Probing the Specifics of Substrate Binding for Cytochrome c Oxidase

A Computer Assisted Approach

VB Sampson, T Alleyne, D Ashe

ABSTRACT

A deficiency of cytochrome c oxidase (COX) is associated with a number of diseases but details of the enzyme's mechanism of action especially the interaction with its substrate, ferrocytochrome c, remain unclear. It is known that the transfer of electrons from ferrocytochrome c to COX is facilitated by the formation of enzyme-substrate (ES) complexes which are stabilized by intermolecular salt bridges, however the identity of residues participating in the salt bridges remains obscure. Using the published structures of the two proteins, computer simulations were employed to model their interactions and to attempt to identify residues that participate in intermolecular salt bridges. The simulation process was guided in the main by cross-linking studies which proposed that Lys-13 of cytochrome c is paired with Asp-158 of COX.

The initial enzyme-substrate complex, created by computer assisted manipulation of the two structures exhibited three salt bridges; following the application of energy minimization procedures, the number of salt bridges increased to seven and there were twenty-four intermolecular hydrogen bonds. The salt bridges emanated from: Glu-119 and Asp-221 of subunit I; Glu-114, Asp-115 and Asp-158 of subunit II and Asp-73 and Glu-78 of subunit VIb. These were paired with Lys-87, 8, 25, 27, 13, 22 and 100 respectively of cytochrome c. These results suggest that subunits I, II and VIb play direct roles in substrate binding. The results also suggest that hydrogen bonds contribute significantly to the stability of the ES-complex.

Investigación de las Especifidades de la Unión de Substrato para la Citocromo-c-Oxidasa

Un Enfoque Asistido por Computadora

VB Sampson, T Alleyne, D Ashe

RESUMEN

La deficiencia de la citocromo-c-oxidasa (COX) se halla asociada con un número de enfermedades, pero los detalles del mecanismo de acción – especialmente la interacción con su substrato, el ferrocitocromo c – no está aún claro. Se sabe que la transferencia de electrones del ferrocitocromo c a la COX, es facilitada por la formación de los complejos enzima-substrato (ES), los cuales son estabilizados por puentes intermoleculares de sal. No obstante, la identidad de los residuos que participan en los puentes sigue sin estar clara. Recurriendo a las estructuras publicadas de dos proteínas, se emplearon simulaciones por computadora a fin de obtener un modelo de sus interacciones, en un intento por identificar los residuos que toman parte en los puentes de sal. El proceso de simulación fue guiado principalmente por estudios de reticulación, que proponen que el Lys-13 del citocromo c está pareado con el Asp-18 de la COX. El complejo enzima-sustrato inicial creado mediante la manipulación asistida por computadora de las dos estructuras, exhibía tres puentes de sal. Tras aplicar los procedimientos de minimización de la energía, el número de puentes de sal aumentó a siete y hubo veinticuatro enlaces intermoleculares de hidrógeno. Los puentes de sal emanaron de: Glu-119 y Asp-221 de la subunidad I; Glu-114, Asp-115 y Asp-158 de la subunidad II y Asp-73 y Glu-78 de la subunidad VIb. Estos fueron pareados con Lys-87, 8, 25, 27, 13, 22 y 100 respectivamente del

From: Faculty of Medical Sciences, St Augustine Campus, University of the West Indies, Trinidad and Tobago.

Correspondence: Dr T Alleyne, Biochemistry Unit, Faculty of Medical Sciences, The University of the West Indies, Eric Williams Medical Sciences Complex, Uriah Butler Highway, Champs Fleurs, Trinidad and Tobago. Fax: (868) 662-1873, e-mail: trevor.alleyne@gmail.com.

citocromo c. Estos resultados sugieren que las subunidades I, II y VIb juegan un papel directo en la unión del substrato. Los resultados también sugieren que los enlaces de hidrógeno contribuyen significativamente a la estabilidad del complejo-ES.

West Indian Med J 2009; 58 (1): 55

INTRODUCTION

Whereas a significant portion of the general public is familiar with the oxygen carrying protein haemoglobin and its mechanism of action is well understood (1–2) few, even among scientists, are familiar with cytochrome c oxidases (COX), the protein that actually uses the vast majority (> 80%) of the oxygen we breathe in. COX reduces molecular oxygen to water in a crucial step of oxidative phosphorylation (3). Therefore, a deficiency or absence of this enzyme leads to a range of diseased states both in man and other animals. In man, for example, COX has been shown to play a role in Alzheimer's disease (4), Parkinson's disease (5), Menke's disease (6) and a range of myopathies (7–8). Primarily because of its critical role in energy production, scientists have dedicated much of the last fifty years to studying the mechanism of action of this enzyme. The interaction between this enzyme and its normal substrate, cytochrome c has been of particular interest.

