Review Letter

ON THE FUNCTION OF MULTIPLE SUBUNITS OF CYTOCHROME *c* OXIDASE FROM HIGHER EUKARYOTES

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Received 7 September 1981

1. Introduction

Cytochrome *c* oxidase (ferrocytochrome *c*: oxygen oxidoreductase, EC 1.9.3.1) complex IV of the respiratory chain of mitochondria [1], catalyzes the transfer of 4 electrons from reduced cytochrome *c* via 4 redox centers (2 heme *a* and 2 copper atoms) to dioxygen. The reaction occurs without accumulation of free toxic intermediates such as H_2O_2 or the superoxide radical O_2^-, and was identified as the third coupling site in mitochondria for the formation of ATP [2].

According to the chemiosmotic hypothesis, the electrochemical gradient of protons is the driving force for ATP formation and for ion and metabolite transport across the mitochondrial membrane [3]. In the original chemiosmotic hypothesis [3] and from later experiments [4] Mitchell denied a proton translocation through cytochrome *c* oxidase, but depicts it as the electron-carrying arm of the last loop in the respiratory chain. Original experiments with mitochondria by Wikström [5,6] and subsequent work with isolated cytochrome *c* oxidase, reconstituted in liposomes in different laboratories [7–10], have now established that cytochrome *c* oxidase pumps protons in addition to the formation of a membrane potential and concomitant alkalinisation of the matrix side, due to the transfer of electrons from cytochrome *c* to oxygen. The data of Wikström [5–7] and of Carafoli and coworkers [10,11] are consistent with an H^+/e^- stoichiometry of 2. The reaction can be summarized as follows [10]:

$$2\ \text{cyt}\ c_o^{2+} + 4\ H_i^+ + 1/2\ O_2 \longrightarrow 2\ \text{cyt}\ c_o^{3+} + 2\ H_o^+ + H_2O_i$$

The molecular mechanism of electron transfer from cytochrome *c* to oxygen has been intensively studied by spectroscopic methods (review [12–16]). Although progress has been made, in particular due to EPR studies [16], concerning the electron transfer from cytochrome *c* via the 4 redox centers to oxygen, very little is known on the arrangement and subunit location of the 4 metal centers within the enzyme complex. In addition almost nothing is known on the mechanism of coupling between electron transport and proton pumping, although some speculations have been presented [15]. This is in particular due to the complicated structure and multisubunit composition of the enzyme complex. In the following, all protein components which are found tightly associated to the enzyme complex are denoted 'subunits' in default of a proven specific function which could be catalytically or regulatory.

2. Subunit composition

The protein subunit composition of cytochrome *c* oxidase from *Neurospora crassa* and from yeast was thoroughly investigated in connection with the identification of the mitochondrial or cytoplasmic site of synthesis of the various subunits. The enzyme from *N. crassa* was found to contain 8 [17,18], that from yeast 7 different polypeptide chains, designated 'subunits' [19–21]. The apparent M_r-values, estimated by SDS gel electrophoresis, were from 4600–40 000 [21]. All protein components seem to occur in the enzyme complex in stoichiometric amounts (1:1) as was deduced from the [^{3}H]leucine radioactivity incorporated into the subunits in intact *N. crassa* cells and corrected for the leucine content of the subunits [17].

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.75

Since the 7 subunit composition of the yeast enzyme was established by a number of different criteria [22], the 'similar protein profile' of beef heart cytochrome *c* oxidase if 'arranged to show correspondence with those of the yeast enzyme' [20], lead to the general suggestion 'that the 7 polypeptides form a specific complex that may function in the oxidation of cytochrome *c* in vivo' [22].

However, already in 1975–1977 more than 7 protein components could be identified in cytochrome *c* oxidases from beef heart [23–25] and rat liver [26] if a high resolution SDS gel electrophoretic system was applied. Nevertheless, in many publications on mammalian cytochrome *c* oxidase which appeared during the last year, the authors claim to confirm the 7 subunit composition of cytochrome *c* oxidase [27–36], although more than 7 protein components often could be separated.

