caa3). The substrate is ferrocytochrome *c* (*B taurus*, *S. cerevisiae*, *P. denitrificans*, *B. subtilis* $aa_3$–605, *T. thermophilus*) or quinol (*E. coli*, *S acidocaldarius*, *B. subtilis* $aa_3$–600). These sequences have been chosen deliberately to demonstrate the range of variation in these highly conserved regions. Residues identical to those in the bovine sequence are indicated by dots, and dashes indicate omitted intervening sequences. Arrows mark the start (▽) or end (▲) of helices. References to sources of sequences are given by Saraste [2], except for *H halobium* [68], *S. acidocaldarius* [69] and *B. subtilis* $aa_3$–600 [70].

from hydropathy, other folding models devised for this and analogous enzymes [2–4,6,37], sequence conservation, transmembrane charge distribution and the position specificities of different amino acids [38]. These helices are no longer than they need to be in order to span the bilayer (≈ Å). The transmembrane helices have negligible hydrophobic moments ($<\mu_H> < 0.22$ residue$^{-1}$), consistent with the absence of any particular structure in the charge distribution around the helical wheel (Table I). There are only two charged residues within the putative transmembrane helices (E242 and K319), both of which are highly conserved (E242 is absent only in *T. thermophilus* and *Sulfolobus acidocaldarius*, K319 is absent only in *S. acidocaldarius*), and the remaining charged residues are outside the helical regions. The charged residues are distributed with a 'net positive charge' on the electrically negative matrix side and a 'net negative charge' on the positive side of the membrane (Fig. 1A, Table II), as has been observed for other membrane proteins [39,40].

There is significant variation in mutability moment* both between helices and within individual helices (Table I). Helix VI has the lowest mean mutability (<M>) and a negligible mutability moment ($<\mu_M>$), but helices VII, VIII and IX have larger $<\mu_M>$ (Table I). Where $<\mu_M>$ is significant, there is an asymmetric distribution of mutability along the length of the helix, as well as around the circumference of the helical projection. Generally, the mutability is least in the vicinity of putative ligands to the redox centres.

The sequence between helices is generally more conserved when it protrudes into the positive side rather than the negative side (Table II). Presumably this reflects the need to maintain the interactions with other subunits necessary for structural integrity and efficient electron transport.

The presence of proline residues in the body of an α-helix may cause a bend [38,41]; this may be the case for helix VI where there is a completely conserved proline at each end of the helix (Fig. 1). Such a structure is reminiscent of helix C of the L subunit of the bacterial reaction centre, [42,43], which is bent.

On the basis of biophysical data, site-directed mutagenesis and sequence comparison, we have devised the model of the bovine enzyme shown in Fig. 1B. Haem *a* is ligated by H61 (helix II) and H378 (helix X) since replacement of either of these residues (with Asn, Ala, Gln, Gly, Leu or Met) leads to the loss of the low-spin haem [3,4,6]. Haem $a_3$ is ligated by H376 (helix X), bringing E242 into the vicinity of the haem, and $Cu_B$ is ligated by H290, H291 (both helix VII) and H240 (helix VI), in accordance with the assumption that there are three ligands. Where a helix has a particularly con-

*M is calculated as a scaled entropy: $M_i = \Sigma_x\, p\ln(p)/\ln(0.05)$, where $p$ is the probability that the amino acid at position i is $x$. When a residue is completely conserved $M_i = 0$, and $M_i = 1$ when all amino acids are equally likely. This measure of mutability correlates well with the variability index employed by Komiya et al. [43] ($r = 0.924$ for the 459 residues considered in Tables I and II.

served face it is oriented towards the binuclear centre. Also shown is helix VIII, which has a single conserved lysine (K319) in the middle of the conserved face. We have proposed previously that this may play some part in the access of protons to the binuclear centre [44].