The results of a wide variety of techniques from early studies (9–13) have led to the conclusion that electrostatic interactions between cytochrome c and cytochrome c oxidases (COX) stabilize a tight enzyme-substrate (ES) complex and facilitate electron transfer from the substrate to the enzyme. While the kinetics of the electron transfer processes (14–15) and proton translocation (16–17) have been intensively researched, there is still a lack of microscopic details concerning the electrostatic interactions between the two proteins. Thus, in spite of the application of sophisticated techniques, resonance Raman spectroscopy (15) and electronic electron paramagnetic resonance EPR relaxation measurements (18) being two examples, little is known about the orientation adopted by the substrate when it docks at the enzyme's binding site and very little is known about the number and identity of the intermolecular salt bridges formed.

In a series of experiments conducted during the 1980s Bisson and coworker (11–13) showed that if Lys-13 of cytochrome c was labelled with 4-fluoro-3-nitophenylazide, this light sensitive group, upon photo activation, became crossed-linked at or near to His-161 located in subunit II of COX. The researchers concluded that a conserved acidic residue Asp-158, which is close to His-161, is the probable partner for Lys-13. In other labelling studies, Millet and coworkers (19) showed that the presence of substrate (cytochrome c) protected three residues of COX, Asp-112, Glu-117 and Glu-198, from labelling by the water soluble compound EDC. They suggested that these residues and probably the nearby Asp-158 participated in substrate binding. Then in 1992, in a study using monoclonal antibodies directed against subunit II of COX, Taha *et al* (20) unearthed strong additional evidence in support of the view that the enzyme's substrate binding site is located predominantly on subunit II. Finally, site-directed mutagenesis of COX isolated from *Paracoccus dentirificans* (21, 22) confirmed that as previously proposed (19) a patch of negatively charged residues on subunit II are indeed critical for the catalytic activity of the enzyme. These studies detected two carboxylic residues (Glu-246 and Asp-206) corresponding to Glu-198 and Asp-158 respectively of subunit II of bovine COX that were of particular significance. With regard to the substrate, cytochrome c, a number of studies (9, 10) had identified Lys-8, 13, 22, 27, 29, 39, 60, 72 and 87 as residues that are to varying extents, important for enzyme binding and/or catalysis. With the exception of Lys-13, however, little is known about the identity of their electrostatic "partners".

In an effort to gain a better insight into the specifics of the enzyme-substrate interactions, Roberts and Pique (23) employed computer simulations to dock cytochrome c, the substrate, on to COX. In that study, which used the published structures of the proteins (24, 25), the workers relied exclusively on energy criteria to guide the simulation of an enzyme-substrate complex. We now report an alternative approach in which previously published experimental data (11–13) rather than energy criteria were used as the starting point of the computer simulation. Following the creation of the initial complex, energy minimization procedures were used to optimize the final ES structure.

Experimental

Coordinates for the molecular structures of oxidised bovine COX and the reduced form of horse heart cytochrome c were obtained from the Brookhaven Protein Data Bank and used for the computer simulation of an ES-complex. For the complex, the critical residues used to guide the simulation were Lys-13 on cytochrome c and His-161 and Asp-158 on COX. Following initial formation of the ES-complex by manual manipulation of the individual structures, energy minimization procedures were employed to yield a refined ES-complex.

Initial Complex formations: Using the Insight II computer programme, only residues His-161, Asp-112, Glu-114 and Asp-158 of subunit II of cytochrome c oxidase were displayed. The remainder of the protein was rendered invisible. Similarly, only Lys-13 of ferrocytochrome c was displayed. Lys-13 was then systematically moved so that it was:

- Close enough (~4 Å) to Asp-158 to form an electrostatic bond
- Close enough to His-161 so that it could theoretically form a covalent bond via an arylazido

extension as described by Bisson and coworker (11–13). The distance involved was estimated to be approximately 11 Å.

The other eighteen lysine residues from cytochrome c were then added to the display, two to three at a time. Keeping the initial alignment of Lys-13 relative to Asp-158 and His-161 relatively fixed, the other lysine residues from the cytochrome c molecule were manoeured to determine whether it was possible to bring any of these in close enough proximity to Asp-112 and Glu-114 to form salt bridges. When the maximum number of complementary pairs was established, all of the residues of cytochrome c (including its haem centre) and all of the residues of subunit II, including Cu_A, were displayed. The position of cytochrome c on subunit II was then systematically adjusted by the minor rotation of one protein relative to the other to observe various docked geometries based solely on the complementarity of pairing of the selected lysine residues. The best position generated was evaluated and ranked according to the minimum distance between the complementary pairs of residues at which the relative propensity of adjacent side chains to collide was also minimal. Finally all of the subunits of COX were displayed and the optimization process completed either manually or by energy minimization.

