EXPERIENTIA

Volume 40/No.9 Pages 901–1024 15 September 1984

Reviews

In memoriam Hugo Aebi 1921-1983

The following two contributions are presented in memory of Hugo Aebi, former editor of Experientia. Professor Aebi died in the Swiss mountains on July 15, 1983 at the age of 62. Hugo Aebi began his career in medicine at the University of Basel, graduating in 1945. His first post was at the Institute of Medical Chemistry in Basel and at 33 he became the Chairman of the Institute of Medical Chemistry at the University of Bern. His scientific interests focused on *nutritional research* and *enzyme interactions*. His role within the university was multifaceted; as a teacher, he ably transmitted his enthusiasm and as an organizer, he never refused responsibility. Hugo Aebi served as Dean of the Medical Faculty, as Rector of the University, as a colonel in the Medical Corps of the Swiss Army, and – during the years of economic recession – as the President of the Swiss Advisory Council for Science. Many younger biochemists have benefited from Hugo Aebi's freely given advice in his function as editor of Experientia, an office he held from 1974 until his untimely death.

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Mitochondria: the utilization of oxygen for cell life

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Key words. Mitochondria; cytochrome *c* oxidase; energy metabolism; ATP synthesis.

A fuel cell

Most of the energy (more than 95%) of animal cells is delivered by oxidative phosphorylation and mitochondria are the cell organelles where this process takes place. The mechanism by which the chemical energy of a substrate is transferred to ATP in the mitochondria is electrical in nature. That transmission of biochemical power occurs in mitochondria through electricity, is surprising because mitochondria do not contain a sufficient density of electron conductors. In these organelles, however, the conduction of protons, in proteinaceous and aqueous regions, substitutes for that of electrons in the electric conductors of metallic power lines of artificial systems[55]. The insulating elements are provided by the low conductors constituted by the membrane bilayer.

The mechanism by which mitochondria generate electricity, or better, 'proticity', is complex but can be essentially considered similar to what occurs in a hydrogen-burning fuel cell. Here there are two elements of the fuel cell: the one in contact with O_2 becomes positively charged and produces O^{2-}; the other, in contact with H_2 becomes negatively charged and produces H^+. The connection between the 2 elements with a H^+ conductor (H_2O) results in a source of electricity; the connection with an electric conductor results in a source of 'proticity'[55]. Mitochondria function essentially in this 2nd form. They have an electron transport chain which allows separation of OH^- in one compartment from H^+ in another compartment and in this way proticity is produced. This form of electrical energy can be utilized to synthesize ATP.

The terminal respiratory enzyme of mitochondria, cytochrome *c* oxidase, represents an element of the respiratory chain capable of electric conduction through its metal centers and separation of H^+ from OH^- through a proton pump. Such a system has an extreme complexity whose present understanding will be reviewed below.

The terminal respiratory enzyme

The terminal oxygen-reducing enzyme of the respiratory chain in mitochondria and in many bacteria is cytochrome *c* oxidase; cytochrome *c* reduces through it molecular oxygen to produce water and in so doing it allows electron flow in the respiratory chain to proceed associated with energy conservation. The enzyme itself is, in addition, capable of storing the energy associated

with the difference in oxidation reduction potential between electron donor and acceptor. If not in all bacteria, in higher organisms cytochrome *c* oxidase is an enzyme of fundamental importance for life: it is, in fact, the last and essential oxidative step of the entire oxidative metabolism.

Cytochrome *c* oxidase from mammalian mitochondria is a membrane-bound, multi-subunit enzyme, having a molecular weight of approximately 200,000[2] and containing 2 heme A groups and 2 copper ions per catalytic unit. Due to the fact that the 2 heme groups have different environments, they also exhibit differing spectral properties and reactivity with ligands, enabling them to be resolved as hemes *a* and a_3. Heme a_3, but not heme *a*, can bind ligands which may be oxygen, CO, HCN and HN_3 (see Lemberg[41]). The 2 copper ions of cytochrome *c* oxidase are located in different environments within the enzyme and thus are also distinguished due to the resulting difference in their spectral properties in Cu_A (detectable by electron spin resonance and also called 'visible') and Cu_B ('invisible'). The exact electron transfer pathway in cytochrome *c* oxidase is not yet fully clarified but it appears that electrons are donated from cytochrome *c* to heme *a*, this component being in rapid redox equilibrium with Cu_A. Electrons then pass to the heme a_3-Cu_B complex and from this to oxygen (see Wikström et al.[80] for a more detailed treatment).