In most investigations the protein composition of cytochrome *c* oxidase was analyzed by SDS–polyacrylamide gel electrophoresis. In parallel with the improvement of the gel electrophoretic technique, beginning from 3 [37] to 5 [38] (a sixth component with app. M_r 66 000 probably represents an impurity), 6 [20,39,40], 7 [25,41–44], 8 [24], 9 [26,45] and finally 12 different protein components [47,48] could be identified in cytochrome *c* oxidase preparations from mammalian sources. The occurrence of 12 components, identified on analytical SDS gels [47], was corroborated by preparative isolation of 12 [49] or 11 [50] different polypeptides by gel chromatography.

The separation of cytochrome *c* oxidase into 12 protein bands by SDS gel electrophoresis is difficult and depends on specific properties of the separation system [47]. In fig.1 the protein pattern of the same preparation of rat liver cytochrome *c* oxidase, separated by 2 different SDS gel electrophoretic systems, is presented. In fig.1A, the discontinuous gradient gel system as described by Cabral and Schatz [51], in fig.1B the gel system of Merle and Kadenbach [47], was applied. Both systems separate subunits I–V quite well. But in fig.1A subunits VIa–c appear as a single band, previously denoted subunit VI, and also subunits VIIa–c and VIII appear as a second single band, previously denoted subunit VII.

In previous studies subunits VIIa–c were usually not separated [40–42,52,53] even with the well-resolving system of Downer et al. [25], because of their very similar apparent M_r-values [47,49]. In most

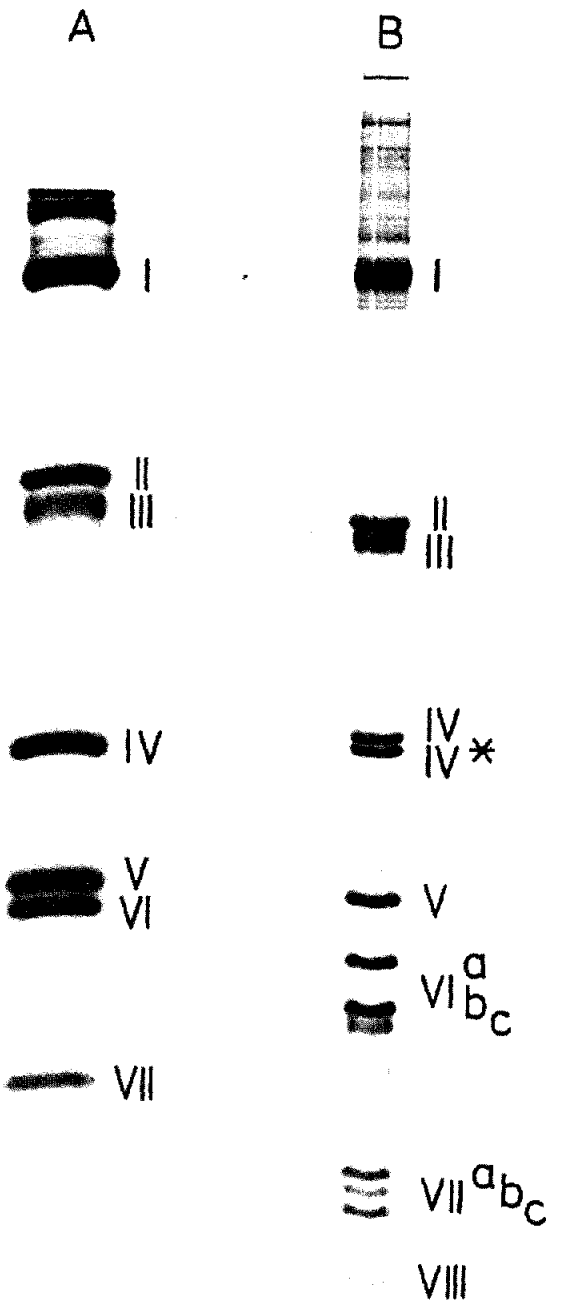

Fig.1. Comparison of the protein pattern of rat liver cytochrome *c* oxidase separated by 2 different systems of SDS gel electrophoresis: (A) that of Cabral and Schatz [51]; (B) that of Merle and Kadenbach [47], 18% acrylamide, 3 M urea and 10.6% glycerol.

published gel patterns, subunit VIII is not visible, due to either insufficient separation, or its weak staining intensity and/or broadening of bands in the lower M_r region [34,40–42,46,52,53].