Energy minimization: Given the large number of atoms present in COX and the exceedingly complex nature of the process, it was not practical to use the entire enzyme for the minimization study: Instead, as done by Roberts and Pique (23), we used only a fraction of the enzyme. A region of ~25 Å in radius which encompassed all of the amino acid residues of ferrocytochrome c and a segment of COX of approximately twice the size of cytochrome c, was selected for minimization. The COX region selected contained those atoms of subunits I, II, III and Vlb which lie along the cytochrome c-COX interface: This region contained 327 amino acid residues and 5144 atoms (Table 1).

First, a working pH of 7.4 was set and hydrogen atoms automatically introduced to satisfy the pK of each amino acid. Next the force field parameters were chosen (26) and the formal oxidation state of each metal atom set. In the next stage, all of the heavy atoms of the residues in the selected zone were fixed to first allow hydrogen atoms to adjust to reasonable positions within the structural environment. Following this, constraints were gradually removed to progressively relax parts of the model. The constraints of the heavy atoms were released and only the main chain atoms were fixed to allow movements of the side chains of all the residues. As the system became more relaxed, these backbone constraints were eventually removed, allowing the entire system to be minimized freely. Finally, the energy minimized cut segment, with cytochrome c attached, was reattached to the remainder of the COX molecule.

Table 1: Residues of the zone of the COX-cytochrome c ES-complex selected for energy minimization computations

Selected Component	Residues/ Metals	No. of Residues
COX, Sub–I	49–53, 55–56, 119–125, 128–140, 142–143 209, 211–232, 294–296, 368–369, 437–441, Mg	63
COX, Sub–II	95–128, 135–144, 150–165, 170–182, 184, 193–212, 219, 222–227, Cu_A, Cu_B	103
COX, Sub–III	36, 108–114, 119, 189–192	13
COX, Sub–VIb	3, 23–34, 36–37, 56–83, 85	44
Cytochrome c	1–104 and heme c	104

The residues of the segment of the COX-cytochrome c ES-complex selected for energy minimization computations are listed. The region contained 327 amino acid residues and 5144 atoms; it included all of the residues of cytochrome c and residues located in subunits I, II, III and VIb of COX.

RESULTS

A general but somewhat surprising feature of the model was that the alignment of Lys-13 with Asp-158, while maintaining a distance of approximately 11 Å from His-161, Bisson *et al* (11–13) required that the cytochrome c molecule be rotated 180° clockwise from the orientation in which it is traditionally displayed; any other orientation led to extensive unfavourable interactions between the two proteins. In this orientation, residues 4 and 86 would be positioned below while residue 44 would be positioned above the haem (Fig. 1).

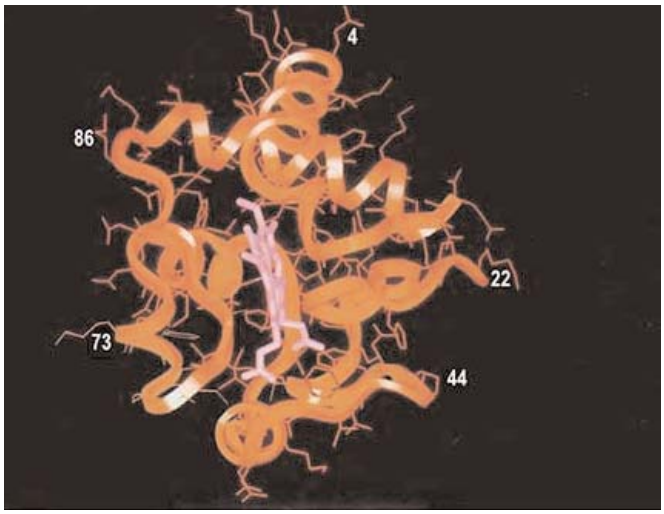


Fig. 1: A ribbon representation of ferrocytochrome c (orange) with its haeme in pink. Simulation of COX-cytochrome c Es-complexes which were free of unfavourable protein-protein interactions, required the cytochrome c to be rotated 180° clock-wise from the orientation shown in the figure.

Using the step by step approach outlined in the methods, it was found that following the alignment of Lys-13 with Asp-158, two additional salt bridges involving ferrocytochrome c and subunit II could be formed by suitable rotation of cytochrome c. The salt bridges formed involved, Lys-25 and Lys-27 of cytochrome c with Glu-114 and Glu-115 respectively of subunit II (Figs. 2A, 2B). The distance between Lys-13 and Asp-158 was 3.49 Å while Lys-25 was located at a distance 4.91 Å from Glu-114 and Lys-27 was at 2.41 Å from Asp-115 (Table 2). In this position, the haem of cytochrome c was positioned at a distance of 21.32 Å from the Cu_A centre of COX.