As already mentioned, cytochrome *c* oxidase possesses an additional capacity namely, the ability to couple the energy derived from electron flow to the outward translocation of protons across the mitochondrial membrane, leading to the formation of a transmembrane electrochemical gradient of H^+ which is used as an energetic intermediate in the synthesis of ATP.

A highly complex quaternary structure

The highly complex quaternary structure of cytochrome *c* oxidase requires a highly integrated and controlled system of biogenesis. Mitochondria possess their own biosynthetic apparatus[76] having their own genome, ribosomes and transfer RNA. This system produces only a limited number of mitochondrial proteins, among which are subunits 1, 2 and 3 of cytochrome *c* oxidase (coded for by the Oxi 3, 1 and 2 genes respectively of the mitochondrial genome). The remainder of the oxidase subunits are products of nuclear genes and there is evidence that at least some of these are synthesized as precursors[42,53] which then undergo an energy-dependent processing[65] involving specific mitochondrial proteases during their integration into the membrane-bound form of the enzyme.

The primary structure of the beef heart cytochrome *c* oxidase has been elucidated to a great extent. Together with the information available from electron diffraction analysis on two-dimensional crystals[31–33,35], subunit cross-linking[6,7], chemical[8,20,22,26,27,44,59] and immunochemical labeling[31,47,50]) it has been possible to construct a structural model of cytochrome *c* oxidase. Cytochrome c oxidase is embedded in the mitochondrial inner membrane with several highly hydrophobic segments in contact with the alkane chains of the phospholipid and hydrophilic segments exposed to the aqueous phases on each side of the membrane.

Two-dimensional crystals of cytochrome *c* oxidase from bovine heart suitable for electron microscopy and image reconstruction[32,33,35] have been obtained. Using several different types of crystalline arrays for the three-dimensional reconstruction, Fuller et al.[32] determined the general size and shape of the cytochrome *c* oxidase molecule. The maximal resolution reached up to now is in the range of 20 Å. This does not provide information about the tertiary structure or the topology of the subunits since for the identification of the amino acid residues a resolution higher than 3 Å is needed.

A suitable crystalline form of cytochrome *c* oxidase is obtained by its extraction from mitochondria using deoxycholate. Negative staining with phosphotungstate or uranyl acetate shows that the enzyme is monomeric (two-heme complex) arranged in detergent-rich sheets with no continuous bilayer[32,33,68].

The cytochrome *c* oxidase monomer is seen as an asymmetric 'Y' with a length of about 110 Å giving rise to 3 domains. The 2 arms of the Y (M_1- and M_2-domains) are approximately 50 Å in length with a center to center separation of 40 Å, each being surrounded by phospholipids. These 2 M-domains separated on the cytosolic side of the membrane span the bilayer and protrude out of the inner membrane by about 15–20 Å on the matrix side. The stalk of the Y is a single domain (C-domain) and extends about 50–55 Å from the cytoplasmic side of the mitochondrial inner membrane.

Low angle X-ray studies show reflections characteristic of helical structures perpendicular to the plane of the membrane. The M_1-domain with a surface area in projection of about 900 $Å^2$ is large enough to contain 8–12 helices of the same average dimensions as those in bacteriorhodopsin. The smaller M_2-domain with about 650 $Å^2$ could contain some 5–8 helices.

