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SHORT REVIEW

Interactions in Cytochrome Oxidase: Functions and Structure[1]

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Abstract

Mitochondrial cytochrome *c* oxidase is an exceedingly complex multistructural and multifunctional membranous enzyme. In this review, we will provide an overview of the many interactions of cytochrome oxidase, stressing developments not covered by the excellent monograph of Wikström, Krab, and Saraste (1981), and continuing into early 1983. First we describe its functions (both in the nominal sense, as a transporter of electrons between cytochrome *c* and oxygen, and in its role in energy transduction). Then we describe its structure, emphasizing the protein (its structure as a whole, the number and stoichiometry of its subunits, their biosynthetic origin, and their interactions with each other, with other components of the enzyme complex, and with the membrane as a whole). Finally, we present a model in which the protein conformation serves as the focus for the dynamic interaction of its two major functions.

Key Words: Cytochrome oxidase; electron transport; oxidative phosphorylation; lipid–protein interactions; digenomic biosynthesis; structural–functional interactions.

I. Introduction

Cytochrome *c* oxidase (EC 1.9.3.1) is the terminal electron transport protein in the mitochondrial inner membrane of eukaryotes (for a comprehensive review, see Wikström *et al.*, 1981) and the plasma membrane of many prokaryotes (Ludwig, 1980). In the former, it arises from the cooperation of

[1]Abbreviations: DCCD, *N*,*N*′-dicyclohexylcarbodiimide; E_m, midpoint potential; EPR, electron paramagnetic resonance; F_1, soluble portion of the ATP synthetase complex; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SUPAGE, SDS–urea–PAGE.

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two genomes, and is an exceedingly complex multisubunit enzyme in which the protein and four prosthetic groups (two hemes *A* and two copper ions) (reviewed, for example, in Wikström *et al.*, 1981; Azzi, 1980; Malmström, 1974; 1979; Erecińska and Wilson, 1978) act in concert as the controlling site (Forman and Wilson, 1982) of energy conservation in oxidative phosphorylation. Four features of cytochrome *c* oxidase fascinate researchers of diverse interests. (1) It is the electron donor to oxygen in the mitochondrial respiratory chain and, in the process of electron transport from cytochrome *c*, it is the locus of complex metal–metal redox interactions. (2) As a major site of oxidative phosphorylation, it is the key to understanding the complex mechanisms underlying the energy transformations of aerobic life. (3) It is a widely studied model for membrane proteins, and a source of data on lipid–protein interactions in typical biological membranes. (4) Its digenomic origin, from dual systems of hereditary information, presents a rare opportunity to study mechanisms for interactive control of transcription and translation in the biosynthesis and assembly of its subunits. The shape of the protein in its dimeric form (Section IIIA.1) is symbolically appropriate, for the four fingers of its M-domains extend into different slices of the biochemical pie, attracting physical chemists, bioenergeticists, molecular biologists, and geneticists to study in common its intricate interactions.

It is our purpose to lead the reader through these diverse fields, united by this enzyme, by providing an overview of these interactions. We will begin with a description of some of the interactions involved in the functions of electron transport and energy transduction. We will then describe recent advances in the study of its structure, emphasizing both the origin of this protein and its relationship with other components within and around it. Finally, we discuss a model in which the shape of the protein, beyond being a symbol of the diversity of its appeal, is the conduit for interaction between its primary functions.

II. Function

IIA. Electron Transport—The Redox Metallocenters

The body of literature treating the various aspects of electron transport through cytochrome *c* oxidase is voluminous, and we will not attempt to cover it exhaustively here. Instead we will introduce some of the issues (especially those which pertain to site–site interactions) which have occupied researchers in the past few years. Since the rediscovery of the enzymes by Keilin in 1925 (see Keilin, 1966), the wealth of literature on the electromagnetic properties of transition-state elements has made possible numerous ingenious studies of the redox metallocenters, their intermediate conformations during turnover

and liganding, and their mutual interactions (reviewed in, for example, Wikström *et al.*, 1981; Malström, 1974; 1979; Erecińska and Wilson, 1978). In fact, as pointed out by Malmström (1974), "The relation between spectroscopic properties and chemical structure of metalloproteins . . . represents one of the few branches of present-day biochemistry to which quantum mechanical calculations can profitably be applied. . . ."

IIA.1. Hemes and Heme–Heme Interaction. Cytochrome *c* oxidase is a metalloprotein which has two types of transition metal prosthetic groups: heme and copper.

The two hemes are 12 to 16 Å apart (Ohnishi *et al.*, 1982), and are chemically identical, since only one heme A can be isolated from cytochrome aa_3 (Caughey *et al.*, 1975). But cytochromes *a* and a_3 have historically been differentiated on the basis that only the latter can form ligands to oxygen, carbon monoxide, cyanide, azide, etc. (Keilin and Hartree, 1939), and the former is generally believed to be the immediate acceptor of electrons from cytochrome *c* (e.g., Wikström *et al.*, 1981; Lemberg, 1969).

The assignment of spectroscopic parameters and midpoint redox potentials (E_m values) to the two hemes has been clouded by controversy about whether the interaction between them (reviewed by Malmström, 1974) shows up primarily as mutual effects on intrinsically similar alpha-band extinction coefficients of two hemes of different E_m values, the "50/50 hypothesis" of Wilson *et al.* (1972), or as mutual effects on intrinsically similar E_m values from components having different spectroscopic parameters, the "neoclassical hypothesis" (Nicholls and Petersen, 1974; Wikström *et al.*, 1976). Erecińska and Wilson (1978) have pointed out that the negative cooperativity of electron affinities (E_m values), assumed by the neoclassical hypothesis, would predict a shift in the E_m of cytochrome *a*, depending on whether the liganded cytochrome a_3 is reduced or oxidized, and that such a shift is not observed. The neoclassicists argue that the shift could be obscured if the affinity of a_3 for the ligand were dependent on the redox state of *a* (Wikström *et al.*, 1981). Furthermore, if the reduced a_3–CO compound were photodissociated in the absence of oxygen at $-100°C$, the difference spectrum in the alpha-band region should depend on the oxidation state of cytochrome *a* according to the 50/50 hypothesis, since the molar absorptivity of heme *a* should be altered some 60% by changing the liganding state of heme a_3, but this is not the case (Wikström *et al.*, 1981). A similar study involving the effects of several ligands on the difference spectra of both the alpha- and gamma-band regions confirms the absence of any strong influence of the other redox centers on the spectrum of heme *a*. However, there does appear an effect on the spectrum of a_3, not only from *a*, but also from Cu_B (see below) (Blair *et al.*, 1982). A more recent study, analyzing the quantum yield of the photodissociation reaction (Boelens *et al.*, 1982), failed to support the strong

spectral interaction predicted by the 50/50 hypothesis. So, for the moment, we may let stand the historical assignment (Lemberg, 1969) (recently reconfirmed in model studies by Carter and Palmer, 1982) of the dominant species of the alpha-band to cytochrome *a*.