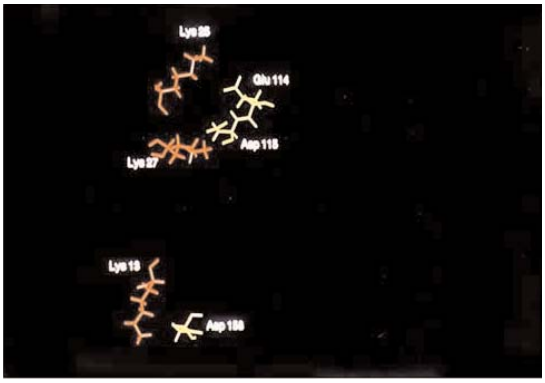
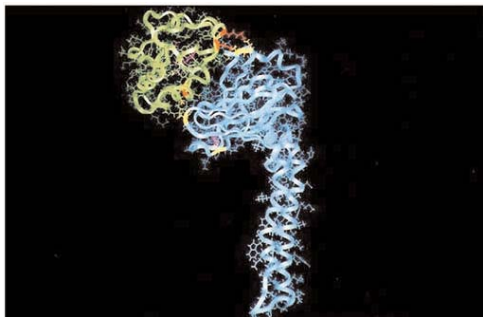
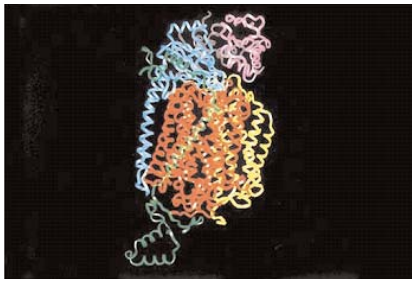


Fig. 2 (A–C): The initial computer simulated COX-cytochrome c ES-Complex
The computer assisted alignment of Lys-13 of cytochrome c with Asp-158 of subunit II of COX, followed by the appropriate rotation of the two molecules produced three salt bridges
A: The residues participating in the three salt bridges are shown; lysine residues from ferrocytochrome c are shown in red with complementary carboxylate residues from subunit II of COX in yellow.



B: The resulting position of cytochrome c (green) relative to subunit II of COX(blue); lysine residues from ferrocytochrome c are shown in red and carboxylates from COX in yellow.



C: The resulting position of cytochrome c (pink) relative to COX. For clarity, only 5 of the 13 COX subunits are shown. The subunits shown are; I(red), II(blue), III(yellow), IV(green) and VIb(grey). The complex has been rotated approximately180^{o} horizontally relative to figures A and B.

Table 2: Salt bridges formed in a computer simulated COX-cytochrome c ES-complex

	Cyt c	Cytochrome c oxidase		
Process	**Lysine**	**Subunit**	**Carboxylate**	**Distance (Å)**
A:	13	II	Asp-158	3.49
Manual Docking	25	II	Glu-114	4.91
	27	II	Asp-115	2.41
B:	8	I	Asp-221	3.13
Energy Minimized	13	II	Asp-158	4.81
	22	VIb	Asp-73	1.92
	25	II	Glu-114	2.63
	27	II	Asp-115	1.53
	87	I	Glu-119	1.62
	100	VIb	Glu-78	2.69

Details of salt bridges formed in the computer simulation of a COX-cytochrome c ES-complex are shown. Details include the participating lysine residues on cytochrome c and the corresponding residues on COX. For COX, the subunit of each participating residue is shown. Also shown is the non-bonded distance for each salt bridge. **A**: Initial complex formed by manual manipulation. **B**: Complex after energy minimization.

When the remainder of cytochrome c and COX were introduced, no additional salt bridges were observed but a total of six intermolecular hydrogen bonds were formed between cytochrome c and two enzyme subunits, namely, subunits II and VIb (Table 3).

Following energy minimization of the complex, there was a shift in the relative positions of the two proteins, resulting in more intimate contact. The net result was an increase in the number of salt bridges from 3 to 7; an increase in the number of intermolecular hydrogen bonds, from 6 to 24 and a shortening of the non-bonded distances for two of the three pre-existing salt bridges (Tables 2 and 3). The residues from COX along with the complementary lysine residues from cytochrome c and the corresponding non-bonded distances for the seven salt bridges were: in subunit II, Asp-158 with Lys-13 (4.81 Å); Glu-114 with Lys-25 (2.63 Å) and Asp-115 with Lys-27 (1.53 Å); in subunit I, Asp-221 with Lys-8 (3.13 Å) and Glu-119 with Lys-87 (1.62 Å) and in subunit VIb, Asp-73 with Lys-22 (1.92 Å) and Glu-78 with Lys-100 (2.69 Å) (Figs. 3, 4A and 4B).

Of the twenty-four intermolecular hydrogen bonds, six were formed from residues located in subunit I, ten mediated from residues within subunit II and eight were formed from residues located in subunit VIb (Table 3). The distance between the haem edge of cytochrome c and the bimetallic Cu_A centre of COX was 21.91 Å and the closest COX residues to the haem edge were Trp-104 and Tyr-105 (< 6 Å).