3. Stoichiometry of subunits and impurities

After isolation of beef heart cytochrome *c* oxidase subunits by gel chromatography, Buse and Steffens [49] observed that some components (denoted VIa–c and VIIIa–c) appeared in sub-stoichiometric amounts. Similar observations were made by Verheul et al. [50]. However, in both investigations a non-quantitative isolation of the components could not be excluded, and no quantitative protein estimation was performed. Yu and Yu [41] estimated the stoichiometry of beef heart cytochrome *c* oxidase subunits from the amount of amino acids measured in the hydrolysed stained gel bands and found an almost 1:1 ratio for 7 separated bands. The applied gel system, however, could not separate subunits VIa–c and VIIa–c and VIII. We have estimated the stoichiometry of the 12 separated protein components of rat liver cytochrome *c* oxidase by quantitation of the Coomassie blue stain and by measuring the fluorescamine fluorescence of amino acids after hydrolysis of the gel bands, and concluded a 1:1 ratio for all 12 components [47].

Ludwig et al. [55] studied beef heart cytochrome *c* oxidase by SDS–polyacrylamide gel electrophoresis and observed in addition to the 7 main components less intensive bands denoted a,b and c (between subunits IV and VI) and further low M_r peptides (below subunit VII). They suggested that these polypeptides represent impurities, since they could be removed or reduced by treatment of the enzyme with trypsin. We have studied the effect of trypsin on the isolated beef heart enzyme in more detail and found also a reduction of subunits VIa–c and in addition a partial decrease of the apparent M_r-value of subunit I. The kinetic analysis of the trypsin-treated enzyme, however, revealed a decreased affinity of the enzyme for cytochrome *c* and an increased V_{max} [56]. From this we suggested that subunits VIa–c are not directly involved in electron transfer between cytochrome *c* and oxygen but may influence the kinetic parameters of the enzyme.

The reason for the occurrence of sub-stoichiometric amounts of subunits VIa and b observed in beef heart cytochrome *c* oxidase preparations may be 2-fold:

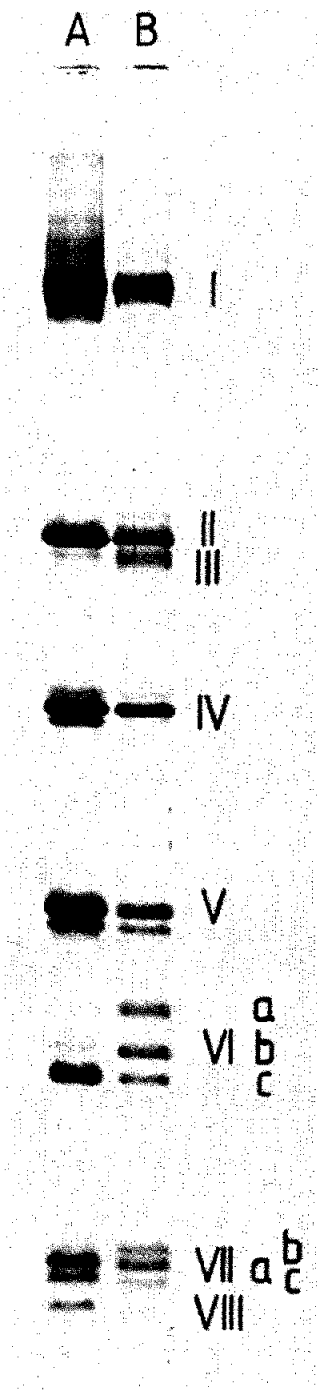

Fig.2. Effect of alkali-treatment on the subunit pattern of beef heart cytochrome *c* oxidase: (A) cytochrome *c* oxidase after alkaline treatment [57] (sample kindly supplied by Professor Wikström); (B) untreated beef heart cytochrome *c* oxidase (isolated in Marburg). Gel system: 18% acrylamide, 6 M urea.

