INTRODUCTION

Although in recent years it has emerged that cytochrome c oxidase (Cox) plays a critical role in the pathogenesis of Alzheimer’s disease, diabetes and several other diseases (1, 2), interest in this enzyme extends back for more than fifty years. Indeed, early activity studies have established that Cox catalyses the reduction of oxygen to water in a reaction that is critical to the production of adenosine triphosphate (ATP) in cells (3, 4). Much is now known about the structure of Cox. Early predictions based on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), covalent modifications, electron paramagnetic resonance (EPR) and other studies (5–11) have been confirmed by X-ray analysis of crystals of the enzyme (12–14). It is therefore now established that the eukaryotic forms of the enzyme consist of thirteen subunits [SUs] per monomer (12) and that each monomer of the enzyme contains eight metal ions, five of which are redox active (12, 15, 16).

The kinetics of Cox is understood with less certainty. In each cycle of activity, the enzyme accepts four electrons from four molecules of its substrate cytochrome c; it passes these electrons on to its second substrate, oxygen, which, as previously stated, is reduced to water (3, 4). The precise route taken by the electrons as they move from cytochrome c to oxygen is not known with certainty. Kinetic evidence suggests that the pair of copper ions, referred to as CuA, is the first of the Cox redox active centres to receive electrons (17). There is rapid equilibration of these electrons with a second centre, haem a, after which the electrons are transferred to oxygen via a third copper and a second haem but the sequence of events has not been agreed on (18–21).

Several studies have demonstrated that quite apart from the uncertainty surrounding the route followed by electrons, the kinetics of oxygen reduction is complicated. On one hand, the steady state assay of Cox exhibits non-hyperbolic characteristics so that the resulting Eadie Hofstee plots generate two intersecting straight lines instead of a single line (22); there is, however, no consensus regarding the signi-
A number of researchers have reported a five-fold increase in Cox activity once the enzyme had undergone a complete cycle of reduction and reoxidation (25–27). The slow form of the enzyme is referred to as the resting enzyme while the highly active form is referred to as the pulsed. Initially, Antonini et al (25) suggested that different rates of internal electron transfer might be responsible for the ‘resting-to-pulsed’ transition. Subsequently, based on cyanide inhibition studies, Jones et al (28) suggested that a conformational change in the region of the ligand/oxygen binding site determined whether ligand binding was fast or slow. The group proposed that the binding site for cyanide and similar ligands was closed and inaccessible in the resting enzyme, but opened and readily accessible in the pulsed (28). To date, however, direct structural evidence of this resting to pulsed transition has not been detected. Also, in spite of recent studies pointing to the involvement of an oxygen channel (29), exactly how oxygen and such ligands reach the Cox binding site is not well understood. In our recent kinetic and EPR studies (18), we observed the different rates of cyanide binding reported by others (27, 28) and thought that a biocomputing approach might provide some answers.

In 1996, the first complete crystal structure of bovine Cox in its fully oxidized form was published (12). Since then, in addition to significant improvements in the resolution, structures of the fully reduced and some ligand bound forms of the enzyme have been published (12–14). Because the production of a Cox crystal is a slow process, and because the pulsed enzyme in the absence of reducing substrate always relaxes back to the resting state (26), no structure of the fully oxidized pulsed enzyme has been reported to date. In this paper, we employed computer modelling to study and compare the structures of the fully oxidized resting, the fully reduced, and the carbon monoxide (CO)-bound form of bovine Cox. We also compared some features of the Thermus thermophilus cytochrome ba 3 oxidase with those in the bovine. Our objective was to analyse these different forms of Cox in the hope of detecting those conformational changes thought to be associated with its resting to pulsed transitions. We also hoped to gain an insight into the process by which oxygen reaches its binding site. Although we studied the whole 13-SU bovine enzyme, we focussed particularly on SU-I, SU-II and SU-III, ie the region regarded as the core of the enzyme, looking at those regions in the vicinity of oxygen binding site.

