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Structural models of the redox centres in cytochrome oxidase

Liisa Holm[1], Matti Saraste and Mårten Wikström

Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10A, SF-00170 Helsinki, and [1]Biotechnical Laboratory, V.T.T., SF-02150 Espoo, Finland

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Evolutionary conservation, predicted membrane topography of the subunits, and known chemical and physical properties of the catalytic metals in cytochrome oxidase provided the basis for plausible structural models of the enzyme's redox centres. Subunit II probably binds one of the copper ions (Cu_A) whilst subunit I is likely to bind the two haems (*a* and a_3) and the other redox-active copper (Cu_B). Two cysteine and two histidine residues of subunit II are the likely ligands of Cu_A, forming a centre that may be structurally similar to that in azurin. The two haems may be sandwiched between two transmembranous segments of subunit I, one of which also provides a histidine ligand to Cu_B. A third segment may provide two more histidine ligands to the latter. The model was constructed with a 4 Å Fe–Cu distance in the binuclear haem a_3–Cu_B centre, and a 14 Å distance between the haem irons. The subunit I model involves only three transmembranous helices which bind three catalytic metal groups. The fit of this model to several known physicochemical properties of the redox centres is analysed.

Key words: cytochrome aa_3/electron transfer/cell respiration

Introduction

Cytochrome oxidase (EC 1.9.3.1) is the enzyme responsible for cell respiration in eukaryotes and many aerobic prokaryotes. It is a multi-subunit complex that contains four redox centres, two haems (*a* and a_3) and two redox-active copper ions (Cu_A and Cu_B). Although the protein structure of the eukaryotic enzyme is very complicated (see Capaldi *et al.*, 1986), it has been known for some time that two of the major subunits (I and II) contain the four redox centres (see Wikström and Saraste, 1984; Wikström *et al.*, 1985). This argument originates largely from the work on the cytochrome oxidase isolated from *Paracoccus denitrificans* (Ludwig and Schatz, 1980; Ludwig, 1987), which is very similar in functional and spectroscopic properties to the eukaryotic enzyme, but contains only subunits I and II. In the accompanying paper (Raitio *et al.*, 1987) it is shown that the primary structures of the *Paracoccus* subunits I and II are indeed strongly homologous to the corresponding subunits in the eukaryotic enzyme.

The two haems, both of which have the haem A structure (Figure 1), and the two redox-active coppers all have distinct properties when bound to the enzyme (Wikström *et al.*, 1981; Blair *et al.*, 1983). Electrons originating in cytochrome *c* are transferred via haem *a* and Cu_A to the binuclear centre formed by haem a_3 and Cu_B, which catalyses the reduction of dioxygen to water. The electron transfer is further associated with translocation of protons across the mitochondrial (or bacterial) membrane (Wikström, 1977; Wikström and Krab, 1979).

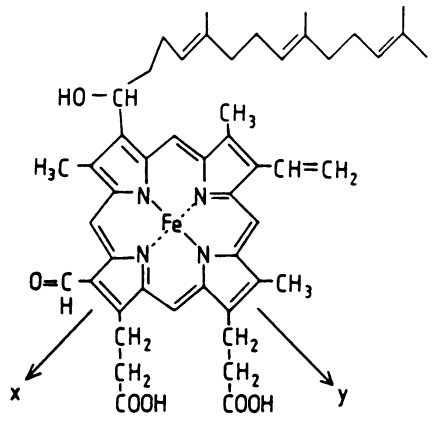


Fig. 1. Structure of haem A. x and y are the two pyrrole N–Fe–pyrrole N axes in the haem plane; x is through the formyl- and vinyl-bearing rings; the z axis runs through Fe perpendicular to the haem plane.