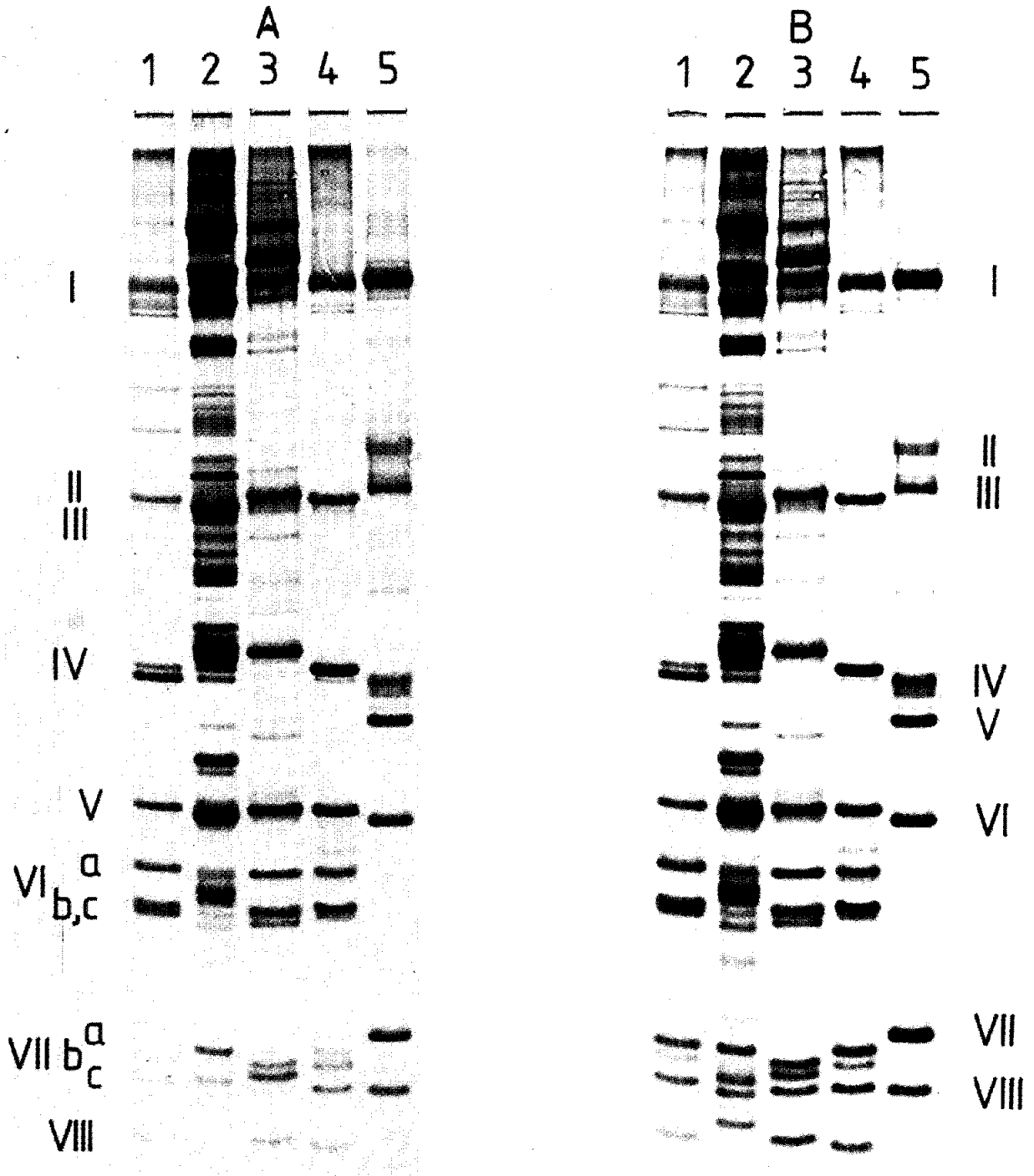

Fig.3. Comparison of relative intensities of cytochrome *c* oxidase subunits from various sources after different staining times. After SDS gel electrophoresis (18% acrylamide, 3.6 M urea and 10.6% glycerol) the gel was stained for 2 h (A), and then again overnight (B). Cytochrome *c* oxidases were isolated by standard procedure [47] from different sources: (1) rat liver; (2) impure preparation from chicken heart; (3) beef liver; (4) rat kidney; (5) yeast (sample kindly supplied by Professor Schatz, Basel).

(i) During isolation of the enzyme some proteases, present in the mitochondrial fraction, may specifically split these subunits;
(ii) The binding of subunits VIa and b to the complex may be less tight in the beef heart enzyme than in cytochrome *c* oxidases from other sources.

This follows from the easy release of subunits VIa and b (and VIIa) from the complex during alkaline treatment (pH 9.5) which also removes subunit III [29], as shown in fig.2. The figure compares an alkali-treated sample of beef heart cytochrome *c* oxidase [57], kindly supplied by Professor Wikström, with our standard preparation. With the isolated rat liver enzyme, however, we have never observed a variation in the relative amounts of the 12 subunits out of more than 50 preparations.