Labeling and cross-linking experiments have allowed the location of the different subunits of the cytochrome *c* oxidase within the Y-shaped profile discussed above[8,22,26,44,59]. The major part of the C-domain should be formed by the subunits I, II, III and V. Subunit I, II and III having 10^{51}, 2^{67} and 6^{51} transmembrane stretches respectively are likely to contribute as to the M_1- as well as to the M_2-domain each of which are embedded in the lipid bilayer. These conclusions are inferred from labeling experiments using immunolabeling[22], or chemical labeling using arylazidophospholipids[21,44] and iodonaphthylazide[59]. Subunit IV, VII_{Ser} and VII_{Ile} each having a single stretch of hydrophobic amino acids[13,15] are also labeled by arylazidophospholipids[8,21]. All these 21 transmembrane segments contribute to the bilayer-intercalated part of the M_1 and M_2-domains. Since neither the M_1 nor the M_2-domain is large enough to contain the 16 helices indicated in subunit I and III, Capaldi et al.[3] suggested placing them in separate domains (subunit I in M_1 and subunit III in M_2).

From studies in which cytochrome c oxidase is a dimer in the vesicle crystal[25,35] (using Triton X-100) it was inferred that the contact between monomers occurs exclusively through the C-domain with a closest approach of 36 Å between the M_2-domains (center to center) of each monomer.

Specific functions can be assigned to the different subunits

In prokaryotes the isolated enzyme complex contains only two[43,45,72] or 3 polypeptides[36,84]; in lower eukaryotes such as *Saccharomyces cerevisiae* and *Neurospora crassa,* 7 and 8 polypeptides were found respectively[58,62,67,79]. In plants, such as pea or sweet potato, a 5-polypeptide cytochrome *c* oxidase has been found[46,49] while in mammalian tissues the enzyme consists of 12–13 polypeptides. The conclusion that the mammalian enzyme contains 12–13 subunits is not, however, unanimous[2,3,12,15,38,51,73,78].

It appears probable that the 3 largest subunits, to a great extent homologous and immunologically cross reactive, are the ones which, in both prokaryotes and eukaryotes, are responsible for the known catalytic functions of the enzyme[2,3,15,23,83]. The other subunits may be involved in: specific interactions with other proteins or complexes; the assembly of the enzyme; or control of the reactivity of the enzyme. The differences observed in the primary structure of some of the low molecular weight polypeptides in different mammalian tissues would point towards their regulatory role[5,37,52]. Similarly the finding that the accessibility of subunit V to antibodies[29] depends on the redox state of the enzyme is also indicative of a control role exerted by this subunit.

Analogy with bacterial oxidases, cross-linking of the hemes to the yeast complex[66], proteolytic removal on non-heme-binding subunits[10,85], location of potential heme-binding amino acids sequences[24,74], electrophoresis under non-disrupting conditions[30,34,37] and resolution of the enzyme by controlled denaturation provide evidence of the location of the hemes in subunits I and II in the native enzyme[23]. The location of the copper(s) at the level of subunit II has been also suggested by Winter et al.[83], by Darley-Usmar et al.[24] and by Buse et al.[11].

Electron-spin resonance and EXAFS (extended X-ray absorption fine structure) studies indicate that Cu_A has 2 sulfur ligands and 2 nitrogen (or oxygen) ligands[75]. A cystein sulfur ligand bridging Fe_{a3} (oxidized) and Cu_B has been suggested by Powers et al.[57]. The only conserved cysteines in cytochrome *c* oxidase (from beef, human placenta, yeast, mouse and maize) are those corresponding in the bovine heart enzyme to residues 196 and 200 of subunit II and they have been shown to become reactive to sulfydryl reagents only after denaturation of the enzyme and loss of copper[24]. Methionine 206 which was suggested to be involved (analogously to blue-copper-proteins) in copper binding has been shown not to be conserved in maize, where it is replaced by a threonine[28]. Other possible copper ligands are the conserved histidine residues of subunit II, 24, 161 and 204. It is clear that if two of them are utilized for liganding Cu_A, besides the cysteines 196 and 200, the best candidates would be 161 and 204 just in the same loop which contains the cysteines. Only histidine 24 would be left for the Fe_a, suggesting that the 6th axial ligand should come from a different subunit. The coordination of Fe_a by hystidine 24 is indicated[54], since this residue is at the very beginning of one of the 2 alpha-helical segments of subunit II, in contact with the lipid bilayer. Such a location would also facilitate the positioning of the heme plane normal to the plane of the bilayer as indicated by Blasie et al.[9].