A consequence of both this model (Fig. 1B) and the alternative assignment of the ligands [3,4,6] is that the three redox centres are located close to the positive side of the membrane. The bilayer surface is estimated to be about 15 Å from the middle of the enzyme (overall height is about 110 Å) [45]. Therefore, it seems likely that the redox centres are situated approximately in the middle of the enzyme. If the effective dielectric distance corresponds approximately to the bulk of the enzyme between the redox centres and the nearest aqueous surface of the enzyme, this is roughly consistent with measurements which place haem *a* about halfway across the membrane dielectric and $Cu_B$ slightly nearer the negative side [15,46–48].

## 4. PROTON CHANNELS INTO AND OUT OF THE BINUCLEAR CENTRE

During enzyme turnover, oxygen intermediates (P and F) are formed which must be protonated from the negative side [44,49,50]. Protons are also translocated across the membrane. It seems reasonable, at present, to postulate a common proton channel into the binuclear centre for both protonation requirements.

Several amino acids have a side chain with a p*K* which is likely to be accessible in the vicinity of the binuclear centre, where it has been estimated that pH ≈ 10 under energized conditions [50], but only two are present in those helical regions of subunit I facing the redox centres: Y244 (for the free amino acid, p*K* = 10.1) and K319 (p*K* = 10.5). We have proposed previously [12] that the proton channel into the binuclear centre might involve Y244 on helix VI (completely conserved among the 35 sequences analyzed) and K319 (replaced with a threonine in *S. acidocaldarius*, but otherwise completely conserved), which is situated between Y244 and the binuclear centre. A third conserved residue (E243, p*K* = 4.1 for the free amino acid) may be involved in protonation reactions, but it is oriented towards the edge of the high-spin haem in our model (Fig. 1B). We have suggested that this residue might be involved in generation of the 'slow' form of cytochrome *c* oxidase [12], because it is the only conserved carboxylate residue in the putative helices and carboxylates are likely candidates for the 'slow' ligand [11].

There are several highly conserved serine (p*K* ≈ 13.6) and threonine (p*K* ≈ 13.6) residues in the vicinity of the binuclear centre (amongst these are T309, T316 and T326 on helix VIII and T294 on helix VII, Fig. 1A), which might be deprotonatable in the environment of the protein. For example, a serine or a threonine at position 223 in the L subunit of the photosynthetic reaction centre of *Rhodobacter sphaeroides* is necessary for rapid protonation of $Q_B$ [51]. The face of helix VIII containing T326, K319, T316 and T309 is oriented to-

Table I

Characteristics of the putative transmembrane helices shown in Fig. 1A.

| Helix | Region | <H>[a] | $<\mu_H>$[b] | $<\mu_H>/<H>$ | <M>[c] | $<\mu_M>$[d] | $<\mu_M>/<M>$ |
|---|---|---|---|---|---|---|---|
| I | 15–34 | 0.65 | 0.20 | 0.30 | 0.37 | 0.054 | 0.14 |
| II | 55–76 | 0.74 | 0.11 | 0.15 | 0.28 | 0.039 | 0.14 |
| III | 100–118 | 0.63 | 0.10 | 0.16 | 0.37 | 0.030 | 0.08 |
| IV | 145–162 | 0.66 | 0.22 | 0.34 | 0.31 | 0 014 | 0.04 |
| V | 182–203 | 0.76 | 0.04 | 0.05 | 0.29 | 0 031 | 0.11 |
| VI | 234–254 | 0.73 | 0.04 | 0.06 | 0.13 | 0.030 | 0.22 |
| VII | 272–294 | 0.67 | 0.08 | 0.13 | 0.24 | 0.083 | 0.34 |
| VIII | 307–329 | 0.50 | 0.14 | 0.27 | 0.25 | 0.071 | 0.29 |
| IX | 335–355 | 0.68 | 0.03 | 0.05 | 0.31 | 0.081 | 0.26 |
| X | 375–397 | 0.64 | 0.07 | 0.12 | 0.26 | 0.059 | 0.23 |
| XI | 412–432 | 0.59 | 0.13 | 0.21 | 0.30 | 0.040 | 0.13 |
| XII | 451–473 | 0.63 | 0.13 | 0.21 | 0.48 | 0.077 | 0.16 |

The hydrophobicity was calculated using the normalized consensus index and the hydrophobicity moments were calculated according to Eisenberg [67]. Mutability was calculated as $M = \Sigma_i\{P(residue = x)\ln(P(residue = x))/\ln(0.05)\}$ on the basis of an alignment of 35 sequences of cytochrome *c* and quinol oxidases.