Finally it should be stressed that the staining intensity of protein bands after SDS-gel electrophoresis represents no quantitative measure of protein amount, because it depends on the staining conditions. Fig.3 compares the staining patterns of cytochrome *c* oxidase preparations from different sources after incubation of the gels for 2 h and for an additional 16 h. A large change of staining intensity is observed after prolonged staining duration, however, only for some selected proteins in the lower M_r region.

4. Nomenclature of subunits

Buse and coworkers determined the N-terminal amino acid sequence for 12 different polypeptides of the beef heart cytochrome *c* oxidase complex [58]. In addition the complete amino acid sequence was obtained for subunits II [59,60], IV [61], V [62], VII [63,64] and VIIIa [49]. Whereas the nomenclature of Buse is based on separation of subunits by gel chromatography, most investigators use a numbering system according to the apparent M_r-values of subunits after SDS gel electrophoresis. The identity of subunits I–V is not in doubt, but much confusion exists concerning the lower M_r subunits (review [65]). The confusion is due partly to the observation that the relative mobility of some subunits depends on the applied gel electrophoretic system [66]. We have analyzed the relative mobility of subunits VIIIa and VIIIb + c isolated and kindly supplied by Professor Buse, in our gel electrophoretic system as shown in fig.4.

Subunits VIIIa and VIIIb + c (mainly containing VIIIc) from Buse seem to correspond to subunits