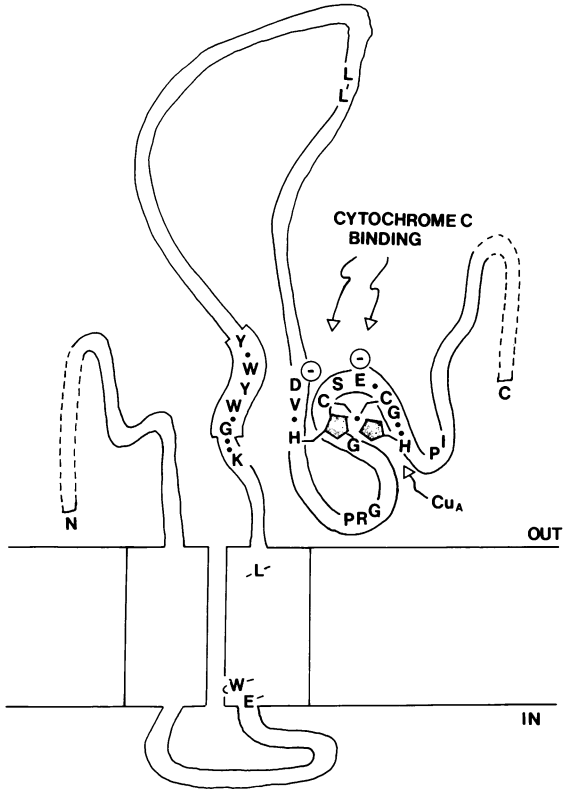


Fig. 2. Schematic presentation of subunit II. Two hydrophobic segments form a 'hairpin' structure that anchors the subunit to the membrane. An invariant aromatic sequence is shown as a widened ribbon. The two invariant carboxylic acids are marked with encircled minus signs, and the proposed ligands to Cu_A are indicated. Dashed lines show the N- and C-terminal extension in the *Paracoccus* protein (Raitio *et al.*, 1987). Only strictly invariant amino acid residues are shown (see Materials and methods). IN and OUT refer to the matrix and cytoplasmic (or cytoplasmic and periplasmic in bacteria) sides of the membrane.

Ps. azurin	41-52	K N V M G H N W V L S
Bovine CO II	156-167	S E D V L H S W A V P
Ps. azurin	106-127	E Q Y M F F C T F P G H S A L M K G T
Bovine CO II	190-210	G L Y Y G Q C S E I – C G S N H S F M

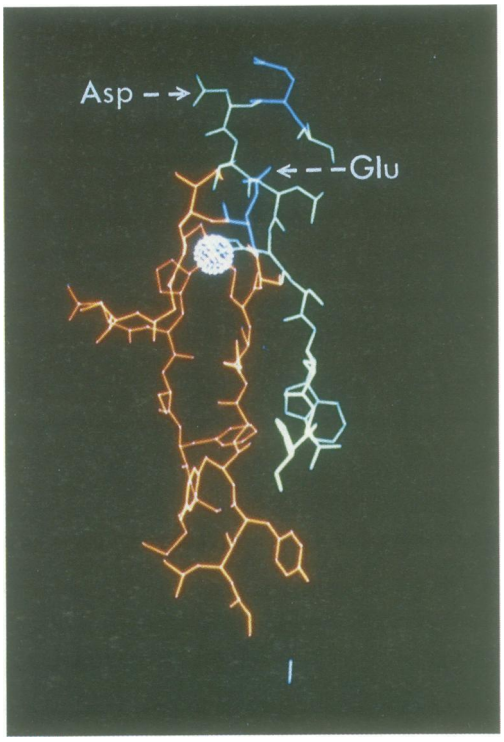


Fig. 3. Model for the Cu_A-binding site in subunit II. **Top:** alignment of the copper-binding segments in *Pseudomonas* azurin with two internal sequences from bovine subunit II of cytochrome oxidase. The copper-binding ligands in azurin (and suggested ligands in subunit II) are indicated by stars. Underlined residues are invariant in subunit II. A deletion is shown with –. The numbering refers to sequence position in the respective protein. **Bottom:** the two internal peptides of subunit II were folded on top of the *Pseudomonas* azurin 3-D structure, after which the latter was removed for clarity. The longer peptide is shown red and the shorter is green. The copper is shown as a white sphere. The labels indicate the invariant Asp and Glu residues (Figure 2). Prior to the Asp in the sequence there is another Glu that is conserved in many mitochondrial enzymes. Both glutamic acids are coloured blue. Side chains but no hydrogens are shown.