Table 3: Hydrogen bonds formed in a computer simulated COX-cytochrome c ES-Complex

Model	Donor	Acceptor	Distance (Å)
A:	Subunit Vlb: 67: HG	Cyt c 15:O	2.25
	Subunit 11: 102: ND	Cyt c 16: OE1	2.47
Manual	Cyt c: 16: HE21	Subunit II: 102: ND1	1.46
Docking	Subunit Vlb: 70: HG	Cyt c: 19: OG1	1.57
	Subunit Vlb: 73: HN	Cyt c 21: OE2	1.86
	Cyt c: 27: HZ2	Subunit II: 115: OD2	1.39
B:	Cyt c: 7: HZ2	Subunit 1: 223:O	2.34
	Cyt c: 8: HZ2	Subunit 1: 221: OD1	1.56
Energy	Cyt c: 8: HZ3	Subunit 1: 135: OD1	1.83
Minimized	Cyt c: 13: HZ3	Subunit II:103: O	2.36
	Cyt c: 14: HN	Subunit II: 157: OE2	2:08
	Cyt c: 16: HN	Subunit II 157: OE1	1.96
	Subunit II: 117: HG	Cyt c: 16: O	1.55
	Subunit II: 107: HG	Cyt c: 16: OE1	1.58
	Cyt c: 16: HE21	Subunit II: 119: OD1	1.75
	Cyt c: 20: HN	Subunit Vlb: 70: OG	2.26
	Subunit Vlb: 61: HZ3	Cy c: 21: OE1	1.53
	Cyt c: 22: HN	Subunit Vlb: 61: OD2	1.92
	Subunit Vlb: 76: HH21	Cyt c: 22: O	1.94
	Cyt c: 22: HZ2	Subunit Vlb: 76: O	1.74
	Cyt c: 25: HZ3	Subunit II: 114: OE1	1.58
	Cyt c: 27: HZ3	Subunit II: 115: OD2	1.53
	Cyt c: 28: HG1	Subunit II: 227: OXT	1.40
	Cyt c: 33: HD1	Subunit Vlb: 74: OD1	1.91
	Cyt c: 86: HZ2	Subunit II: 103: OE1	1.77
	Cyt c: 86: HZ3	Subunit I: 137: O	1.81
	Cyt c: 87: HZ1	Subunit I: 119: OE1	1.62
	Cyt c: 87: HZ2	Subunit I: 139: O	1.80
	Cyt c: 97: HH	Subunit Vlb: 74: OD2	1.42
	Cyt c: 100: HZ1	Subunit Vlb: H78: OE2	1.64

Details of hydrogen bonds formed in the computer simulation of a COX-cytochrome c ES-complex are shown. Details shown include the respective residue number of each hydrogen bond as well as the identity of the specific atoms involved. For COX, the subunit of each participating residue is shown. Also shown is the non-bonded distance for each hydrogen bond. **A**: Initial complex formed by manual manipulation. **B**: Complex after energy minimization.

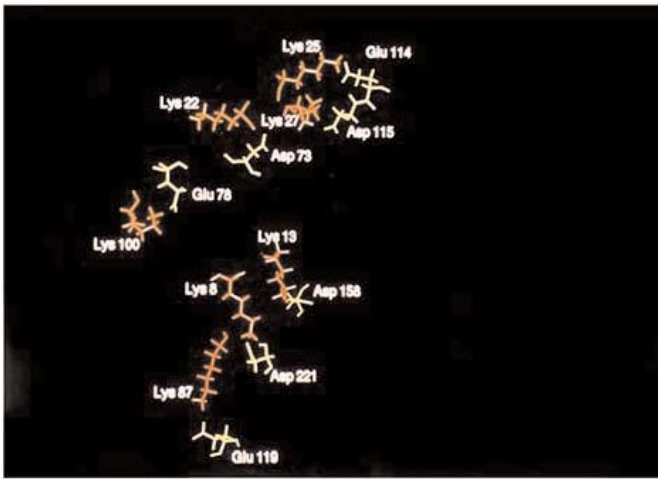


Fig. 3: The computer simulated COX-cytochrome c ES-Complex obtained after energy minimization. Following application of energy minimization to the initial COX-cytochrome c complex seven salt bridges were obtained. A: The lysine residues from ferrocytochrome c are shown in red with complementary carboxylate residues from subunit II of COX in yellow.