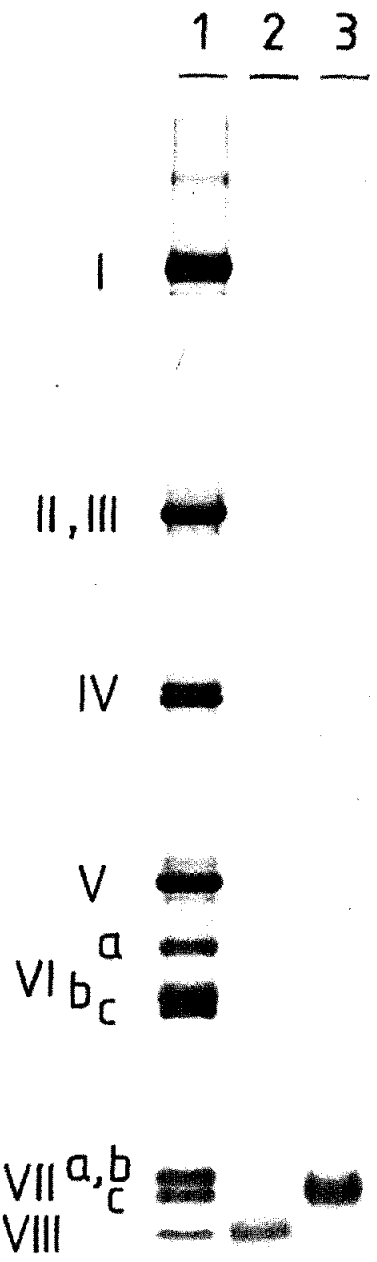

Fig.4. SDS-gel electrophoresis of total beef heart cytochrome *c* oxidase and of isolated subunits VIIIa and VIIIb + c from Professor Buse. The separation gel contained: 18% acrylamide and 6 M urea. (1) Total beef heart cytochrome *c* oxidase; (2) isolated subunits VIIIb + c; (3) isolated subunits VIIIa (nomenclature from Buse).

VIIc and VIII of our nomenclature, respectively. However, there remains disagreement concerning the number of polypeptides in that M_r-region. Whereas Buse finds 3 polypeptides (VIIIa–c), we observed always 4 different protein bands (VIIa–c and VIII) in all investigated enzyme preparations, isolated in the presence or absence of protease inhibitors. In collaboration with Professor Kühn (Martinsried) the N-terminal amino acid sequence of beef heart subunit VIa (our nomenclature), extracted from SDS gels, was obtained as follows: Ala–n.d.–Ala–Ala–Lys–Gly–Asp (Dieringer, Kühn and B. K., unpublished), which is identical with the sequence of subunit VIb of Buse (Ala–Ser–Ala–Ala–Lys–Gly–Asp) (Buse, personal communication). From these data the subunit nomenclature can be correlated as given in fig.5. Subunit VIb of our nomenclature corresponds to subunit VII of Buse and 'impurity c' of Capaldi (Buse, personal communication). The amino acid sequences of subunit VII of Buse [63] and subunit VI of King [64] are identical. Subunit VIa (Buse) according to its app. M_r of 14 000 [58] correlates with a band which we obtained only under certain conditions with the beef heart enzyme below subunit V and which probably corresponds to 'impurity a' of Capaldi's group [55]. This polypeptide has not yet been considered as a subunit by our group and would represent a 13th subunit.

5. Number and apparent M_r-values of subunits from different species and organs and from bacteria

Cytochrome *c* oxidases from mammals and birds can be shown to contain 12 different polypeptides, if analyzed by a suitable SDS-gel electrophoretic system. This was shown for the enzymes isolated from rat liver, heart and kidney, beef liver and heart, pig liver and heart, chicken liver and heart, rabbit, stone-marten and deer liver, and of human placenta [47,56, 67].

From a total rat liver mitochondrial lysate all 12 polypeptides are specifically precipitated with antisera against the holoenzyme [47] or the isolated subunit IV [48].

Variations of the apparent M_r-values were observed between enzymes from different species, which were more pronounced for the low M_r polypeptides. In addition, the occurrence of tissue-specific isoenzymes has been suggested. Cytochrome *c* oxidases isolated