A further interesting feature of the cytochrome *a* redox center is the possible presence of hydrogen bonding between the formyl group of the heme *a* and H-donor group of the protein. The interactions are stronger in the reduced, rather than in the oxidized, form of the center. This energy shift may provide a pathway by which electron transfer events at the redox active cytochrome *a* iron may be communicated to the surrounding protein matrix[14].

The development of cross-linking techniques and especially of photoaffinity labeling[21] has permitted identification of subunit II of cytochrome *c* oxidase as the one in functional contact with cytochrome *c*[4]. Birchmeier et al.[6] and Fuller et al.[33] have shown that the region of cytochrome *c* corresponding to the C-terminus is in contact with subunit III of cytochrome *c* oxidase, although such a contact should not be implicated in electron transfer, since this subunit is devoid of Cu and of heme[56,64] and its removal does not abolish electron transfer. The carboxylate groups which are involved in the binding of cytochrome *c* at the level of subunit II are glutamate 18, aspartate 112, glutamate 114 and 198, as judged from the labeling experiments described above[54]. It is interesting to notice that a cluster of aromatic residues, -Trp-Tyr-Trp-Ser-Tyr-Glu-Tyr- included in the beef heart sequence positions 104–110, is completely invariant. This special structure has been suggested to be involved in electron conduction[15,34,54].

The molecular characteristics of the proton pump remain unrevealed

The enzyme turnover leads to the formation of a proton-motive force (inside negative and alkaline) across the mitochondrial membrane which exerts a 'control' on the enzyme's activity which may be released by a protonophore or by the opening of a proton channel physiologically produced by ADP.

It was proposed by Wikström[81] and demonstrated by experiments with intact mitochondria, sub-mitochondrial particles (cf. Winter et al.[83]) and reconstituted cytochrome *c* oxidase in phospholipid vesicles, that electron transfer through the enzyme is coupled to outwardly directed H^+ translocation. There is some disagreement concerning the number of protons extruded per cytochrome *c* molecule oxidized. While most laboratories have found this value to be close to one (both for the enzyme in mitochondria[69,81] and in reconstituted vesicles[16,39,70]), Lehninger and co-workers have consistently reported values higher than this.

The mechanism of proton pumping is as yet relatively obscure. A prerequisite of any H^+-translocating apparatus is the presence of one or more H^+ transferring devices along which protons may go from the inside of the membrane to the outside, and not vice versa. A number of lines of evidence have indicated that in the case of the cytochrome *c* oxidase proton pump, subunit III may be involved in this function. The first indications came from studies with dicyclohexylcarbodiimide (DCCD)[17,18]. These showed that when cytochrome *c*

oxidase vesicles or mitochondria were incubated with DCCD, the H^+-pumping activity is strongly inhibited though there are only minor effects on the electron transfer rate. In addition, the DCCD-induced decrease in size of the cytochrome *c*-induced extravesicular acidification in oxidase vesicles is not caused by a decreased rate of H^+ extrusion but by a lowering of the number of H^+ translocated per electron (see Casey et al.[19]) i.e., the reaction with DCCD induces a molecular decoupling of the H^+ pump. It has also been found that following incubation of cytochrome *c* oxidase in reconstituted vesicles or mitochondria with DCCD, there is a specific labeling of subunit III[18, 19, 60, 61] at a single glutamic acid residue[60]. The sequence of amino acids flanking this residue is remarkably similar to those of the DCCD-binding peptides from H^+-conducting proteolipids of a number of H^+-translocating ATPases (see Prochaska et al.[60]).

These results are interpreted as indicating that subunit III of cytochrome *c* oxidase is involved in some aspect of the coupling of electron flow to H^+ translocation.

These proposals are strengthened by the following observations[64]: Removal of subunit III from cytochrome *c* oxidase has negligible effects on the electron transfer rate but leads to loss of H^+-pumping activity following reconstitution; subunit III is not involved in electron transfer[83] since the hemes and coppers are not located there.