MATERIALS AND METHOD

The structures of oxidized, reduced and CO-bound bovine Cox were obtained from the Brookhaven Protein Data Bank and analysed as described below, using the Swiss PDB Viewer programme. Both the oxidized and reduced structures were at 2.8 Å resolution, while for the CO, the closest available resolution was 2.35 Å. For part two of the study, the structure of the fully oxidized Thermus thermophilus cytochrome ba 3 oxidase was downloaded from the bank.

Comparative studies on bovine Cox

First, one monomer of either the oxidized, reduced or CO Cox was displayed, showing haem a, haem a 3, CuA and CuB. This monomer was manipulated so that it could be viewed from different angles. Next, all of the SUs except those of interest (SUs I, II or III) were hidden (made to disappear). Starting with SU-II for all three forms of the enzyme, the proteins were viewed from all angles and relevant distances between residues recorded. Then SU-III was added to the display and the viewing from different angles was repeated, paying particular attention to residues in the vicinity of haem a, haem a 3 and CuB. Next, SUs II and III for all three forms of Cox were superimposed on each other using the oxidized form as the reference molecule. When the analysis of SUs II and III was completed, SU-I of each of the three forms of Cox was manipulated and viewed. Finally, SU-I of the oxidized and the CO Cox and then the reduced and the CO Cox were superimposed and each pair studied for alignment.

Comparative studies on bovine and T thermophilus Cox

The structure of SU-I of Thermus thermophilus ba 3 Cox was first viewed from the top and side and then superimposed on that of the bovine (aa 3) enzyme for comparison. The objective here was to determine if the region of the ba 3 oxidase, identified as the oxygen diffusion channel (29), exists in the bovine enzyme.

RESULTS

Bovine studies: In Fig. 1, which shows a monomer of bovine Cox, SUs I, II and III are seen to span the membrane with SU-I extending slightly above the surface of the surrounding membrane as previously reported (13). When viewed from the top (overhead), which corresponds to perpendicular to the intermembrane space, SU-I has the appearance of a propeller shaped funnel; wide open at the top and becoming more restricted toward the centre (Fig. 1 inset). When the complete monomer is viewed from the top (Fig. 1B), it becomes evident that except for a small region, about 25–30% covered by the extra-membrane portion of SU-II, the surface of SU-I is exposed to the contents of the intermembrane space. The exposed region includes that part which stretches from the surface and extends to the haem a 3-CuB centre and designated as ‘pore B’ by Iwata et al (13).

When all of the SUs, except first SU-II and then III, of oxidized Cox were rendered invisible, it became evident that neither SU-II nor SU-III made any direct or close contact with the body, ie the tetrapyrole component, of haem a or haem a 3; also, neither SU came close to CuB (Fig. 2A). Interestingly, the tail of the long hydrophobic side chain of haem a 3 came very close to the two transmembrane segments of SU-II. The tail was sandwiched between the two chains, possibly forming hydrophobic interactions with the two.
closest residues, Ile-34 and Pro-69, which were 4.38 Å and 3.85 Å away, respectively (Fig. 2A–C). Also, the oxidation state did not appear to influence the conformation of SUs II or III, as the SUs of the reduced protein were perfectly overlaid on the SUs of the oxidized (Fig. 2D). Similarly, SUs II and III of the CO Cox were perfectly overlaid on the SUs of the oxidized (Fig. 2E).

Examination of SU-I of oxidized Cox showed that while haem a, haem a₃ and CuB were totally buried within the protein matrix, the end of the long hydrophobic side chain of haem a₃ protrudes out of the SU (Fig. 3A). When the SU was tilted and viewed from the top, suitable rotation showed that haem a and the haem a₃–CuB centre were located in totally unhindered regions of the protein backbone (Fig. 3B) corresponding to pores C and B, respectively (13). Similarly, the top view for the CO-bound Cox showed the haem a₃–CuB ligand binding site to be well clear of the protein backbone (Fig. 3C).