The assignment of EPR signals is generally agreed on. The high-spin signal (at $g = 6$), which occurs on partial reduction, corresponds to cytochrome a_3, which would be expected to have, like the high spin hemes of hemoglobin, a free coordination site available to bind molecular oxygen; the low-spin signal ($g = 3$), seen in the oxidized enzyme, is assigned to heme *a*, which would be expected to resemble the low spin iron in cytochrome *c*, since, like *c*, heme *a* is merely an electron-transfer component of the enzyme (Malmström, 1979). Because of anomalies in the transient appearance of the high-spin signal, some authors have offered other attributions for part of the high-spin signal under certain conditions [to heme *a* in the presence of reduced a_3 (Wikström *et al.*, 1981) or to a conformational state not involved in the catalytic cycle (Wilson *et al.*, 1982)].

IIA.2. The a_3*–*Cu_B *Site.* However, attribution of *any* EPR signal to heme a_3 raises an interesting point, since there is strong ($-J \geq 200$ cm^{-1}) coupling between heme a_3 and one of the coppers (Tweedle *et al.*, 1978; Moss *et al.*, 1978) [in eukaryotic oxidase, but not in the primitive version of *Thermus thermophilus* (Kent *et al.*, 1982)], indicating a distance between them of less than 4 Å (Clore and Chance, 1979). In fact the two copper ions are distinguished from one another in that only one of them, the "visible" copper (Cu_A), can be observed by EPR ($g = 2$) (Malmström, 1974; Van Gelder and Beinert, 1969) and optical spectroscopy (830 nm) (Beinert *et al.*, 1980), while the other, the "invisible" copper (Cu_B), cannot be observed by these techniques, presumably because of the coupling. It should be pointed out that, recently, EPR signals, obtained by selectively reducing a_3 and probably holding it that way as an oxygen adduct, have been attributed to some portion of the "invisible copper" (Reinhammar *et al.*, 1980; Karlsson and Andréasson, 1981). If partial reduction (to $a^{+2}Cu_A{}^{+1}a_3{}^{+3}Cu_B{}^{+2}$) reveals the unpaired electron of $a_3{}^{+3}$, why does it not also show the $Cu_B{}^{+2}$? An unconventional interpretation was advanced by Seiter and Angelos (1980), and later Hagen (1982), who argued that the $S = 2$ observed (Tweedle *et al.*, 1978; Moss *et al.*, 1978) might stem not from $a_3{}^{+3}$–$Cu_B{}^{+2}$ coupling, but from an uncoupled $a_3{}^{+4}$ and $Cu_B{}^{+1}$, the latter being a nonparticipant in redox events, though other data (e.g., the spectrum of resting oxidase) argue against this (Wikström *et al.*, 1981; Thomson *et al.*, 1982). The usual interpretation is rapid reduction of the Cu_B on uncoupling (Wilson *et al.*, 1982), perhaps after disruption of a bridged oxygen atom (Brudvig *et al.*, 1981; Reed and Landrum, 1979) or peroxy intermediate (Clore and Chance, 1979). The notion of a peroxy

intermediate is attractive, because it can be arrived at via a two-electron reduction of O_2, avoiding the energetically unfavorable one-electron reduction to the dangerous superoxide anion (Malmström, 1974). Chance's work at low temperatures provides spectral evidence (e.g., Chance, 1981) for the existence of the $a_3{}^{+2}O_2Cu_B{}^{+1}$, and oxidase will bind to hydrogen peroxide with high affinity (Bickar *et al.*, 1982). In fact, the peroxy intermediate is one of the few generally agreed on features in the events at the a_3–Cu_B site (Wikström *et al.*, 1981; Erecińska and Wilson, 1978; Reed and Landrum, 1979; Chance and Waring, 1979; Antonini *et al.*, 1977).

More recent studies by Chance and co-workers (Chance, 1981; Powers *et al.*, 1981, 1982) using X-ray edge absorption and EXAFS (extended edge absorption fine structure) methods on molecular structure around the heme a_3–Cu_B site in the fully oxidized state indicate a sulfur bridge with three ligands 2.60 Å from the heme a_3 iron and 2.18 Å from Cu_B. The distance between the heme a_3 iron and Cu_B is 3.75 ± 0.05 Å. The remarkable conclusions from these EXAFS studies are that the first shell of Cu_B is identical to that of the copper in oxidized stellacyanin, with two nitrogens and a bridging sulfur.Upon reduction with CO, the first shell of the iron of heme a_3 is identical to that in oxyhemoglobin, but has CO instead of O_2. These structures provide further support for the dioxygen reduction mechanism involving oxy and peroxy intermediates proposed earlier by Chance *et al.* (1975), based on spectroscopic evidence.

IIA.3. The a–Cu_A Site. At the port of electron entry, there are two prosthetic groups: the heme *a* and Cu_A. The latter represents 40% of the copper content (about 10% of the total is extraneous copper, probably due to denaturation (Greenaway *et al.*, 1977), and is separated from the former by a distance of 7 Å (Greenaway *et al.*, 1977). As reviewed by Wikström *et al.* (1981), if the resting oxidized oxidase is pulsed with cytochrome c^{+2}, there is an initial burst of electron transfer in which one e^- is admitted per aa_3. Cytochrome *a* is the primary electron acceptor, but the Cu_A is quickly reduced also, so that half of each of these two sites is reduced before the electrons are passed on to the a_3–Cu_B site. Wikström *et al.* (1981) believe, however, because electrons enter and leave *a* much faster than Cu_A, that the transfer is not from *a* to Cu_A to a_3–Cu_B, but that, though the Cu_A equilibrates with heme *a*, the actual exit to the oxygen reduction site is from heme *a*. This view is not shared by others (e.g., Blair *et al.*, 1982; Clore and Chance, 1979; Nicholls and Chanady, 1982), who believe that Cu_A is the direct electron donor to the oxygen site. One may even question whether the copper itself is the actual redox participant; the locus of activity may be an associated cystine sulfur in the Cu_A site (Stevens *et al.*, 1982). In either case, whether Cu_A is on the direct electron transfer path or is merely an electron sink, it aids in paired transfer of

electrons to a highly cooperative two-electron acceptor—the a_3–Cu_B site (Malmström, 1974).

IIB. Energy Conservation: Metal–Protein Interaction

Classically, there were three hypotheses to explain oxidative phosphorylation, the coupling between electron transport and the phosphorylation of ADP, to produce the aerobic cell's energy coinage, ATP. The coupling was thought to be produced (1) by chemical intermediates (Slater, 1953), (2) by chemiosmosis (Mitchell, 1966), and (3) by conformational interaction (Boyer, 1965). There is an excellent overview composed jointly by their champions (Boyer *et al.*, 1977). Over the years, the nature of the controversy has shifted as increasing numbers of research (Mitchell, 1976) accepted the major role in energy coupling assigned by the chemiosmotic theory to the electrochemical potential gradient of the proton across the inner mitochondrial membrane. There are, however, still questions about the details of the production and utilization of that gradient, and as reviewers of an electron transport enzyme we are most interested in the former. The chemiosmotic hypothesis would assign proton transport capability only to those redox enzymes which can vectorially dehydrogenate a substrate. By this criterion, cytochrome oxidase should not be a direct participant (e.g., Mitchell, 1979a, b).