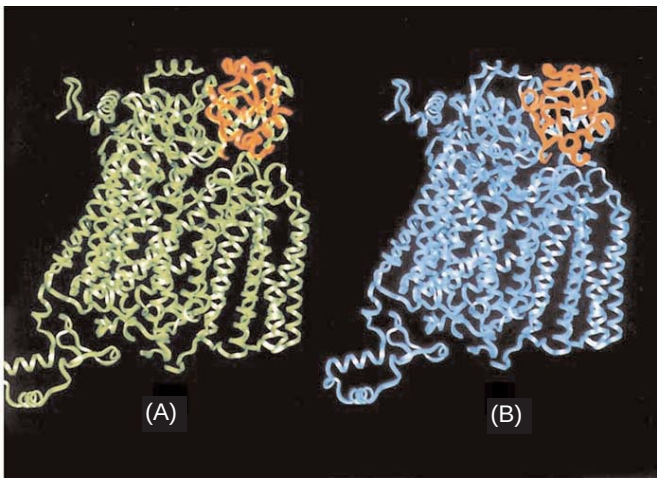


Fig. 4A: The position of ferrocytochrome c (orange) relative to COX before energy minimization of the ES complex. Fig. 4B: The position of ferrocytochrome c (orange) relative to COX after energy minimization of the ES-complex.

The total columbic energy for the simulated complex was -135.5×10^8 kJ mol^{-1}.

DISCUSSION

Even with the assistance of powerful computers, establishing the identity of those residues involved in intermolecular salt bridges formed between a substrate and a large enzyme, particularly when the substrate is itself a protein, is still difficult. Two factors (i) knowledge of the specific cross-linking region for Lys-13 of the substrate and (ii) the ability to selectively display only those residues of interest while rendering the remainder of both proteins invisible, considerably simplified the process in this particular instance.

Quite apart from the discovery that cytochrome c modified at Lys-13 with a photo labile group became cross-linked to COX in a region near to Asp-158 (11–13), Bisson *et al* (27) also showed that this specific modification of the substrate led to total inhibition of COX. A number of other studies, using totally different approaches (19, 20) also identified Asp-158 as being involved in or critical to COX catalysis. By applying energy minimization to an ES-complex created on the basis of interaction between Lys-13 and Asp-158, we believe that we would have simulated a COX ES-complex that is grounded on credible experimental data and which should therefore be of relevance to investigations on substrate binding and enzyme catalysis. A number of researchers have suggested that there might not be a single unique orientation adopted by cytochrome c when it becomes coupled to COX during turnover (22), however, Bertini *et al* (28) conducting computer simulations on the simpler bacterial oxidase noted that not all low energy COX-ES complexes generated by energy minimization procedures had catalytic capabilities. By combining the experimental data with energy minimization procedures it seems certain that we would have avoided complications of the latter type.

Our finding that the simulation of a COX ES-complex in which Lys-13 was paired with Asp-158 and which was free of extensive unfavourable inter-chain interactions could only be achieved if cytochrome c was rotated ~180° from the orientation in which it is traditionally presented, had not been predicted by previous studies, and was therefore totally unexpected. The more than doubling of the number of salt bridges, from 3 to 7, following energy minimization of the initial complex, also came as a pleasant surprise and suggests that induce fit (29) and/or transition state mechanisms (30) may be at work when COX binds to its substrate.

Like Roberts and Pique (23), we found that because of its large size it was not possible, using our computing capabilities, to achieve energy minimization for the entire COX ES-complex. However, since the fraction of COX used in the energy minimization process was substantial, approximately twice the size of cytochrome c and since it encompassed significant proportions of the subunits which interfaced with cytochrome c, we believed that the final fit achieved, with some seven salt bridges, represents fairly well the initial ES complex formed when the two proteins come together. Indeed in our ES-complex, as was the case for Roberts and Pique (23), Trp-104 is in close proximity (<6 Å) from the haem edge of cytochrome c. This finding is consistent with the results of site-directed mutagenesis which have identified the latter residue as the point of entry for electrons from the substrate into COX.

Interestingly, whereas our model and that of Roberts and Pique (23) appear to converge at Trp-104, the pairing of the salt bridges in the two models were totally different. Significantly, in the case of the latter, Lys-13 is paired with Asp 119 of subunit II and does not come close to Asp-158 as suggested by experimental findings (11–13). Also whereas our complex exhibited seven salt bridges, that of Roberts and Pique exhibited six. Moreover one of that six, formed between Lys-72 and Asp-158, was only achieved indirectly and "by undergoing substantial conformational change" (23). In both models, Gln-103, shown to be important to substrate binding (31) forms an important hydrogen bond. In the Roberts-Pique model, it is bonded to Lys-72 while in our case the bond is formed with Lys-13.

The present results suggest that in addition to subunit II, both subunits I and VIb play important, direct roles in substrate binding, contributing between them four of the seven salt bridges and fourteen of the twenty-four hydrogen bonds. This finding is consistent with the proposal of Tsukihara and coworkers (32) who in an analysis of their crystal structure of oxidized COX had directed attention to a concave region defined by subunits II, VIa and VIb which they suggest might form the cytochrome c binding site. Also, in our own cross-linking studies performed under steady-state reducing conditions (33), we had observed that in addition to binding to subunit II, cytochrome c became bound to two other COX subunits, one of which was subunit VIb.