The overlays of subunit-I (Fig. 4 A–D) helped to shed even more light on the picture. Superimposition of the fully reduced on the fully oxidized and of the CO-bound on the fully reduced detected no change in the organization of the protein backbone or the haems when either the oxidation state or ligand state was changed (Fig. 4A, B).

We observed that there were 31 residues (Table) that came within 5 Å of the oxygen/ligand binding site located between haem a₃ and CuB centre. These could be divided broadly into three sections: one to the right of CuB (Fig. 4C), which included Val-243 and Tyr-244, another to the left
which included Val-373 and Gly-355 and the third group adjacent to CuB which included Met-292 and Thr-294. Close inspection of the haem a₃-CuB ligand binding region using expanded views (Fig. 4C, D) did not detect any structures that appear to restrict access to the site. In fact, even when all of the amino acid residues were displayed (Fig. 4D), or all of the SUs were displayed (Fig. 1B), it was still possible to see the unhindered, ‘buried’ ligand binding centre from the top (overhead) view.

T thermophilus: Although SU-I of the Thermus thermophilus ba₃ Cox is known to be some 48 residues bigger than that of the bovine enzyme, these two SUs were found to have the same basic organization (Fig. 5). Thus, when viewed from the top, the alpha helical sections of the ba₃ Cox also gave the appearance of a propeller shaped funnel, wide open at the top but becoming narrow in the vicinity of the haem a₃-CuB centre (Fig. 1 inset, Fig. 5A, B). Superimposition of the two SUs reinforced the idea that although they differ in size, they had the same overall structure (Figs. 5C, D). As observed for the bovine enzyme, haem a₃ in the ba₃ Cox was also seen to be in an unhindered region of the protein matrix (Fig. 5A, B).

Table: Amino acid residues

| Amino acid residues | Amino acid residues |
|---------------------|---------------------|
| Trp 126             | Met 292             |
| Trp 236             | Thr 294             |
| His 240             | Val 295             |
| Val 243             | Thr 294             |
| Tyr 244             | Ala 313             |
| Ile 247             | Thr 294             |
| His 290             | Gly 317             |
| His 291             | Phe 348             |

DISCUSSION

The discovery in the 1970s that Cox catalyses the conversion of oxygen to water at two vastly different rates depending on how recently it had undergone total reduction (25) has thus far been explained only in conceptual terms. Antonini et al (25) described the slow form of the enzyme as ‘resting’ and the more active form as ‘pulsed’. The group proposed that the change in kinetic properties was modulated by a redox-induced conformational change that affected the rates of intramolecular electron transfer. Later, in the 1980s, Jones et al (28) proposed that the perplexingly slow binding of the deadly poison cyanide could be explained in terms of the ‘resting’ and ‘pulsed’ enzyme, if it was assumed that a conformational change switched the enzyme’s binding site from an open and readily accessible position in the pulsed enzyme to a closed and inaccessible position in the resting state.

When recently (18) we observed the different rates of cyanide binding reported previously by others (27, 28), we decided to turn to biocomputing for answers. Since the ‘pulsed’ enzyme always relaxes to the ‘resting’ form on standing (26), we hypothesized that analysis of the existing crystal structures of Cox would reveal the secrets of the ‘closed’ ligand binding site.

In our quest to unearth the conformational change that triggers the resting to pulsed transition of Cox, we compared structures in the vicinity of the oxygen/ligand binding site of three forms of the bovine enzyme, namely, the oxidized, the fully reduced and the CO Cox, which is a form of the fully reduced enzyme. To complete the study, we also compared the ligand binding site region of the bovine and Thermus thermophilus ba₃ Cox.
When the position of SUs II and III relative to haem a and the haem a3-CuB centre was examined, neither SU made any direct contact with either haem but Pro-69 and Ile-34 of SU-II came close, within ~4 Å of the tail of the long hydrophobic side chain of haem a3 (Fig. 2A–C). Equally important, overlays (Fig. 2D, E) showed that neither the positions of SUs II and III nor the redox centres of the oxygen/ligand binding site changed detectibly when the oxidation state of Cox was changed, or when the enzyme bound the CO ligand. These observations suggest that if the resting to pulsed transition of Cox involves a conformational change, the initial change does not involve SUs II or III directly.