A relatively recent conceptual approach to energy coupling is proton-pumping (Wikström *et al.*, 1981; Papa, 1976, 1982; De Pierre and Ernster, 1977). The mechanism of H^+ transport could be by way of conformational changes, (Wikström *et al.*, 1981), subtle alterations in a heme side chain (Ondrias and Babcock, 1980), or Bohr effects (Papa, 1976, 1982). The potential gradient of the chemiosmotic model is, of course, still the coupling device. For some bacterial oxidases, proton pumping has either been shown (Sone and Yanagita, 1982) or offered as the most consistent explanation of the observed data (Ferguson, 1982). In the case of the eukaryotic oxidase, as reviewed by Wikström *et al.* (1981), there have been hints that changes in the configuration about the redox centers associated with transitions between intermediate oxidation states are accompanied by dynamic events in their protein environment. These authors believe that oxidase is using electron transport energy to power shifts in location and pK_a of one or more proton-bearing groups on heme *a* and/or the protein, causing the transmembranous movement of one H^+/e^- *above* that consumed by the scalar reduction of oxygen.

Moyle and Mitchell (1978) account differently for the apparent observation by others of antimycin-insensitive proton translocation by particulate mitochondrial oxidase (of either native or inverted orientation) (e.g., Wik-

ström, 1977; Wikström and Saari, 1977; Krab and Wikström, 1979; Sorgato and Ferguson, 1978; Sigel and Carafoli, 1978; Alexandre *et al.*, 1978; Azzone *et al.*, 1979; Reynafarje *et al.*, 1982). They found a stoichiometrically excessive oxygen uptake over alkalinization in uncoupled particles when an artificial electron donor, such as ferrocyanide, is used to reduce endogenous cytochrome *c*, and argued that the excess oxygen was being consumed by an endogenous reductant, presumably ubihydroquinone (Lorusso *et al.*, 1979), which was being vectorially dehydrogenated by the ferricyanide product. Since they were unable to observe proton translocation in the absence of the stoichiometric anomaly, they inferred that the proton translocation originated from the ubiquinone region of the electron transport chain. However, Wikström and Krab (1978) [see also Krab and Wikström (1978)] *did* demonstrate translocation with the expected stoichiometry. Also, they showed that, when the anomaly occurred, the ferricyanide production paralleled the oxygen, rather than the proton, uptake (i.e., the oxygen was not in excess), casting doubt on the inferred endogenous hydrogen donor. Similarly, Mitchell and Moyle (1983) have suggested that the transient acidification observed when liposomes inlaid with isolated oxidase are pulsed with reductant or oxygen (e.g., Wikström and Saari, 1977; Krab and Wikström, 1978; Casey *et al.*, 1979a; Sigel and Carafoli, 1979; Prochaska *et al.*, 1981; Nicholls *et al.*, 1982) could be explained as an artifact arising from the oxidation of a complex of ferrocytochrome *c* and phospholipid (especially phosphatidylserine). But this argument is weakened by the observation that azide-induced inhibition of the translocation is not counteracted when ferricyanide is supplied to oxidize the putative ferrocytochrome *c*–phospholipid complex (Casey and Azzi, 1983). Perhaps an even more specific probe of the role of oxidase in proton translocation would be the use of antibodies to inhibit the activities of cytochrome oxidase vesicles. In fact, research of this type is presently underway in our laboratory (Chan and Freedman, 1983). Although it may seem disheartening that, despite the volume of literature, the diversity of its sources, and the protracted period of controversy, there is no clear "resolution paper" on the role of oxidase in this matter, the reader is reminded that the present acceptance of the broad outlines of chemiosmotic theory itself was through the steady accumulation of data that, point by point, laid to rest the objections of its detractors.

If oxidase is a pump, there is strong evidence for the involvement of its protein. *N,N'*-dicyclohexylcarbodiimide (DCCD), an inhibitor of proton translocation by the mitochondrial ATPase (Beechey *et al.*, 1967), was shown to inhibit proton translocation by oxidase (Casey *et al.*, 1979b). DCCD was subsequently shown to bind to subunit III of the enzyme (Casey *et al.*, 1980) at a glutamyl residue (Prochaska *et al.*, 1981). Subunit III can be readily removed from the enzyme by two different methods (Penttilä, *et al.*, 1979; Bill

and Azzi, 1982). The remaining enzyme no longer pumps protons across an artificial membrane (Saraste *et al.*, 1980). The possibility that the DCCD effect results from interference with ferrocytochrome *c* complexation with phosphatidylserine (Mitchell and Moyle, 1983) is ruled out by the observation that inhibition by DCCD is totally independent of the content of that phospholipid in the vesicles (Casey and Azzi, 1983). All this implies a dynamic participation of the protein which ought to show up in the details of the enzyme's topography. Clearly there is a need for a thorough understanding of the protein structure of oxidase.

III. Structure—The Protein

IIIA. As a Whole

IIIA.1. Shape and Setting. Cytochrome *c* oxidase is an integral membrane protein in the sense of the word used in the fluid mosaic model of Singer and Nicolson (1972). Its overall shape and setting in the membrane have been determined by the application of the techniques of electron microscopy and image reconstruction to two-dimensional crystalline arrays of monomers (Fuller *et al.*, 1979) and dimers (Deatherage *et al.*, 1982a,b). The monomer [aa_3 (Fuller *et al.*, 1979)] is shaped like a skewed "Y" with a transmembranous length of 110 Å. The arms of the "Y" are 55 Å long and their centers are separated by about 40 Å (see the balsa wood model of Fuller *et al.*, 1979). According to X-ray diffraction studies (Blasie *et al.*, 1978), the stem of the "Y" projects 60 Å into an aqueous phase which has been identified by immunoelectron microscopy (Frey and Chan, 1980) as the cytoplasmic side (Frey *et al.*, 1978). The membrane is 40–50 Å thick (De Pierre and Ernster, 1977), the arms (M-domains) of the "Y" project less than 20 Å (Fuller *et al.*, 1979; Deatherage *et al.*, 1982a,b) or as much as 30 Å (Frey *et al.*, 1982) into the matrix, and, given the length of the M-domains, the separation between them must create a depression (45–55 Å) in the membrane which just about spans the bilayer and culminates somewhere within the "Y"-stem of the cytoplasmic domain (Frey *et al., 1982). If oxidase is a dimer in vivo* (see Wainio, 1983), the depression is further enclosed by an M-domain of the second monomer (Deatherage *et al.*, 1982a,b; Frey *et al.*, 1982). Calculations which place lipid molecules in the depression (Deatherage *et al.*, 1982b) are based on the assumption (Deatherage *et al.*, 1982a) that the ends of the M-domains are level with the membrane surface. If the matrix surface of the membrane is not featureless [and it is not (Frey *et al.*, 1982)], then the accuracy of the data of Deatherage *et al.*, (1982b) and the inferences drawn depend on the faithful coating and the homogeneity of the embedding medium. These workers did express some doubt on this point in the case of

their glucose-embedded vesicles (see also Section IIIB.4d). It is surprising that the inference, primarily from data available since 1979 (Fuller *et al.*, 1979), of a physical channel in oxidase has not previously been drawn in the literature, especially given the controversy about oxidase's role in proton translocation (Section IIB) through the membrane.