Results and Discussion

Subunit II: a membrane-anchored copper protein

Figure 2 shows the predicted folding of subunit II in the membrane (Capaldi, 1982; Wikström *et al.*, 1985), and indicates the possible location of the strictly conserved amino acids. Subunit II has four invariant features. The first is a transmembranous 'hairpin' structure of which the C-terminal segment has three invariable residues while the sequence of the N-terminal segment is variable. The second feature is the aromatic sequence in the beginning of the hydrophilic C-terminal domain. Thirdly, there are two carboxylic acid residues (marked with minus signs in Figure 2), which are strictly conserved. This supports the earlier conclusion from labelling data (Millett *et al.*, 1983) that these may be important in the binding of cytochrome *c* (cf. below). The fourth invariancy is the nature of the four ligands that are probably involved in the binding of Cu_A (Stevens *et al.*, 1982; Blair *et al.*, 1983). Alignment of subunit II sequences with amino acid sequences of azurin suggests that these are two cysteines and a histidine close to one another in the primary structure, and a second histidine that is found earlier in the sequence (Figure 3, top).

Taken together these data suggest that subunit II is a membrane-anchored copper protein. There can be no doubt that this is indeed the site for Cu_A and not for Cu_B. The only conserved cysteines of subunits I and II are the two in this site (Figures 2 and 4; Wikström *et al.*, 1985). Comparison of the primary structures from different species shows that there are no conserved histidines in subunit II apart from the two probable ligands to the copper. Since the three other redox-active metals all require histidine ligands (see below) it may be concluded that subunit II contains no other redox centre. Consequently, the three others should all be found in subunit I (see also Wikström *et al.*, 1985 for an earlier discussion of this point).

Possible structure of the Cu_A site

Figure 3 shows two internal sequences from the bovine subunit II aligned with sequences from the copper protein azurin. These include the proposed ligands to copper (see Chothia and Lesk, 1982; Stevens *et al.*, 1982; Blair *et al.*, 1985; Wikström *et al.*, 1985). In azurin the longer peptide forms two adjacent antiparallel β-strands and a loop between them. Three of the ligands to copper (Cys, His and Met) reside in the loop. The fourth ligand (His) is in the centre of the shorter peptide. As first pointed out by