When SU-I was added to the analysis, we observed that the haem a3-CuB ligand binding centre sat in a clear, unhindered spot (Figs. 3, 4), forming the base of the channel previously designated ‘pore B’ (13). Although Yoshikawa et al (14) reported a very small difference in the distance separating haem a3 from CuB in the oxidized compared to the reduced enzyme, this very small change did not appear to have an impact on our overlays (Fig. 4A, B). In fact, our overlays of SU-I showed that when CO-bound Cox was compared to the fully oxidized and the fully reduced forms, neither the position nor orientation of haem a3 or CuB was affected by the presence of CO (Fig. 4A–D). Consistent with the findings of Yoshikawa et al (14), who reported major redox linked conformational changes of subunit-I occurring in regions remote from the oxygen binding site, our analysis did not detect any movement of the protein backbone or the amino acid residues in regions close to the oxygen binding site (Fig. 4). In these circumstances, the proposed opening up of the ligand binding site as the enzyme switched from the ‘resting’ to the ‘pulsed’ state seems unlikely and unnecessary, as the binding site in all three forms of the bovine appeared totally unhindered (Figs. 3, 4). We therefore concluded that the slow binding of cyanide to oxidized Cox was not due to the existence of a ‘closed’ ligand binding site in that form of the enzyme.

While we continue to support the concept of the resting and pulsed forms of Cox, we think that the paradoxical kinetics of cyanide binding can be explained by consideration of the overall architecture of SU-I. We have already noted that this SU has an overall funnel shape, wide at the top but narrow toward the centre (Fig. 1 inset). Since our fitting showed that SU-I of bovine and the Thermus thermophilus ba3 Cox had the same general structure (Fig. 5A–D), we conclude that the narrowing in the vicinity of the ligand binding site of the bovine enzyme corresponds to the oxygen channel of the ba3 Cox identified by Luna et al (29). We have shown (Fig. 1) that only about 30% of SU-I is covered/protected mainly by SU-II, exposing its internal regions to the contents of the intermembrane space. Under these conditions, it would be reasonable to conclude that pore B (13) is usually filled with oxygen-rich material. If another ligand, eg cyanide, is presented to the enzyme, it cannot access the haem a3-CuB binding site until the ‘queued-up’ oxygen has been used up. If the enzyme is turning over, this would happen quickly, but if it is not turning over, binding of the alternative ligand will be slow. Hence, cyanide binds slowly to the fully oxidized Cox but rapidly if a suitable source of electrons is added.

So did this study take us any closer to understanding the resting to pulsed transition in Cox? It seems that the initial proposal (25, 26) which suggests that the enzyme must first become fully reduced holds the key. As in deoxy-haemoglobin (30), the iron ion at the centre of haem, in this case haem a3, is out of the haem plane (13). We are of the view that, as is the case for haemoglobin (30), the initial trigger for the ‘resting to pulsed’ transition is the binding of oxygen to the ligand binding site, with pore B (13) serving as the oxygen reservoir. We are proposing that the binding of oxygen not only facilitates the full reduction of the enzyme, but also shifts the iron atom into the haem plane, initiating a range of conformational changes. We think that critical conformational changes are communicated from the ligand binding site in SU-I, to the enzyme’s substrate binding site on SU-II, via the long chain hydrophobic tail of haem a3. Recall this haem side chain comes very close to, probably forming hydrophobic bonds with, Pro-69 and Ile-34 of the transmembrane portion of this SU (Fig. 2). Finally, we propose that structural changes are induced in the vicinity of the cytochrome c binding site, leading to enhanced electron transfer rates as previously proposed (25, 26). In previous studies (31, 32), we proposed mechanisms for electron transfer within Cox; the challenge now will be to demonstrate those conformational changes that enhance electron transfer rates.

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