IIIA.2. Interaction with the Lipid Bilayer. The nature of the interactions between that membrane and the complex has been the subject of considerable investigation. Mainly on the basis of EPR work with spin labels, a theory evolved in which a "single boundary layer of immobilized lipid . . . (surrounds an integral membrane) . . . protein" such as oxidase (Griffith *et al.*, 1973) [one study finds a shell three molecules thick (Benga *et al.*, 1981; also see Falk and Karlsson, 1979)], although NMR studies (e.g., Seelig and Seelig, 1978; Kang *et al.*, 1979) have failed to indicate that there is an immobilized layer. Instead, they show that the protein induces a higher state of disorder into the lipid bilayer, and that there is a high rate of exchange between the disordered lipid near the protein and the noninteractive lipids farther away. One group, noting mixed populations of oxidase of different rotational mobilities, has suggested that the observation of an immobilized layer resulted from lipid trapped in a protein aggregate (Swanson *et al.*, 1980), although reconstituted oxidase such as that used in the EPR studies has been reported with a rotational mobility too high to be compatible with such aggregates (Ariano and Azzi, 1980). Two studies, one based on fluorescence depolarization measurements (Kinosita *et al.*, 1981), and one on a combination of ^{2}H-NMR and EPR techniques (Paddy *et al.*, 1981), have attempted to reconcile the differences by arguing that the protein is surrounded by a relatively immobilized but disordered lipid layer which rapidly exchanges with the remainder of the bilayer.

However, at least some of the lipids do not readily exchange. Oxidase, expecially from beef heart, is normally isolated with 10–20% of its weight as bound phospholipid (Caughey *et al.*, 1976), and only recently have isolations (in which the oxidase is not irreversibly denatured) been described (Fry and Green, 1980; Robinson *et al.*, 1980), containing less than 0.5% phospholipids (less than 2 moles phospholipid per aa_3). In both cases, the only remaining lipid was cardiolipin. These preparations are reminiscent of those characterized by Awasthi *et al.* (1971) and Chuang and Crane (1973), containing approximately 3% phospholipids, of which three-fourths was cardiolipin. This phospholipid is asymmetrically distributed in the membrane, but even on the matrix side, where it is richest, it is still only 18% of the total phospholipid content (Krebs *et al.*, 1979), and it is clearly overrepresented in the tightly held lipids about the oxidase. In studies of head-group specificity in the boundary layer, cardiolipin is preferred to other phospholipids (Cable and Powell, 1980), even other anionic lipids (Knowles *et al.*, 1981), although its

binding affinity to delipidated oxidase is only slightly greater than that of similarly charged phosphatidic acid (Gwak and Powell, 1982). This associative specificity for cardiolipin is mirrored in specificity of activation; no other lipid tested is as effective in restoring activity to delipidated oxidase (Awasthi *et al.*, 1971; Chuang and Crane, 1973; Fry and Green, 1980; Robinson *et al.*, 1980; Robinson, 1982a,b), and an antibiotic which specifically complexes with cardiolipin can markedly inactivate the oxidase (Goormaghtigh *et al.*, 1982). But it should be noted that cardiolipin also stimulates the activity (with respect to horse or yeast cytochrome *c*) of *Nitrobacter agilis,* whose membrane does not contain that phospholipid; and the stimulation is not seen with the physiologic electron donor to *N. agilis* oxidase (Fukumori and Yamanaka, 1982).

IIIB. The Subunits

IIIB.1. In General. Bacterial aa_3 type cytochromes are structurally simple, and have been isolated as one- or two-subunit (23–55 kD each) enzymes, sometimes combined with portions of other electron transport complexes (Ludwig, 1980). However, eukaryotic oxidase is a distinct, complex, multisubunit enzyme. The amino acid sequence has been determined (or inferred from gene sequences) for four yeast and at least eight mammalian subunits; the latter are the 56.965-kD subunit I (Anderson *et al.*, 1982), the 26.021-kD subunit II (Steffens and Buse, 1979), the 25.991-kD subunit III (Anderson *et al.*, 1982), the 17.153-kD subunit IV (Sachar *et al.*, 1979), the 12.436-kD subunit V (Tanaka *et al.*, 1977, 1979), the 10.67-kD subunit VIa (Biewald and Buse, 1982; Reimer *et al.*, 1983), the 5.541-kD subunit VII_{ser} (Buse and Steffens, 1978), and the 10.026-kD subunit VII_{ala} (Steffens *et al.*, 1979). From the yeast, the genes for the 55.93-kD subunit I (Bonitz *et al.*, 1980), the 28.48-kD subunit II (Curuzzi and Tzagoloff, 1979), and the 30.340-kD subunit III (Thalenfeld and Tzagoloff, 1980) have been sequenced, and the sequence of the 12.627-kD subunit VI reveals a homology of up to 40% to the mammalian subunit V (Gregor and Tsugita, 1982). It would seem logical to use these kilodalton values for nomenclature in the Sections that follow, but we fear it would be premature at this stage, since much of the work characterizing the subunits preceded their sequencing, and confusion could result. Therefore, we will follow the conventions used by Downer *et al.* (1976), Tracy and Chan (1979), and Azzi (1980), based on the relative rates of subunit migration in the SDS–urea gel system of Swank and Munkres (1971). On the basis of size, it is tempting to identify the largest two or three subunits of the eukaryotic oxidase with those of the prokaryotic versions, and, indeed, there is immunochemical cross-reactivity between subunits II of *Paracoccus denitifricans,* the thermophilic bacterium PS3,

yeast, and beef heart (Ludwig, 1980). But it should be pointed out that the same experiment raises the suspicion that beef heart subunit IV and one of its subunits VII may share antigenic determinants with the more primitive subunits II. Confirmation of this and a similar study of cross-reactivity of subunits I from different sources is certainly called for, since it is possible that homologies between the prokaryotic and eukaryotic oxidases may be more extensive than previously believed.