The isolated cytochrome *c* oxidase from *Paracoccus denitrificans* contains only 2 subunits[45] but when reconstituted exhibits a proton-pumping activity, with half of the H^+/e ratio of that obtained with the reconstituted bovine enzyme[71]. This observation may be interpreted in terms of a H^+ channel less efficient than that in the bovine enzyme.

It has been found[56] that depletion of subunit III from cytochrome *c* oxidase which leads to loss of H^+-pumping activity also results in the loss of the pH-dependence of the heme *a* midpoint potential, and it has been suggested[80] that the proton translocation event is associated with the transfer of electrons to and from heme *a* .

Thus a tentative model for the cytochrome *c* oxidase proton pump would be that reduction of heme *a* leads to the uptake by this heme or by a group in close proximity to it, of a proton which is in electrochemical equilibrium with the protons in the internal phase via a proton channel. Subsequent oxidation of heme *a* would lead to release of the proton into another channel in equilibrium with the external space. While these proton channels may traverse several subunits, the findings described above suggest that part of this activity may be associated with subunit III. A notable feature of such a model is that it would require an inter-subunit communication between subunit III and heme *a* which is located in subunits I or II.

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0014-4754/84/090901-05$1.50 + 0.20/0

Phagocytes use oxygen to kill bacteria

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Key words. Phagocytes; bacteria; respiratory burst enzyme; oxygen-dependent killing.

The blood phagocytes, neutrophils, eosinophils and monocytes, have a main function in common: they attack and kill invading microbes and parasites. The prey is usually phagocytosed by these cells, i.e. fully enclosed in a vacuole where killing and digestion take place without harm to the surrounding tissues. On interaction with the microorganisms and other particles which are recognized as phagocytosable, the phagocytes suddenly increase their oxygen consumption. This phenomenon is called respiratory burst. It was discovered in the 1930's and believed to provide energy for phagocytosis[9]. Its role as a source of microbicidal oxidants became apparent much later, through studies from the laboratories of M.J. Karnovsky[45,38] and J.H. Quastel[26]. The demonstration that oxygen-derived products are of primary importance for microbial killing was then provided by experiments with neutrophils from chronic granulomatous disease patients, a condition characterized by the inherited inability of phagocytes to mount a respiratory burst[25].

The blood phagocytes

Neutrophils, eosinophils and monocytes have several common features[8]. They are formed in the bone marrow, are equipped with storage granules which (in part at least) contain peroxidase and lytic enzymes, are able actively to move from the blood stream to the tissues in response to chemotactic stimuli, and – as their name indicates – are able to phagocytose. Neutrophils and eosinophils are end-cells, suited for brisk but short-lasting interventions. They have abundant deposits of export enzymes (in their granules) and fuel in the form of glycogen, but are unable to replenish these stores. The monocytes, by contrast, are long-lived. Early in their life cycle, they discharge their granules[8], and – unlike the other phagocytes – then continue to function as granule-free phagocytic cells which are called macrophages. Most of what is known about the microbicidal activity of phagocytes has been elaborated in studies with neutrophils, and this cell will be the main object of my review.

Phagocyte activation

Bacteria usually colonize the interstitial space of a tissue. Neutrophils and other phagocytes are attracted to these sites by chemotactic signals which the microorganisms emit or induce[39]. The intervention of the phagocytes takes place in 2 steps. The cells are first activated by the chemotaxins in the micro-vessels which irrigate the affected tissue. As a consequence, they marginate and migrate through the walls of post-capillary venules towards the source of the chemotactic signal. Later, at the site of infection, the phagocytes engulf and kill the bacteria. Diapedesis, migration and phagocytosis are the physical consequences of activation. On the biochemical level, this process is characterized by the release of products necessary to the ultimate goal of phagocyte mobilization, i.e. the killing and disposal of the microorganism. The full extent of the response is displayed during phagocytosis. Experiments in the test tube, in which opsonized particles are added to a suspension of neutrophils and the products are determined in the incubation medium, permit the analysis of the whole release repertoire. Two classes of products are distinguished: those which are pre-formed and already present in the storage organelles of the resting cells; and those which are synthetized following stimulation. The pre-formed products are enzymes and other macromolecules; they are liberated by exocytosis following fusion of the membrane of the storage organelles with the