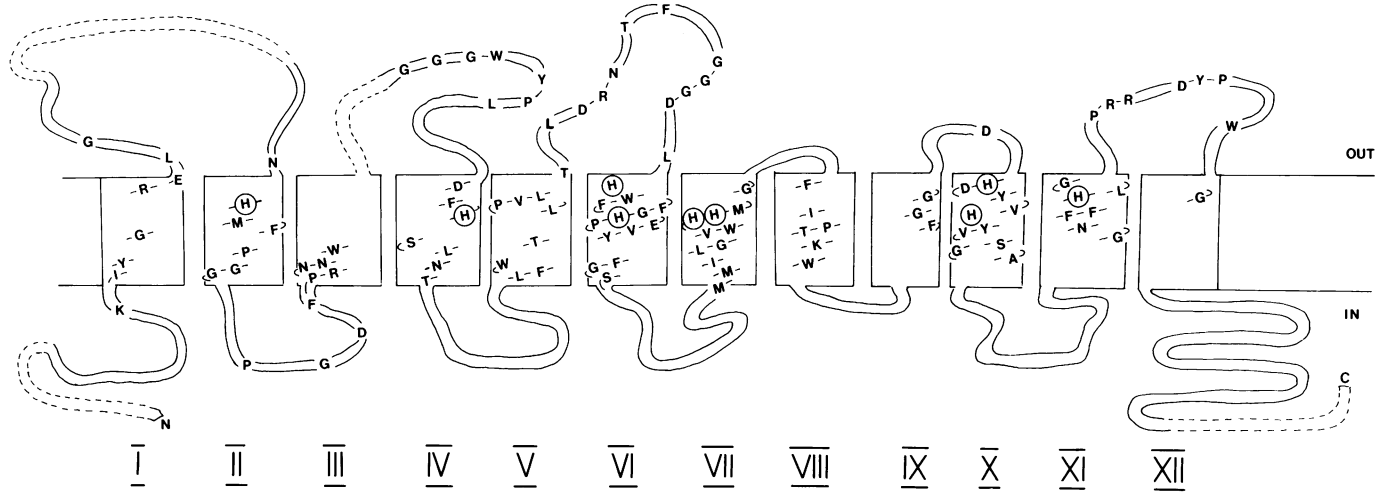
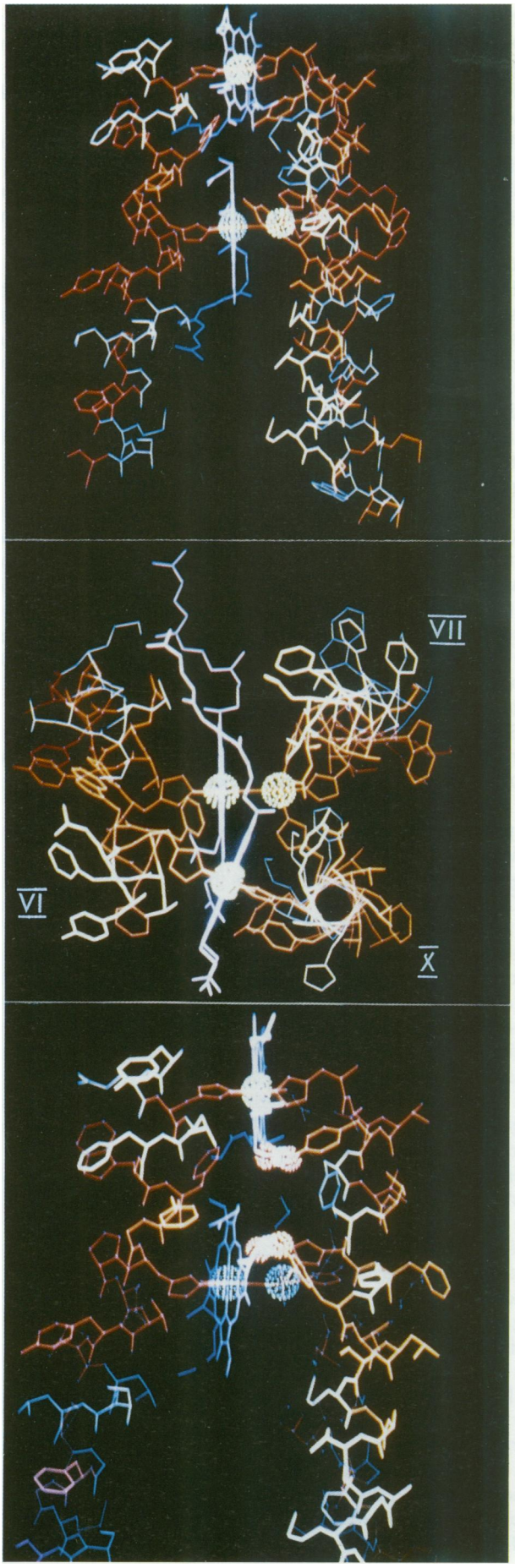


Fig. 4. Schematic presentation of subunit I. Twelve transmembranous segments are numbered I–XII. Dashed lines indicate insertions and terminal extensions in the *Paracoccus* subunit, relative to the mitochondrial counterparts (Raitio *et al.*, 1987). Only strictly invariant amino acid residues are shown. The membrane-traversing segments are assumed to be helical; hence the amino acids are drawn on helical surfaces. Invariant histidines are encircled.



Steffens and Buse (1979) there is a weak homology between the copper-binding region of azurin and plastocyanin, and the likely Cu_A-binding site of subunit II.

We have taken the two subunit II peptides and superimposed them on the known three-dimensional structure of *Pseudomonas* azurin. The copper-binding loop between the β-strands is tighter in subunit II than in azurin due to the deletion of one amino acid. Figure 3 (bottom) shows the resulting subunit II structure (after removal of the azurin backbone to clarify the picture). In cytochrome oxidase there are two cysteine ligands to Cu_A (Stevens *et al.*, 1982; Scott, 1982; Scott *et al.*, 1986), the corresponding ligands of the blue copper proteins being cysteine and methionine. This explains why the Cu_A site does not have the properties of a blue copper (Blair *et al.*, 1983).