[a] Average hydrophobicity of the helix

[b] Average hydrophobici moment per residue in the helix.

[c] Average mutability of the helix.

[d] Average mutability moment of the helix.

Table II

Characteristics of the sequences connecting the putative transmembrane helices shown in Fig. 1A

| Connecting loop | Number of residues | <H>[a] | <M>[b] | <N>[c] | Net charge[d] |
|---|---|---|---|---|---|
| i | 20 | 0.18 | 0.40 ± 0.04 | 6.6 ± 0.6 | −2 |
| ii | 23 | 0.30 | 0.23 ± 0.03 | 4.0 ± 0.3 | 0 |
| iii | 26 | 0.33 | 0.34 ± 0.04 | 5.9 ± 0.6 | −2 |
| iv | 19 | 0.14 | 0.38 ± 0.04 | 6.0 ± 0.4 | +1 |
| v | 30 | 0.18 | 0.26 ± 0.02 | 4.7 ± 0 3 | −2 |
| vi | 17 | 0.08 | 0.38 ± 0.04 | 6.1 ± 0.5 | +1 |
| vii | 12 | 0.08 | 0.29 ± 0.03 | 5.5 ± 0.5 | −1 |
| viii | 5 | 0.08 | 0.50 ± 0.08 | 7.4 ± 1.4 | +1 |
| ix | 19 | 0.42 | 0.25 ± 0.03 | 4.0 ± 0.4 | −2 |
| x | 14 | 0.15 | 0.48 ± 0.04 | 7.4 ± 0.7 | 0 |
| xi | 18 | −0.11 | 0.30 ± 0.05 | 5.5 ± 0.7 | 0 |
| Negative side | 78 | 0.17 | 0.36 | 5.8 | +3 |
| Positive side | 125 | 0.20 | 0.31 | 5.4 | −9 |

The hydrophobocity was calculated using the normalized consensus index [67]. Mutability was calculated as $M = \Sigma_i\{P(residue = x)\ln(P(residue = x)/\ln(0.05)\}$ on the basis of an alignment of 35 sequences. The values for the C- and N-terminal alignments have been omitted.

[a] Mean hydrophobicity.

[b] Mean mutability.

[c] Average number of different amino acids aligned at each residue.

[d] The net charge was obtained by assuming all glutamate and aspartate residues are negatively charged and all lysine and arginine residues are positively charged.

wards $Cu_B$ in Fig. 1B and, together with Y244, might form part of a proton channel into the binuclear centre.

It is also possible that the conserved histidines are themselves involved in protonation/deprotonation reactions. For example, the axial histidine ligand of some cytochrome *c* oxidases undergoes deprotonation to yield an imidazolate anion with a p*K* between 8 and 9 [52], and the histidine ligand in peroxidases undergoes partial deprotonation due to an alteration in the strength of hydrogen bonding [53]. Such a reaction might be expected to alter the spin-state equilibrium of the haem and produce characteristic optical, MCD and EPR features [54], as well as changes in the resonance Raman spectrum [55] and $^1H$ NMR spectrum [56]. It has been suggested that protonation of an axial histidine might account for the structural heterogeneity observed in $aa_3$-type oxidases [29]. On the basis of data from model haem compounds it might be expected that the CO recombination rate would be affected by (partial) deprotonation of the imidazole ligands [57]. Kinetic data, at low temperature, provide some support for a pH dependence of CO recombination [58]. Furthermore, the tetragonal field strength of the low-spin haem from *Paracoccus denitrificans* and *R. sphaeroides* lies midway between that of the alkaline and neutral forms of haem A, suggesting that one histidine ligand might be deprotonated [59]. However, any such effect might be confounded by the sensitivity of CO recombination to the environment of the haem or access to it from the aqueous phase, as has been observed with myoglobin and with cytochrome *bo* from *E. coli* and cytochrome *c* oxidase from *Saccharomyces cerevisiae* (Brown, Gennis and Rich, manuscript in preparation). Furthermore, the pH in the vicinity of the binuclear centre may not be amenable to manipulation by varying the external pH, so that it might be difficult to generate the imidazolate anion.