IIIB.2. Their Number. Cytochrome *c* oxidase isolated from a primitive eukaryote such as *S. cerevisiae* has been convincingly demonstrated to contain seven and only seven different polypeptide subunits by the criteria of size, charge, antigenicity, and amino acid composition (Poyton and Schatz, 1975a,b) (although see Gutweniger *et al.,* 1981). But it became obvious that the mammalian oxidase has a more complicated composition when the yeast and beef heart enzymes were directly compared on a two-dimensional gel system (Poyton *et al.,* 1978). In fact, since 1975, the number of subunits in the mammalian oxidase has been claimed to be as low as 6 (Briggs *et al.,* 1975), as high as 15 (Griffin and Landon, 1981), and nearly every value between (Downer *et al.,* 1976; Tracy and Chan, 1979; Poyton *et al.,* 1978; Kadenbach and Merle, 1981; Yu and Yu, 1977; Steffens and Buse, 1976; Verheul *et al.,* 1979, 1981). While sometimes the issue is the resolving system used (e.g., Downer *et al.,* 1976; Tracy and Chan, 1979; Briggs *et al.,* 1975; Kadenbach and Merle, 1981), there have also been questions about which subunits are genuine components of the complex, prompted by the ease of removal of some of the subunits by particular isolation procedures (e.g., Penttilä *et al.,* 1979) or by limited proteolysis (Ludwig *et al.,* 1979), without affecting redox activity.

It is important to bear in mind that concepts limiting the definition of an enzyme (whose coisolates represent genuine components, and which are contaminants) need not be the same for membrane-bound enzymes as they are for soluble ones. Besides the functions it demonstrates in the solubilized form, a membrane-bound enzyme may have functions which depend on its accurate orientation with respect to some lipid or protein component of its environment. To call "contaminants" all those components which can be removed on isolation and solubilization without affecting the function of the solubilized enzyme is to ignore those capacities which are inherent in the *in vivo* state, e.g., energy translocation. The observation that the removal of subunit III (the third largest subunit) by gentle methods (Penttilä *et al.,* 1979; Bill and Azzi, 1982) does not affect redox capability does not remove it from consideration as a genuine subunit, since the removal destroys the enzyme's H^+ translocation capacity in the reconstituted system. Yet workers (Wikström *et al.,* 1981) who believe that subunit III is a *bona fide* component on these grounds reject the inclusion of three other polypeptides [called "a," "b," and "c" by Capaldi's group (1977)] on the grounds that their removal by proteolysis does

not impinge on redox activity. Recently, one of these subunits, identified as VIa by the Kadenbach group (Kadenbach and Merle, 1981), demonstrated organ-specific differences in primary structure (Kadenbach *et al.*, 1982), which that group believes may be partly responsible for the kinetic differences observed between liver and heart bovine oxidase (Merle and Kadenbach, 1982). Earlier Merle and Kadenbach (1980) had pointed out that the consistency in the subunit number and stoichiometry they had observed in different mammalian species, even in the presence of protease inhibitors, argued for the inclusion of all 12 [and later 13 (Kadenbach and Merle, 1981)] subunits as genuine components. More recently, they added compelling immunochemical data to their argument (Jarausch and Kadenbach, 1982). We would like to go further and argue that the very nature of membrane-bound enzymes makes their definition nebulous. That is, even if its coisolates are *not* consistent [as suggested by the recent crystallization of a seven-subunit version of the beef heart enzyme (Ozawa *et al.*, 1982)], indicating a loose physical association, a coisolate of such an enzyme, if important for proper interaction of the enzyme with a neighbor, may still be defined as part of the enzyme. Should such a component also be found at some future time to copurify with a neighboring protein, its membership could be assigned to either or both complexes. The assignment is, after all, a conceptual convenience for the researchers, and the disputes are problematic.

IIIB.3. Their Biosynthetic Origin. The fascination with the subunits of oxidase stems in part from their origin, since oxidase is one of three complexes in the membrane which derive subunits from both the nuclear and mitochondrial genomes in both lower (Schatz and Mason, 1974) and higher eukaryotes (Koch, 1976; Yatscoff *et al.*, 1977; Bernstein *et al.*, 1978). Aside from homologous regions, evolutionary conservatism is maintained in that the three largest polypeptides are consistently synthesized by the mitochondrial system (Schatz and Mason, 1974; Koch, 1976; Bernstein *et al.*, 1978). Diversity is expressed in the increasing complexity of the cytoplasmic contributions (Poyton *et al.*, 1978; Kadenbach and Merle, 1981), in which, as already noted, differences among the smaller subunits can be seen even between organs of the same species (Kadenbach *et al.*, 1982; Jarausch and Kadenbach, 1982).

In 1978, we constructed a model (Freedman and Chan, 1978) for interactive biosynthesis and assembly of inner membrane complexes, such as cytochrome oxidase, which derive subunits from both the nuclear and the mitochondrial genomes. We speculated that a single precursor protein for all the cytoplasmically synthesized subunits (Poyton and Kavanagh, 1976) was inserted co- or posttranslationally into the membrane and acted as a receptor for each of the mitochondrially synthesized polypeptides. In the intervening years, the new data have been consistent with some aspects of the model, but not others. Initially, the notion of the polysubunit precursor was supported by

the finding of a similar one in rat liver cells (Ries *et al.*, 1978) and by isolation and characterization of Poyton's original polyprotein (Poyton and McKemmie, 1979a,b). Poyton reviewed the evidence for this giant precursor (Poyton, 1980), and speculated on a model in which the precursor was inserted into the membrane, stimulated mitochondrial synthesis, and split into its constituent subunits, which then assembled with the mitochondrially synthesized polypeptides (Poyton, 1980). But the polyprotein precursor was subsequently refuted by the observation that the cytoplasmically made subunits of F_1-ATPase and cytochrome oxidase are synthesized as *individual* precursors, with hydrophobic leader sequences terminated by *N*-formylmethionine (Lewin *et al.*, 1980), which are removed after insertion (Schatz, 1979; Schmelzer *et al.*, 1982; Hatalová and Kolarov, 1983). It was shown that the uptake of the precursors for the cytoplasmically synthesized subunits of the yeast mitochondrial ATP synthetase by the mitochondria is independent of protein synthesis (Maccecchini *et al.*, 1979), implying that insertion was not by vectorial translation. However, finding that the mRNA for rat liver subunit IV of the oxidase occurred as free, loosely, and tightly membrane-bound polysomes (Northemann *et al.*, 1981) left open the possibility of bivectorial synthesis (and assembly) of that enzyme in the rat liver. Recently it was reported that, while mitochondrially bound polysomes in yeast are enriched in some cytoplasmically synthesized mitochondrial proteins, the precursors to cytochrome oxidase subunits V and VI are synthesized predominantly on free polysomes (Suissa and Schatz, 1982). However, the relative sizes of the pools of free and bound polysomes depends on the metabolic state of the organism (Reid and Schatz, 1982). Thus, bivectorial synthesis is not a requirement for assembly in yeast. Insertion of the cytoplasmically synthesized precursors is energy dependent (Nelson and Schatz, 1979), for their insertion can be inhibited by depleting the mitochondrial matrix of ATP. The dependence on ATP is a consequence of a more direct dependence on an electrochemical gradient (Gasser *et al.*, 1982). The important, cytoplasmically synthesized precursors are processed [by a mitochondrial matrix protease which was recently isolated (Böhni *et al.*, 1983; Cerletti *et al.*, 1983)] independent of the energy supply, whereupon the mature products can be found exclusively in their proper part of the mitochondrion. That is, F_1 is found in the matrix, oxidase subunits in the inner membrane, and cytochrome b_5 in the intermembrane space (Gasser *et al.*, 1982). Thus the model of Freedman and Chan (1978) can be modified so that the cytoplasmically synthesized polypeptides are individually inserted, probably posttranslationally in yeast, but cotranslationally in rat liver (Parimoo *et al.*, 1982). They are received into the inner membrane where they can then act as receptors for the nascent chains of their mitochondrially synthesized partners.