An interesting corollary of this structure is the predicted location of the two invariant carboxylic acid residues (Figures 2 and 3), an aspartate that is close to the invariant histidine in the short peptide and a glutamate between the cysteine residues in the loop. The corresponding residues in azurin are on the surface of the molecule. This would fit well with the proposed essential role of the carboxylic residues in binding of cytochrome *c* to cytochrome oxidase (Millett *et al.*, 1983). It would also suggest that Cu_A may be relatively close to the surface of subunit II. The haem edge of cytochrome *c* is surrounded by a cluster of lysine residues that are essential in binding (Osheroff *et al.*, 1980). Interaction between these and the carboxylic residues on subunit II might assist in docking cytochrome *c* to its binding site.

Brunori *et al.* (1981) have summarized the evidence for the prevailing view that haem *a* is the primary electron acceptor from cytochrome *c*. The present structural considerations cannot rule out that possibility, but they do question it for a number of reasons. It seems clear that the Cu_A site lies on the outside of the membrane, which is also the location of cytochrome *c*. In contrast haem *a* is likely to lie at least partially in the membrane (see Wikström *et al.*, 1985), and the binuclear centre even deeper in it. It is difficult to imagine a situation where cytochrome *c* would primarily donate an electron to haem *a* in the membrane, followed by electron transfer outwards to Cu_A, and again into the membrane — to the binuclear centre. In a careful kinetic study Antalis and Palmer (1982) showed, in fact, that the velocities of haem *a* and Cu_A reduction by cytochrome *c* were equal in all tested conditions. It seems possible, therefore, that Cu_A is the primary electron acceptor, and that electron equilibration between Cu_A and haem *a* is much faster than reduction of the former by cytochrome *c*. Very fast Cu_A–haem *a* electron transfer could be facilitated by the highly conserved aromatic segment in subunit II (Figure 2).

The ligands of the cuprous Cu_A may differ from those of the cupric centre described above. S.I.Chan and collaborators (personal communication) have suggested that a tyrosine may replace

Fig. 5. Structural model of the redox centres in subunit I. Invariant amino acid residues are coloured red. The haem irons and Cu_B are shown as large white or blue-white spheres. A red line indicates the Fe/a_3)–Cu_B axis. **Top** and **bottom:** the viewer looks along the membrane plane, at slightly different angles with respect to the haems. Segment VI is to the left; VII and X are superimposed on the right. The cytoplasmic surface of the (mitochondrial) membrane is on the top. In the bottom figure two hydrogen bonds are indicated with small pink spheres; the upper one is between the formyl group of cytochrome *a* and an invariant Tyr in segment X; the lower one is between a propionate carboxyl of haem a_3 and the bottom Tyr in segment X (see text). No other hydrogens are shown. **Middle:** top view from the cytoplasmic surface, looking down along the membrane normal. Transmembranous segments VI, VII and X (see Figure 4) are labelled. They are 25, 26 and 25 residues long, corresponding to the membrane-penetrating lengths. Haem *a* is seen below the binuclear centre.

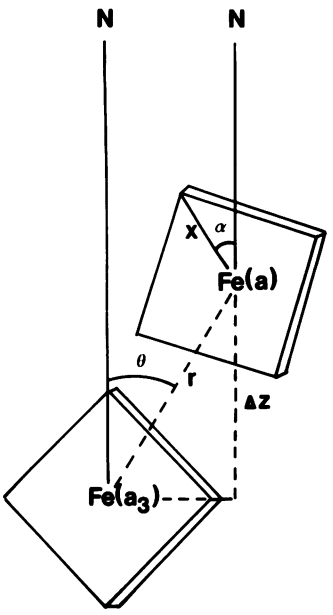


Fig. 6. Scheme of the proposed geometrical relationship between the two haems of cytochrome oxidase. The scheme corresponds to the model in Figure 5. The lines marked N are the membrane normals, r is the Fe–Fe distance, and Δz is the difference in membrane normal co-ordinates of the haem irons. The x axis is defined as in Figure 1 [notice that the definition of x and y axes is reversed in comparison with Blum *et al.* (1978) and Erecinska *et al.* (1979)].

one of the two cysteines. There are two strictly conserved tyrosines in subunit II both of which reside in the aromatic stretch (Figure 2). Hence, this segment might come close to the copper-binding site and facilitate electron transfer to haem *a*.