A proton channel from the binuclear centre to the positive side of the membrane is more difficult to assess because of the interaction between the many subunits of eukaryotic oxidases, which confounds the interpretation of sequence conservation. It may be that this problem is significantly simplified by considering bacterial oxidases which generally have only three or four subunits. Nonetheless, there are several highly conserved protonatable residues which can be suggested as potential participants (Fig. 1A). By analogy with bacteriorhodopsin, and in the absence of appropriately located glutamate residues on the positive side of the membrane, certain aspartate residues may be candidates for involvement. The proximity of D298 and D300 to the $Cu_B$ ligands (H290 and H291), and therefore to haem $a_3$, as well as the proposal that the channel for proton input is concentrated around helix VIII, suggest that these could form part of the proton channel out of the binuclear centre (Fig. 1A). Other conserved, charged residues (D144 at the top of helix IV, D212 in loop v and D364 in loop ix) may interact with other subunits (Fig. 1A).

## 5. ELECTRON TRANSPORT IN SUBUNIT I

In subunit I, electrons flow from the low-spin haem (*a*) to the binuclear centre (haem $a_3$–$Cu_B$). In previous models $Cu_B$ has been situated in various positions relative to the haems [1–6]. The model shown in Fig. 1B differs from all of these in that $Cu_B$ is positioned on the opposite side of haem $a_3$ from haem *a*. Recent data obtained in this laboratory has shown that $Cu_B$ is not required for rapid haem–haem electron transport which provides support for this distribution of the redox centres (Brown, Gennis and Rich, manuscript in preparation). The distance between haem edges in our model of subunit I is of the order of 10 Å. This distance could allow direct electron transport between the haems at a rate of the order of $10^9$ $s^{-1}$ under optimal conditions [60], which would easily accomodate the observed rate of reverse electron transport (haem $a_3$ to haem *a*) of $2 \times 10^5$ $s^{-1}$ [61]. If electron transport from haem *a* to haem $a_3$ requires any intermediary, a candidate is Y379, which protrudes between the haems and is highly conserved. There is a similar arrangement in the *Rhodospeudomonas viridis* reaction centre: the distance between the edges of haem $c_{558}$ and the bacteriochlorophyll special pair (P* is about 11 Å, and YL162 lies about halfway between them [42], although it is not clear whether the tyrosine is involved in electron transport. The rate constant for the reduction of P* by $c_{558}$ is about $3.5 \times 10^6$ $s^{-1}$ [62].

## 6. INTERACTION WITH OTHER SUBUNITS

Bovine cytochrome *c* oxidase has 13 subunits and the proximity between these subunits is defined in only a general way. However, it is a reasonable assumption that the interaction between subunits should be maintained by conserved residues, and that the large proportion of the bulk of the enzyme which protrudes into the intermembrane space [45] is comprised of those subunits which are absent from the bacterial enzymes. Such subunit interaction provides an explanation for the higher degree of conservation, among the 35 sequences analyzed, of the connecting loops which are exposed on the positive side of the membrane relative to those exposed to the negative side (Table II). The asymmetry in conservation of these connecting loops is particularly apparent in loops iv–xi, which might imply that loop iii is relatively unimportant for subunit interactions. Furthermore, the requirement for electron transport between $Cu_A$ in subunit II and haem *a*, ligated between helix II and helix X of subunit I, provides some contraints in attempting to predict those residues which might be close to the region involved in inter-subunit electron transport. The HDTYYVV and PRR se-

quences in loops ix and xi, respectively, are likely candidates for such a role in that both are highly conserved among cytochrome *c* oxidases. However, the HDTYYVV sequence is not conserved among the quinol oxidases, which lack $Cu_A$ (Fig. 2), whereas the PRR sequence is highly conserved in both types of enzyme. For this reason, we suggest that the HDTYYVV region is close to the route of electron transport between $Cu_A$ and haem *a*.