The regulation of synthesis and assembly is a complex process. In the

laboratories of Bertrand and Werner, a series of cytoplasmic and nuclear mutants are being studied which form partial complexes of the oxidase (Bertrand and Collins, 1978; Bertrand and Werner, 1979; Nargang *et al.*, 1979). This implies a stepwise assembly process, as predicted by the model (Freedman and Chan, 1978). Partially supportive is the finding that, on incubation of isolated liver mitochondria with [^{35}S]methionine, subunit II and III are labeled immediately in the assembled enzyme, but, while labeled subunit I can be found in intact mitochondria, it does not integrate into the holoenzyme for 1.5–2 hours (Wielburski *et al.*, 1982). We interpret this to mean that subunit I may be taken up posttranslationally, perhaps after processing, by a preformed assembly of the other subunits. Subunit I does appear to require processing in *Neurospora* (Werner and Bertrand, 1979; Van't Sant *et al.*, 1981), although not at the carboxy terminus, as Darley-Usmar and Fuller (1981) were able to show with the beef heart enzyme. The observation that isolated beef heart subunit I still bears an *N*-formylmethionine (Buse and Steffens, 1978) means that it is not processed at the *N*-terminus either, so different organisms may use different mechanisms of assembly.

The molecular biology of the mitochondrial genome is full of surprises. The most striking finding was that the universal codon dictionary contains mitochondrial variants. The stop codon UGA is used for tryptophan in the oxidase subunit II gene from the mitochondrial DNA of humans (Barrell *et al.*, 1979) and yeast (Fox, 1979), but not *Zea mays* (Fox and Leaver, 1981). The mitochondrial violation of universality is itself nonuniversal. If the original source of mitochondrial DNA was a primeval prokaryotic endosymbiont (Margulis, 1970), plants might have been infested at some different stage of its evolution, or perhaps by an entirely different species. The endosymbiont theory is further undermined by the finding of large intervening sequences in the yeast mitochondrial structural genes for cytochrome oxidase subunit I and cytochrome *b* (Borst and Grivell, 1978). Interestingly, the intron of each exerts regulatory effects on the synthesis of the gene product of the other (De La Salle *et al.*, 1982; Netter *et al.*, 1982). However, such introns were not present in bovine mitochondrial DNA (Anderson *et al.*, 1982). Thus in the tapestry of this immensely complex enzyme, interactive patterns in its function weave through its biosynthetic origin, in the influence of the nuclear genome on the mitochondrial genome and the mutual influences between it and another electron-transport enzyme within the mitochondrial genome.

IIIB.4. Their Interactions. Even though the amino acid sequence is known for only some of the subunits (Section IIIB.1), that the apoprotein is constructed of distinct pieces makes possible numerous studies of the details of the structure of the holoenzyme. Near neighbor relationships among the subunits create a fabric upon which the nonprotein portions of the functional

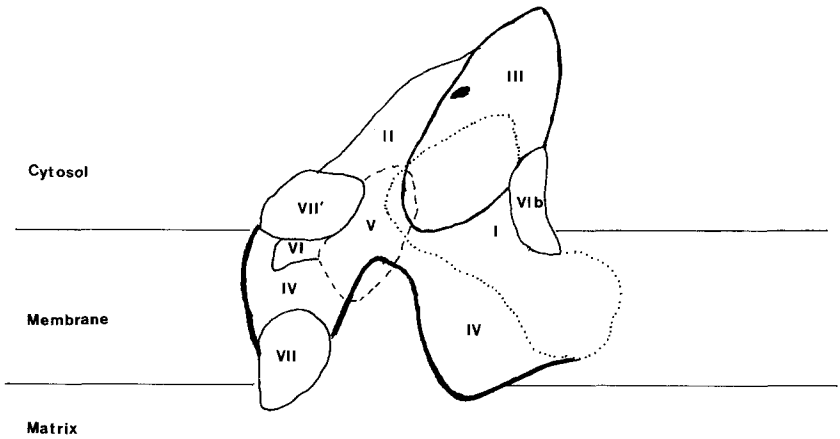

Fig. 1. A rough sketch of the probable locations of some subunits in the open conformer of the monomer, as suggested by the reviewed data. Subunits I and V are shown by dotted and dashed lines, respectively. Subunit VI is inaccessible to both aqueous phases (Chan and Tracy, 1978) and adjacent to both IV and II (Briggs and Capaldi, 1977, 1978) (below the plane of the paper). Two small subunits (Bisson and Montecucco, 1982) could abut on a substrate nesting in the cytoplasmic crevasse between two members of a dimer. The second monomer would be displaced above the plane of the paper and to the left. Subunit IV would contribute the bulk of the M-domains.

entity are arrayed. The reader may wish to use Fig. 1 as a frame of reference for the following sections. For the historical reasons already explained, we will continue with the nomenclature of Section IIIB.1.

IIIB.4a. With Each Other. Using an 11-Å bifunctional cross-linker, Briggs and Capaldi (1977, 1978) were able to link V with I, II, III, or VI; IV with VI or VII; and II with VI. With a 5-Å cross-linker, Kornblatt and Lake (1980) linked III to VI. These subunit numbers are based on the nomenclature of Downer *et al.* (1976) and Tracy and Chan (1979), but it is now known that their VI and VII are each several polypeptides (Azzi, 1980; Buse and Steffens, 1978; Kadenbach and Merle, 1981; Verheul *et al.*, 1981), so the above sets of dimers do not establish that one subunit, either VI or VII, links both V and IV. A study of the differential susceptibility of the subunits in the whole enzyme to proteases would suggest that I is on the outside and V–VII are buried within the complex, except that, unfortunately, the experimenters (Nagasawa-Fujimori *et al.*, 1980) neglected to establish the relative susceptibility of the isolated subunits. Still, cross-linking studies that show V surrounded by I, II, III, and VII (Briggs and Capaldi, 1977, 1978) would at least in part predict their results. Fluorescence resonance energy transfer measurements (with assumptions about the relative orientation of the fluorophore and chromophore) indicated a distance of 35 Å between the heme of cytochrome *c* covalently bound to yeast subunit III and a fluorophore bound to subunit II

(Dockter *et al.*, 1978). Correlation of this study with the others is difficult, if only because subunit structural parallels between yeast and mammalian oxidase are questionable (see Sections IIIB.2 and IIIB.4b).