The present study also highlights the possible importance of hydrogen bonds to the stabilization of the COX ES-complex. Indeed, there were more than three times as many hydrogen bonds as there were salt bridges. Witt *et al* (34) have suggested that such non-ionic bonds play a vital role in fine tuning the final fit between the enzyme and the substrate. With respect to the salt bridges, the present model suggests that Glu-119 and Asp-221 of subunit I; Glu-114, Asp-115 and Asp-158 of subunit II and Asp-73 and Glu-78 of subunit VIb form salt bridges with Lys-87, 8, 25, 27, 13, 22 and 100 respectively of cytochrome c (Table 2). With the exception of Lys-100, the other six lysine residues listed are among those identified by Ferguson-Miller *et al* (10) and Osheroff *et al* (35) as being particularly important for binding to COX. While we do not rule out the possibility that COX and its substrate cytochrome c may dock effectively in more than one way (22), this idea that COX behaves differently to all other enzymes will require the support of experimental evidence to gain acceptance. In the mean time, we believe that the alignment reported here is one of the more important, if not the most important for catalysis. Having established a

hypothetical structure for a COX-cytochrome c ES-complex, it might now be possible to turn ones attention to the question of intermolecular electron transfer between these two proteins and how this might be affected in disease states.

ACKNOWLEDGEMENTS

We wish to thank Professor Ernest Feytmans for his assistance with the computer simulations, in particular for his assistance with the energy minimization. We also thank Mr Richard Spence and Mr Dexter Superville for photography and Mr Michael Khan and Ms Heather Woodroffe for preparation of figures.