The roles of those subunits which do not bind redox centres are unknown, but many appear to be important for assembly of the eukaryotic enzyme. However, the deletion of the subunit III gene from *P. denitrificans* resulted in a reduction of enzyme activity and defective assembly [63], although the enzyme was still able to translocate protons [64], suggesting the subunit is not essential for turnover. In contrast, strains of yeast carrying mutations in the genes coding for subunits II and III of cytochrome *c* oxidase have been obtained recently [65]. These strains do not grow on glycerol, suggesting that the enzyme is inactive, and yet they have an optically detectable amount of cytochrome oxidase [66]. Work is currently underway to define these mutations in order to clarify the functional significance of these loci and the subunit interactions of the enzyme.

## 7. CONCLUSIONS

We have described a structural model for subunit I of the proton motive terminal oxidases which is consistent with much current genetic and biophysical data. It indicates a relatively compact site which carries out the chemistry and provides the driving force for proton translocations. This site is connected by proton channels or wells to the aqueous phases in a manner reminiscent of the structure and mechanism of bacteriorhodopsin. Although the oxidase model is by no means a unique interpretation of the available data, it shows a number of distinct and testable features which should help in designing new investigations of important features of structure/function.

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## REFERENCES

[1] Holm, L., Saraste, M. and Wikström, M. (1987) EMBO J. 6, 2819–2823.
[2] Saraste, M. (1990) Q. Rev. Biophys. 23, 331–366.
[3] Minagawa, J., Mogi, T., Gennis, R.B. and Anraku, Y. (1992) J. Biol. Chem. 267, 2096–2104.
[4] Lemieux, L.J., Calhoun, M.W., Thomas, J.W., Ingledew, W.J. and Gennis, R.B. (1992) J. Biol. Chem. 267, 2105–2113.
[5] Wrigglesworth, J.M. (1991) Biochem. Soc. Trans. 19, 258S.
[6] Shapleigh, J.P., Hosler, J.P., Tecklenburg, M.M.J., Younkyoo, K., Babcock, G.T., Gennis, R.B. and Ferguson-Miller, S. (1992) Proc. Natl. Acad. Sci. USA 89, 4786–4790.
[7] Powers, L., Chance, B., Ching, Y. and Angiolillo, P. (1981) Biophys. J. 34, 465–498.
[8] Cline, J., Reinhammar, B., Jensen, P., Venters, R. and Hoffman, B.M. (1983) J. Biol. Chem. 258, 5124–5128.
[9] Li, P.M., Gelles, J., Chan, S.I., Sullivan, R.J. and Scott, R.A. (1987) Biochemistry 26, 2091–2095.
[10] Scott, R.A., Li, P.M. and Chan, S.I. (1988) Ann. NY Acad. Sci. 550, 53–58.
[11] Moody, A.J., Cooper, C.E. and Rich, P.R. (1991) Biochim. Biophys. Acta 1059, 189–207.