Haem a and the binuclear haem a_3–Cu_B centre

From the considerations above we concluded that subunit I should contain both haem *a* and the binuclear centre. These metal groups require six histidines as ligands; two for haem *a* (Peisach, 1978; Babcock *et al.*, 1981), one for haem a_3 (Blokzijl-Homan and van Gelder, 1971; Stevens and Chan, 1981; cf. Wikström *et al.*, 1981), and probably three for Cu_B (Cline *et al.*, 1983; Blair *et al.*, 1983; Scott *et al.*, 1986). Subunit I contains nine invariant histidines (Figure 4).

We will now search for a compact structure that might accommodate all three metal groups. This seems well motivated because the distances between the haem irons (12–20 Å; Ohnishi *et al.*, 1982; Mascarenhas *et al.*, 1983; Scholes *et al.*, 1984; Brudvig *et al.*, 1984) and haem iron of a_3 and Cu_B (<5 Å; Powers *et al.*, 1981; Blair *et al.*, 1983; Scott *et al.*, 1986) are short. *Ad hoc*, it is therefore plausible that all three metal groups may bind to common segments in the protein.

Like subunit I of cytochrome oxidase, cytochrome *b* of the cytochrome *bc*-type complexes also contains two haems in the same subunit. It has been suggested that these haems are 'sandwiched' between two common transmembranous helices (Saraste and Wikström, 1983; Saraste, 1984; Widger *et al.*, 1984; Link *et al.*, 1986). By analogy, the haems of cytochrome oxidase might also be arranged in this fashion.

In our model of subunit I (Figure 4) there are twelve predicted transmembranous helical segments. Three of them (labelled VI, VII and X) contain two invariant histidines each. These pairs of histidines are separated by six residues in segment VI, by seven in segment X, and are adjacent in segment VII. These three segments also have a particularly high degree of amino acid invariance in the subunit (Figure 4). Clearly the most compact model of binding the three metal centres would be one using these segments.

First, the bisimidazole complex of haem *a* was constructed by placing haem A between segments VI and X and with the top histidines co-ordinating the iron (Figure 5, top). This puts haem *a* close to the membrane interphase on the cytoplasmic side (see Wikström *et al.*, 1981, 1985). We define the membrane normal as parallel to the haem plane (Erecinska *et al.*, 1977). Segment X was placed parallel to the membrane normal (Figure 5, middle).

Haem A (Figure 1) is unusual in having a formyl group in position 8 of the porphyrin ring and a long isoprenoid chain in position 2. On the basis of laser Raman spectroscopy Babcock *et al.* (1981) have shown that the formyl of haem *a* (but not of a_3) is hydrogen-bonded to an amino acid side chain. Babcock and Callahan (1983) have suggested that this hydrogen bond may be a tyrosine residue, and that its strength varies with the redox state of the iron. They further proposed that this bond may be important in the mechanism of proton-pumping.

From the conserved tyrosine below the top histidine in segment X (Figure 4) a hydrogen bond was constructed to the carbonyl of the haem (Figure 5, bottom). To form this bond the haem must be rotated around its z-axis (perpendicular to the haem plane) so that the x-axis (Figure 1) forms an angle of ~30° with the membrane normal (α in Figure 6). It is interesting that this is the orientation of haem *a* suggested earlier on the basis of spectroscopic studies of membrane multilayers of cytochrome oxidase (Blum *et al.*, 1978; Erecinska *et al.*, 1979).

Several attempts were then made to position the haem of cytochrome a_3 into the model. The proximal histidine of this haem could *a priori* be either one of the bottom histidines in segments VI and X. In our model we chose the histidine of segment VI as proximal ligand (Figure 5, top). If the histidine of segment X is used, the conserved tyrosine below it in this segment (see Figures 4 and 5) sterically hinders the positioning of the haem.

Cu_B was placed distally to haem a_3 on the histidine nitrogen–Fe axis at a distance of 4 Å from the iron (Powers *et al.*, 1981). A shorter distance of 3 Å, as determined recently from copper X-ray absorption spectroscopy by Scott *et al.* (1986), would also be accommodated. The lower histidine of segment X was then co-ordinated to the copper (Figure 5, middle). Finally, the third helical segment (VII; Figure 4) was brought in so that its two adjacent invariant histidines co-ordinate the Cu_B. The three N–Cu bonds are all roughly at right angles to one another, which requires a slight tilt of segment VII with respect to the membrane normal (Figure 5, middle).