REFERENCES

1. Perutz M. Stereochemistry of cooperative effects in Haemoglobin: Haem-Haem Interaction and the Problem of Allostery. Nature 1970; **228:** 726–34.
2. Arnone A, Perutz M. Structure of inositol hexaphosphate-human deoxyhaemoglobin complex. Nature 1974; **249:** 34–6.
3. Babcock GT, Wikstrom M. Oxygen activation and the conservation of energy in cell respiration. [Review] Nature 1992; **356:** 301–9.
4. Kish SJ, Mastrogiacomo F, Guttman M, Furukawa Y, Taanman JW, Dozic S et al. Decreased brain protein levels of cytochrome oxidase subunits in Alzheimer's disease and in hereditary spinocerebellar ataxia disorders: a non specific change? J Neurochem 1999; **72:** 700–7.
5. Itoh K, Weis S, Mehraein P, Muller-Hocker J. Defects of cytochrome c oxidase in the substantia nigra of Parkinson's disease: an immunohistochemical and morphometric study. Mov Disord 1997; **12:** 9–16.
6. Sparaco M, Hirano A, Hirano M, DiMauro S, Bonilla E. Cytochrome c oxidase deficiency and neuronal involvement in Menkes' kinky hair disease: immunohistochemical study. Brain Pathol 1993; **3:** 349–54.
7. Lee WT, Wang PJ, Young C, Wang TR, Shen YZ. Cytochrome c oxidase deficiency in fibroblasts of a patient with mitochondrial encephalomyopathy. J Formosan Med Assoc 1996; **95:** 709–11.
8. Eshel G, Lahat E, Fried K, Barr J, Barash V, Gutman A et al. Autosomal recessive lethal infantile cytochrome c oxidase deficiency. Am J Dis Child 1991; **145:** 661–4.
9. Smith HT, Hildebrandt P, Rosell FI, von Mauk AG, Walter M, Buse G, et al. Use of specific lysine modifications to locate the reaction site of cytochrome c with cytochrome c oxidase. Biochemistry 1977; **16:** 4971–4.
10. Ferguson-Miller S, Brautigan DL, Margoliash E. Definition of cytochrome c binding domains by chemical modification. III. Kinetics of reaction of carboxydinitrophenyl cytochromes c with cytochrome c oxidase. J Biol Chem 1978; **253:** 149–59.
11. Bisson R, Gutweniger H, Montecucco C, Colonna R, Zanotti A, Azzi A. Covalent binding of arylazido deravitives of cytochrome c to cytochrome c oxidase. FEBS Lett 1977; **81:** 147–50.
12. Bisson R, Azzi A, Gutweniger H, Colonna R, Montecucco C, Zanotti A. Interaction of cytochrome c with cytochrome c oxidase. Photoaffinity labeling of beef heart cytochrome c oxidase with arylazido-cytochrome c. J Biol Chem 1978; **253:** 1874–80.
13. Bisson R, Steffens GC, Capaldi RA. and Buse G. Mapping of the cytochrome c binding site on cytochrome c oxidase. FEBS Lett 1982; **144:** 359–63.
14. Alleyne TA, Wilson MT, Antonini G, Malatesta F, Vallone B, Sarti P. et. al. Investigation of the electron-transfer properties of cytochrome c oxidase covalently cross-linked to Fe- or Zn-containing cytochrome c. Biochem J 1992; **287:** (Pt 3): 951–6.
15. Hrabakova J, Ataka K, Heberle J, Hildebrandt P, Murgida DH. Long distance electron transfer in cytochrome c oxidase immobilised on electrodes. A surface enhanced resonance Raman spectroscopic study. Phys Chem Chem Phys 2006; **8:** 759–66.
16. Prochaska LJ, Reynolds KA. Characterization of electron-transfer and proton-translocation activities in bovine heart mitochondrial cytochrome c oxidase deficient in subunit III Biochemistry 1986; **25:** 781–7.
17. Branden G, Pawate AS, Gennis RB, Brzezinski P. Controlled uncoupling and recoupling of proton pumping in cytochrome c oxidase. Proc Natl Acad Sci USA 2006; **103:** 317–22.
18 Lyubenova S, Siddiqui MK, Vries MJ, Ludwig B, Prisner TF. Protein-protein interactions studied by EPR relaxation measurements: cytochrome c and cytochrome c oxidase. J Phys Chem B 2007; **111:** 3839–46.
19. Millett F, de Jong C, Paulson L, Capaldi RA. Identification of specific carboxylate groups on cytochrome c oxidase that are involved in binding cytochrome c. Biochemistry 1983; **22:** 546–52.
20. Taha TS, Ferguson-Miller S. Interaction of cytochrome c with cytochrome c oxidase studied by monoclonal antibodies and a protein modifying reagent. Biochemistry 1992; **31:** 9090–7.
21. Witt H, Zickermann V, Ludwig B. Site-directed mutagenesis of cytochrome c oxidase reveals two acidic residues involved in the binding of cytochrome c. Biochim Biophys Acta 1995; **1230:** 74–6.
22. Witt H, Zickermann V, Ludwig B. Cytochrome-c-binding site on cytochrome oxidase in *Paracocus denitrificants*. Eur J Biochim 1998; **251:** 367–73.
23. Roberts VA, Pique ME. Definition of the interaction domain for cytochrome c on cytochrome c oxidase. III. Prediction of the docked complex by a complete, systematic search. J Biol Chem 1999; **274:** 38051–60.
24. Yoshikawa S, Shinzawa-Itoh K, Tsukihara T. Crystal structure of bovine heart cytochrome c oxidase at 2.8 A resolution. J Bioenerg Biomembr 1998; **30:** 7–14.
25. Takano T, Dickerson RE. Conformation change of cytochrome c. II. Ferricytochrome c refinement at 1.8 A and comparison with the ferrocytochrome structure. J Mol Biol 1981; **153:** 95–115.
26. Dauber-Osguthorpe P, Roberts VA, Osguthorpe DJ, Wolff J, Genest M, Hagler AT. Structure and energetics of ligand binding to proteins: Escherichia coli dihydrofolate reductase-trimethoprim, a drug-receptor system. Proteins 1988; **4:** 31–47.
27. Bisson R, Jacobs B, Capaldi RA. Binding of arylazidocytochrome c derivatives to beef heart cytochrome c oxidase: cross-linking in the high- and low-affinity binding sites. Biochemistry 1980; **19:** 4173–8.
28. Bertini I, Cavallaro G, Rosato A. A structural model for the adduct between cytochrome c and cytochrome c oxidase. J Biol Inorg Chem 2005; **10:** 613–24.
29. Koshland DE, Jr. Nemethy G, Filmer D. Comparison of experimental binding data and theoretical models in proteins containing subunits1. Biochemistry 1966; **5:** 365–85.
30. Hammond GS. A correlation of reaction rates. J Am Chem Soc 1955; **77:** 334–8.
31. Lappalainen P, Watmough NJ, Greenwood C, Saraste M. Electron transfer between cytochrome c and the isolated Cu_A domain: identification of substrate-binding residues in cytochrome c oxidase. Biochemistry 1995; **34:** 5824–30.
32. Tsukihara T, Aoyama H, Yamashita E, Tomizaki T, Yamaguchi H, Shinzawa-Itoh K, et al. The whole structure of the 13-subunit oxidized cytochrome c oxidase at 2.8 A. Science 1996; **272:** 1136–44.
33. Sampson, V. Alleyne T. Cytochrome c/cytochrome c oxidase interaction. Direct structural evidence for conformational changes during enzyme turnover Eur J Biochem 2001; **268:** 6534–44.
34. Witt H, Malatesta F, Nicoletti F, Brunori M, Ludwig B. Tryptophan 121 of subunit II is the electron entry site to cytochrome-c oxidase in Paracoccus denitrificans. Involvement of a hydrophobic patch in the docking reaction. J Biol Chem 1998; **273:** 5132–6.
35. Osheroff N, Brautigan DL, Margoliash E. Mapping of anion binding sites on cytochrome c by differential chemical modification of lysine residues 209. Proc Natl Acad Sci USA 1980; **77:** 4439–43.