IIIB.4b. With the Prosthetic Groups. Since its isolation in association with the metal in 1965 (MacLennan and Tzagoloff, 1965), there has been growing evidence that subunit II is the copper-binding protein. Although Mason's group (Gutteridge *et al.*, 1977) found copper predominantly in subunits V and VII by Cu-specific staining of gels of the mildly dissociated enzyme, when they controlled the dissociating conditions more carefully, the only copper-bearing subunit was subunit II (Winter *et al.*, 1980). Buse *et al.* (1978) have pointed out sequence homologies between other copper-containing protein and fragments of the subunit [or its complete sequence (Steffens and Buse, 1979)]. Evidence has been presented that at least one of the two cysteines in subunit II is liganded to copper (Darley-Usmar *et al.*, 1981).

The heme story is less clear. They were reported to have been isolated in association with subunit V (Tanaka *et al.*, 1977, 1979; Yu *et al.*, 1977), although we found subunit V to be the one most easily separated from the hemes (Freedman *et al.*, 1979), and sequence homologies with hemoglobin (Tanaka *et al.*, 1977) claimed for the beef heart subunit were not seen in the yeast homolog (Gregor and Tsugita, 1982). Sequence homologies to heme-containing proteins have been reported for one of the subunit VII polypeptides (Buse *et al.*, 1978; Steffens *et al.*, 1979). In gels of mildly dissociated oxidase, hemes travel with subunits I and II (Gutteridge *et al.*, 1977; Winter *et al.*, 1980). Indeed, between these sources and those reviewed by Azzi and Casey (1979), association with heme has been claimed for every subunit of the enzyme. The facts that (1) radiation inactivation studies (e.g., Thompson *et al.*, 1982) are consistent with a target having a molecular weight of only 70 ± 15 kD (subunits I, II, and possibly III), and (2) the more primitive prokaryotic cytochromes aa_3 contain only the larger subunits (reviewed by Ludwig, 1980) would both make a cytoplasmically synthesized primary heme locus unlikely. However, cytoplasmically synthesized oxidase subunits have been found to associate with porphyrin (Keyhani and Keyhani, 1978), and if eukaryotic cytoplasmically synthesized subunits do bear antigenic relationships with the large bacterial subunits (see Section IIIB.1), sandwiching of the prosthetic groups between subunits is an attractive possibility.

IIIB.4c. With Cytochrome c. At the time Azzi (1980) reviewed the oxidase interaction with cytochrome *c*, the most likely candidate for a *c*-binding subunit was subunit II, with the possible assistance of subunit III. The one report of participation by a low-molecular-weight subunit (Erecińska, 1977) is as yet, unconfirmed. Azzi also reviewed the evidence concerning the pertinent part of the cytochrome *c* molecule. The area which interacts with oxidase is at the front (exposed hemeedge) and top left part of the molecule,

centered around lysines 8, 13, 72, 73, 86, and 87, the same locus on cytochrome *c* which interacts with reductase (Azzi, 1980). A few comments on this are in order.

Oxidase subunit II is most certainly involved (Briggs and Capaldi, 1978; Bisson *et al.*, 1978a,b; 1982; Millett *et al.*, 1982), although cytochrome *c*'s point of interaction on the beef heart oxidase (upper left part of the cytochrome *c* molecule) is not the same as in the yeast enzyme. Only in the latter case is interaction seen with cytochrome *c* lysine 22, to the *lower right* of the heme crevice (Bisson *et al.*, 1982). Oxidase subunit III from both beef heart (Fuller *et al.*, 1981) and yeast (Birchmeier *et al.*, 1976; Birchmeier, 1977) interacts (e.g., through beef heart subunit III cysteine 115 (Malatesta and Capaldi, 1982) with the back side of cytochrome *c* near its cysteine 107 (for the relevant domains on the cytochrome *c* surface, consult the diagrams of Speck *et al.*, 1979). Capaldi *et al.* (1982) believe the small *c* molecule sits in the crevasse between subunit II of one oxidase member of the dimer, and subunit III of the other. The reports of participation by a smaller oxidase subunit have persisted (Seiter *et al.*, 1979; Erecińska *et al.*, 1980). But these researchers have used gel systems which may lead to a different nomenclature than that of Downer *et al.* (1976) and Tracy and Chan (1979), and so the identity of the polypeptide is unclear. In 1978, Bisson *et al.* (1978a) seemed to suspect subunit V. But more recently, using carbodiimides of different size and hydrophobicity, they have been able to demonstrate that subunits VII and b (Downer *et al.*, 1976) [also known as VIb (Kadenbach and Merle, 1981)] form part of the high-affinity binding site when a sufficiently small and hydrophilic cross-linker was used. These small polypeptides do not appear to be as crucial for electron transport activity as subunit II (Bisson and Montecucco, 1982). It will be interesting to see which of the subunits VII is involved. In mammalian oxidase, polypeptide VII b is organ-invariant, but one of the subunits VII is not (Kadenbach *et al.*, 1982). If a subunit which exhibits an organ-specific primary structure does form part of the substrate binding domain, it could be the source of organ-specific kinetics observed by Kadenbach's group (Merle and Kadenbach, 1982).

IIIB.4d. With the Lipid Bilayer. Because oxidase is completely transmembranous (Section IIIA.2), its topography is divided into three areas: that in contact with the lipids of the membrane, that which projects into the intermembrane space (the cytosolic side of the inner membrane), and that which is exposed to the matrix. The first is the easiest to examine, since errors in vesicle preparation leading to inhomogeneity of orientation will not affect the results. And so it is that investigations using hydrophobic affinity probes agree that, in the yeast enzyme (Gutweniger *et al.*, 1981; Cerletti and Schatz, 1979), subunits I–III and VII were labeled, V was labeled lightly [near one terminus (Gutweniger *et al.*, 1981)] and IV and VI were not labeled at all. In

the beef heart enzyme, there is agreement about the strong hydrophobicity of subunits I, III, and VII, and the milder hydrophobicity of subunits II and IV (Prochaska *et al.*, 1980; Georgevich and Capaldi, 1982; Bisson *et al.*, 1979), but subunit VI was only labeled in one study (Bisson *et al.*, 1979). Beef heart V was never labeled by a hydrophobic probe, and neither was the yeast subunit VI, in keeping with their homology (Gregor and Tsugita, 1981). Cerletti and Schatz (1979) reported some inconsistent labeling of yeast subunit IV, which resembles the inconsistencies in the Capaldi and Bisson data on beef heart subunit VI (Prochaska *et al.*, 1980; Georgevich and Capaldi, 1982; Bisson *et al.*, 1979).