[12] Brown, S., Moody, A.J., Jeal, A.E., Bourne, R.M., Mitchell, J.R. and Rich, P.R. (1992) EBEC Short Rep. 7, 39 (Abstract).
[13] Blum, H., Harmon, H.J., Leigh, J.S., Salerno, J.C. and Chance, B. (1978) Biochim. Biophys. Acta 502, 1–10.
[14] Salerno, J.C. and Ingledew, W.J. (1991) Eur. J. Biochem. 198, 789–792.
[15] Ohnishi, T., LoBrutto, R., Salerno, J.C., Bruckner, R.C. and Frey, T.G. (1982) J. Biol. Chem. 257, 14821–14825.
[16] Ingledew, W.J. and Bacon, M. (1991) Biochem. Soc. Trans. 19, 613–616.
[17] Powers, L., Blumberg, W.E., Chance, B., Barlow, C.H., Leigh, J.S., Smith, J., Yonetani, T., Vik, S. and Peisach, J. (1979) Biochim. Biophys. Acta 546, 520–538.
[18] Thomson, A.J., Greenwood, C., Gadsby, P.M.A., Peterson, J., Eglinton, D.G., Hill, B.C. and Nicholls, P. (1985) J. Inorganic Biochem. 23, 187–197.
[19] Goodman, G. and Leigh, J.S. (1985) Biochemistry 24, 2310–2317.
[20] Blair, D.F., Ellis, W.R., Wang, H., Gray, H.B. and Chan, S.I. (1986) J. Biol. Chem. 261, 11524–11537.
[21] Myer, Y.P. and Pande, A. (1978) The Porphyrins, Vol. III (Dolphin, D., ed.) pp. 271–322, Academic Press, London.
[22] Degli Esposti, M., Palmer, G. and Lenaz, G. (1989) Eur. J. Biochem. 182, 27–36.
[23] Rich, P.R., Jeal, A.E., Madgwick, S.A. and Moody, A.J. (1990) Biochim. Biophys. Acta 1018, 29–40.
[24] Widger, W.R., Cramer, W.A., Herrmann, R.G. and Trebst, A. (1984) Proc. Natl. Acad. Sci. USA 81, 674–678.
[25] Saraste, M. (1984) FEBS Lett. 166, 367–372.
[26] Carter, K. and Palmer, G. (1982) J. Biol. Chem. 257, 13507–13514.
[27] Wang, H., Sauke, T., Debrunner, P.G. and Chan, S.I. (1988) J. Biol. Chem. 263, 15260–15263.
[28] Stevens, T.H. and Chan, S.I. (1981) J. Biol. Chem. 256, 1069–1071.
[29] Rusnak, F.M., Münck, E., Nitsche, C.I., Zimmermann, B.H. and Fee, J.A. (1987) J. Biol. Chem. 262, 16328–16332.
[30] Thomson, A.J., Johnson, M.K., Greenwood, C. and Gooding, P.E. (1981) Biochem. J. 193, 687–697.
[31] Surerus, K.K., Oertling, W.A., Fan, C., Gurbiel, R.J., Einarsdóttir, O., Antholine, W.E., Dyer, R.B., Hoffman, B.M., Woodruff, W.H. and Fee, J.A. (1992) Proc. Natl. Acad. Sci. USA 89, 3195–3199.
[32] Zimmermann, B.H., Nitsche, C.I., Fee, J.A., Rusnak, F. and Münck, E. (1988) Proc. Natl. Acad. Sci. USA 85, 5779–5783.
[33] Wu, W., Chang, C.K., Varotsis, C., Babcock, G.T., Puustinen, A. and Wikström, M. (1992) J. Am. Chem. Soc. 114, 1182–1187
[34] Aronoff, S. (1966) The Chlorophylls: Physical, Chemical, and Biological Properties (Vernon, L.P. and Seely, G.R., eds.) pp. 3–20, Academic Press, New York.
[35] Allen, J.P., Feher, G., Yeates, T.O., Komiya, H. and Rees, D.C (1987) Proc. Natl. Acad. Sci. USA 84, 5730–5734.