In constructing the binuclear centre haem a_3 was allowed to translate along and rotate around the membrane normal through the iron.

The model is somewhat flexible with respect to the Fe–Fe distance. If segments VI and X are kept strictly helical this distance is only ~12 Å. However, the amino acid residue following the bottom histidine in segment VI is an invariant proline. This allows a bend in the helix whereby the distance between the haem irons can be increased, up to a maximum of ~16 Å. In Figure 5 the Fe–Fe distance is 14 Å (r in Figure 6), and the helix is bent slightly at the proline. This still allows the haem normals to be almost perfectly in the same plane (the plane of the membrane). If the haems are forced further apart they become gradually more tilted with respect to one another whereby the haem normals depart from a common plane.

The haem planes are not parallel in our model, but form an

angle of 25° (Figure 5, middle). The Fe−Fe axis could not be put along the membrane normal, mainly due to steric hindrance exerted by the aforementioned bottom tyrosine of segment X. In our model the Fe−Fe axis forms an angle of about 27° with the membrane normal (θ in Figure 6). This is near the lower limit for this angle, as estimated in the e.p.r. study of Ohnishi *et al.* (1982) with orientated cytochrome oxidase membranes.

The resonance X-ray diffraction data of Blasie *et al.* (1982) predicted a difference of 8 ± 1.4 Å between the membrane normal co-ordinates of the two irons (cf. Ohnishi *et al.*, 1982). This is less than the corresponding value in our model, which is about 12 Å (Δz; Figure 6). The picture of the haem arrangement based on these X-ray data (see Ohnishi *et al.*, 1982) is in fact hard to reconcile with a model where both haems are axially ligated by residues from common transmembranous helices, and with a compact structure involving only three transmembranous helices (as assumed here).

If the invariant tyrosine below the bottom histidine in segment X (Figure 4) is arranged to make a hydrogen bond to one of the propionate carboxyls of haem a_3 (Figure 5, bottom), the haem becomes orientated with either its x- or y-axis in the membrane plane. This is the orientation found by Erecinska *et al.* (1979).

Conclusions

We wish to emphasize that our models must be considered tentative. They are intended to illustrate that it is possible to arrange the redox centres of cytochrome oxidase in subunits I and II following certain outlined assumptions. Thus we conclude that it is possible to construct a model of the Cu_A site in subunit II on the basis of the structure of azurin, and that this model leads to an interesting prediction for the interaction of subunit II and Cu_A with cytochrome *c*. It is also possible to construct a compact model of three transmembranous helices of subunit I, which accommodates both the haem of cytochrome *a* and the binuclear centre, and in which a large number of known structural features can be realized simultaneously.

The model presented here will be tested experimentally as soon as the cloned genes of the *Paracoccus* cytochrome oxidase (Raitio *et al.*, 1987) can be used for site-directed mutagenesis.

Materials and methods

The primary structures of cytochrome oxidase subunits I and II were taken from the following sources: the human, bovine, mouse, rat, maize, *Saccharomyces* and *Neurospora* sequences of subunit II, and human, bovine, mouse, *Saccharomyces* and *Neurospora* sequences of subunit I were aligned by Wikström *et al.* (1985). Additional sequences for the *Drosophila* (de Bruijn, 1983), *Xenopus* (Roe *et al.*, 1985), *Leischmania* (de la Cruz *et al.*, 1984), *Trypanosoma* (Hensgens *et al.*, 1984) and *Paracoccus* (Raitio *et al.*, 1987) subunits I and II are included in the comparisons presented here.

Hydropathic profiles of the polypeptides were calculated using the Kyte and Doolittle (1982) index and a window length of 11 residues. The predicted hydrophobic subunits were further analysed by calculating the hydrophobic moments (Eisenberg *et al.*, 1984) and, as a rule, the most uniformly hydrophobic regions were centred on the middle of the membrane.

An Evans & Sutherland PS300 graphics terminal was used for construction of protein structures; the computer program employed was Chem-X (January 1987; Chemical Design Ltd, Oxford, UK). The three-dimensional structure of *Pseudomonas* azurin (Adman and Jensen, 1981) was taken from the Brookhaven Protein Data Bank.

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