The examination of the other two areas presents some difficulties. To date, it has not been possible to separately explore the subunit accessibility on the two faces of the yeast inner membrane because of the problem of obtaining homogeneous inside-out inner membrane preparations (Eytan and Schatz, 1975). There are indications that simple sonication of beef heart mitochondria does not yield homogeneously inverted particles either (Chance *et al.*, 1975). But for this problem there are solutions available (Tracy, 1978; Eytan *et al.*, 1975; Huang *et al.*, 1973; Freedman and Chan, 1983), and it is unfortunate that precautions, including testing for homogeneity of orientation of the oxidase itself, were not observed in all studies of beef heart subunit sidedness. Moreover, as we have indicated earlier, there is some confusion about the identity of the subunits resolved by different gel systems. Earlier subunit sidedness studies (Eytan *et al.*, 1975; Kornblatt *et al.*, 1975) used the Weber and Osborn (1969) SDS polyacrylamide gel electrophoresis (PAGE) system, but it has since been shown (Downer *et al.*, 1976; Tracy and Chan, 1979) that the Swank and Munkres (1971) SDS–urea–PAGE (SUPAGE) offers superior resolution. Comparison of results using the two systems is complicated for three reasons. The two systems have different resolving power (subunits II and III are separated by SUPAGE, but not by SDS–PAGE (Downer *et al.*, 1976). Subunits resolved by the different systems have different relative mobilities [e.g., subunits V and VI migrate in different orders (Tracy and Chan, 1979; Capaldi *et al.*, 1977)]. It was found that the subunits separated by the SUPAGE system can be further resolved (see Section IIIB.2), so that bands in either system could contain constituents of noncorresponding bands from the other.

In spite of these difficulties, a partial picture is possible. Subunits II and/or III are labeled only on the outside of the inner membrane (Eytan *et al.*, 1975; Kornblatt *et al.*, 1975; Chan and Tracy, 1978). Since both have been implicated in binding to cytochrome *c* (Section IIIB.4c), which is known to bind exclusively on the cytoplasmic side (e.g., Schneider *et al.*, 1972), the inability of SDS–PAGE to resolve the two is of no consequence. That Ludwig *et al.* (1979) found II and III on the surface of sonicated submitochondrial particles is insufficient evidence that these subunits are transmembranous,

since homogeneity of orientation was checked only by the accessibility of the F_1 knobs. A particle in which the constituents have been dislocated relative to one another (see, for example, the diagrams of Harmon *et al.*, 1974) could appear inverted by this criterion, and yet still have exposed cytosol-facing cytochrome *c* binding sites on some cytochrome oxidase molecules. Studies taking this into account (Eytan *et al.*, 1975), or using oxidase reconstituted into vesicles in which the cytochrome *c* binding sites are homogeneously accessible (Kornblatt *et al.*, 1975), find no matrix accessibility of the two subunits. Subunit IV faces the matrix only (Ludwig *et al.*, 1979; Tracy, 1978; Eytan *et al.*, 1975; Chan and Tracy, 1978). Subunits I and VI are nearly buried in the membrane or the interior of the enzyme (Ludwig *et al.*, 1979; Tracy, 1978; Eytan *et al.*, 1975; Chan and Tracy, 1978). But subunits V and VII were found on the cytoplasmic side only in one chemical probe study (Eytan *et al.*, 1975), the matrix side only in another (Ludwig *et al.*, 1979), and on both sides by immunochemical probes (Tracy, 1978; Chan and Tracy, 1978).

The inconsistency in the case of "subunit VII" is to be expected. Band VII, after all, is acknowledged to be heterogenous (Azzi, 1980; Buse and Steffens, 1978; Steffens *et al.*, 1979; Kadenbach and Merle, 1981; Steffens and Buse, 1976; Verheul *et al.*, 1981; Merle and Kadenbach, 1980), and it is possible that the confusion about its sidedness may relate to the distribution of its component oligopeptides in different resolving systems. But band V (or its

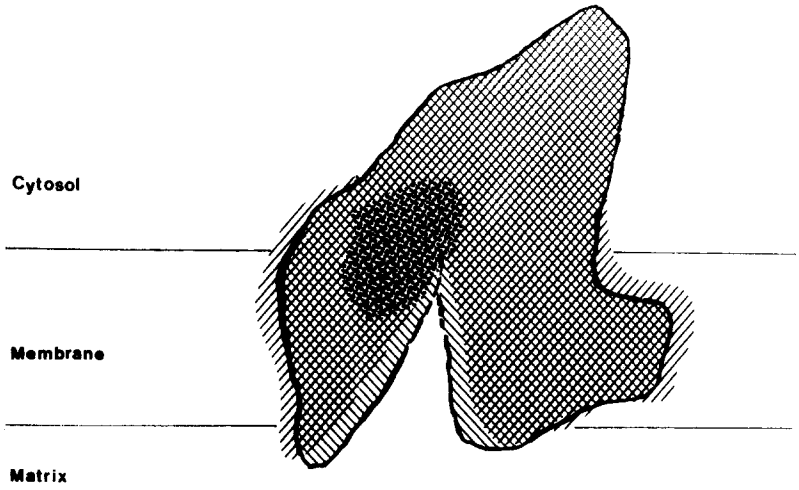

Fig. 2. A model of the topology of subunit V in membranous beef heart cytochrome *c* oxidase and its conformational dynamics (Freedman and Chan, 1983). The outlined hatched area is a tracing of the balsa wood model of the monomer (Fuller *et al.*, 1979). Hatching in the other direction depicts a conformational change which provides access for antibodies to subunit V (stippled region).

yeast subunit VI homologue) has been assumed to be a single constituent (Downer *et al.*, 1976; Tracy and Chan, 1979; Tanaka *et al.*, 1977; Buse and Steffens, 1978; Gregor and Tsugita, 1982; Yu and Yu, 1977; Verheul *et al.*, 1981; etc.), albeit occasionally plagued by a contaminant (Kadenbach and Merle, 1981; Ludwig *et al.*, 1979). The puzzle was recently resolved (Freedman and Chan, 1983). A monospecific, enzyme-linked immunosorbent assay designed for use on intact membranes was applied to highly homogeneously oriented inner membrane particles, and the accessibility of the subunit (matrix side only) was shown to be redox sensitive. Such conditional accessibility to a hydrophilic probe is consistent with a channel (see Section IIIA.1) having variable dimensions. This was taken as direct evidence of a redox-associated conformational shift (see Fig. 2). Given the subunit's location and its probable redox-unrelated functional importance (implied by an evolutionary longevity which does not include homologies to heme-containing proteins (Gregor and Tsugita, 1982), the study could be evidence for a conformational link between electron and proton translocation by the enzyme.

IV. Conclusion

One can hypothesize that, during turnover *in vivo*, the protein subunits of cytochrome *c* oxidase exhibit a conformational mobility which can be perceived as a dynamic variability in the detailed topography of the membrane-bound enzyme. As the enzyme performs its functions of electron transport and energy transduction, the protein undergoes changes in shape by means of which it serves as a conduit for the interaction of the two functions. This is the last part in our picture of the interactive aspects of oxidase. We first discussed the mutual influence of the components responsible for its redox functions. Then, we described the fit of this enzyme to schemes to explain how electronic and protonic energies are intraconverted, the interactive aspects of its biosynthesis, the interactions of its protein components with each other and with nonprotein elements within and without the enzyme, and lastly, the central role of the protein as integrator of structural and functional aspects of the complex. We hope we leave our readers with an image of cytochrome *c* oxidase as a tapestry woven of a multitude of intricate patterns, whose systematic unraveling will continue to challenge researchers of diverse and imaginative investigative talent.

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