[36] Moore, T.A., Gust, D., Mathis, P., Mialocq, J.-C., Chachaty, C., Bensasson, R.V., Land, E.J., Doizi, D., Liddell, P.A., Lehman, W.R., Nemeth, G.A. and Moore, A.L. (1984) Nature 307, 630–632.
[37] Chepuri, V. and Gennis, R.B. (1990) J. Biol. Chem. 265, 12978–12986.
[38] Richardson, J.S. and Richardson, D.C. (1988) Science 240, 1648–1652.
[39] Deisenhofer, J. and Michel, H. (1989) Science 245, 1463–1471.
[40] Von Heijne, G. (1992) J. Mol. Biol. 225, 487–494.
[41] Lemmon, M.A. and Engelman, D.M. (1992) Curr. Opin. Struct. Biol. 2, 511–518.
[42] Allen, J.P., Feher, G., Yeates, T.O., Komiya, H. and Rees, D.C. (1987) Proc. Natl. Acad. Sci. USA 84, 6162–6166.
[43] Komiya, H., Yeates, T.O., Rees, D.C., Allen, J.P. and Feher, G. (1988) Proc. Natl. Acad. Sci. USA 85, 9012–9016.
[44] Mitchell, R., Mitchell, P. and Rich, P.R. (1992) Biochim. Biophys. Acta 1101, 188–191.
[45] Valpuesta, J.M., Henderson, R. and Frey, T.G. (1990) J. Mol. Biol. 214, 237–251.
[46] Hinkle, P. and Mitchell, P. (1970) Bioenergetics 1, 45–60.
[47] Rich, P.R., West, I.C. and Mitchell, P. (1988) FEBS Lett. 233, 25–30.
[48] Rich, P.R. (1988) Ann. NY Acad. Sci. 550, 254–259.
[49] Wikström, M. (1988) FEBS Lett. 231, 247–252.
[50] Wikström, M. and Morgan, J.E. (1992) J. Biol. Chem. 267, 10266–10273.
[51] Paddock, M.L., McPherson, P.H., Feher, G. and Okamura, M.Y. (1990) Proc. Natl. Acad. Sci. USA 87, 6803–6807.
[52] La Mar, G.N., Jackson, J.T., Dugad, L.B., Cusanovich, M.A. and Bartsch, R.G. (1990) J. Biol. Chem. 265, 16173–16180.
[53] La Mar, G.N., De Ropp, J.S., Chacko, V.P., Satterlee, J.D. and Erman, J.E. (1982) Biochim. Biophys. Acta 708, 317–325.
[54] Quinn, R., Nappa, M. and Valentine, J.S. (1982) J. Am. Chem. Soc. 104, 2588–2595.
[55] Spiro, T.G. (1985) Adv. Protein Chem. 37, 111–159.
[56] La Mar, G.N. and Walker, F.A. (1979) The Porphyrins, Vol. IV (Dolphin, D., ed.) pp. 61–157, Academic Press, New York.
[57] Schwartz, J.C., Stanford, M.A., Moy, J.N., Hoffman, B.M. and Valentine, J.S. (1979) J. Am. Chem. Soc. 101, 3396–3398.
[58] Harmon, H.J. and Stringer, B.K. (1990) FEBS Lett. 267, 167–170.
[59] Hosler, J.P., Tecklenburg, M.M.J., Atamian, M., Revzin, A., Ferguson-Miller, S. and Babcock, G.T. (1991) Biophys. J. 59, 470a (abstract).
[60] Moser, C.C., Keske, J.M., Warncke, K., Farid, R.S. and Dutton, P.L. (1992) Nature 355, 796–802.
[61] Oliveberg, M. and Malmström, B.G. (1991) Biochemistry 30, 7053–7057.
[62] Holten, D., Windsor, M.W., Parson, W.W. and Thornber, J.P. (1978) Biochim. Biophys. Acta 501, 112–126.
[63] Haltia, T., Finel, M., Harms, N., Nakari, T., Raitio, M., Wikström, M. and Saraste, M. (1989) EMBO J. 8, 3571–3579.
[64] Haltia, T., Saraste, M. and Wikström, M. (1991) EMBO J. 10, 2015–2021.
[65] Meunier, B., Lemarre, P. and Colson, A.M. (1992) Eur. J. Biochem. (submitted).
[66] Brown, S., Colson, A.-M., Meunier, B. and Rich, P.R. (1992) Eur. J. Biochem. (in press).
[67] Eisenberg, D. (1984) Annu. Rev. Biochem. 53, 595–623.
[68] Denda, K., Fujiwara, T., Seki, M., Yoshida, M., Fukumori, Y. and Yamanaka, T. (1991) Biochem. Biophys. Res. Commun. 181, 316–322.
[69] Lübben, M., Kolmerer, B. and Saraste, M. (1992) EMBO J. 11, 805–812.
[70] Santana, M., Kunst, F., Hullo, M.F., Rapoport, G., Danchin, A. and Glaser, P. (1992) J. Biol. Chem. 267, 10225–10231.