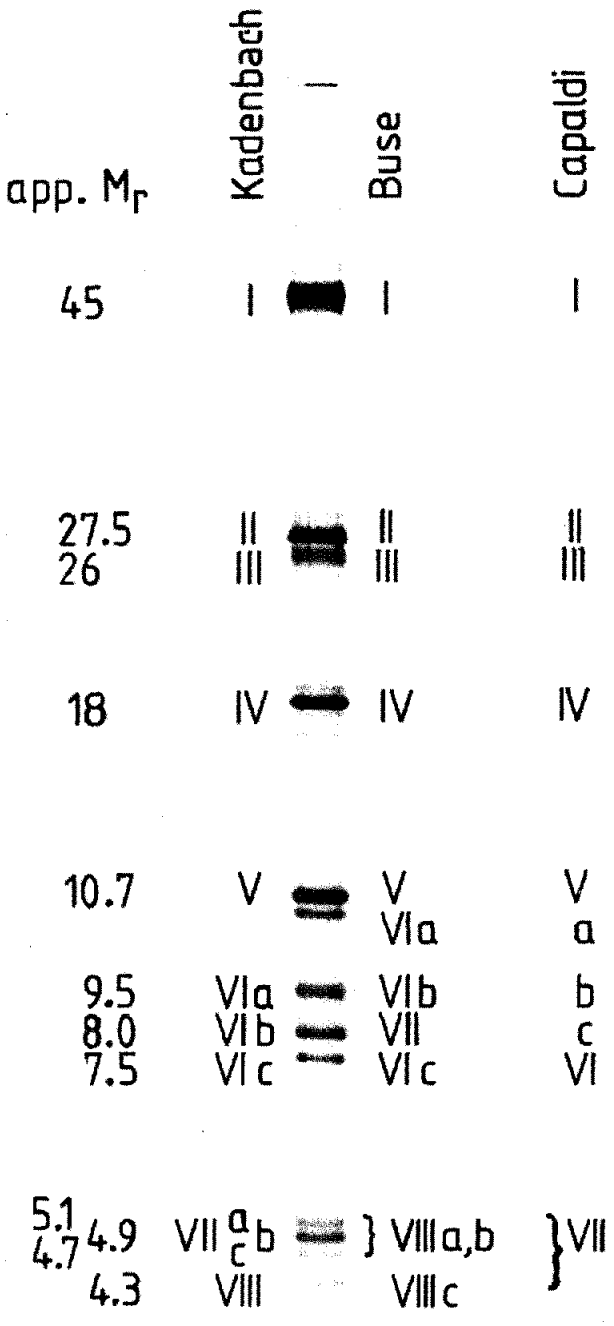

Fig.5. Correlation of the nomenclature of cytochrome *c* oxidase subunits used by Buse with that of Capaldi and of our laboratory. Beef heart cytochrome *c* oxidase was separated by the gel system in [47] containing 18% acrylamide and 6 M urea. Nomenclature was taken from Kadenbach [47], Buse [49] and Capaldi [66].

from liver and heart of the same species show differences in the apparent M_r-values of subunits VIa, VIIa and VIII [56,67]. The enzymes from rat differed in the apparent M_r of subunit VIa, the enzymes from chicken in subunit VIII, the enzymes from pig in subunits VIIa and VIII and those from beef in subunits VIa, VIIa and VIII.

Cytochrome *c* oxidase from yeast was reported to contain 7 subunits [21], but with a high-resolution gel electrophoretic system 8 different polypeptides can be separated [56] (see fig.3). Although the enzyme from *N. crassa* contains also 8 subunits [18], the apparent M_r-values are quite different from the corresponding subunits of yeast [56]. Except for subunit I, very little correlation of the apparent M_r of subunits is found between the enzymes from fungi and from vertebrate sources [56] (fig.3).

A cytochrome *c* oxidase, containing 2 heme *a* and 2 copper atoms has also been isolated from bacteria. Only 2 protein subunits were detected after SDS gel electrophoresis in the enzymes from *Paracoccus denitrificans* [68], *Thiobacillus novellus* [69], *Thermus thermophilis* and *Nitrobacter agilis* [70]. The apparent M_r-values of the 2 polypeptides are in the range of subunits I and II of the eukaryotic enzyme. Cytochrome *c* oxidase from the thermophilic bacterium PS3 showed only 1 protein band after SDS gel electrophoresis [71]. But it was suggested to consist also of 2 different polypeptides of equal apparent M_r [72].

6. Location of redox centers and the proton channel

The location of the 2 heme *a* groups and 2 copper atoms within the enzyme complex has been the subject of many investigations (see table 1 in [34]). All of the '7 subunits' have been claimed to bind in the native complex copper or heme *a*. Subunit V has been isolated as a dark green heme *a*–protein complex and denoted 'heme binding subunit' [73]. Its amino acid sequence was obtained and discussed on the assumed function to bind heme *a* [62]. Since a redistribution between subunits of non-covalently bound heme *a* and copper during dissociation of the complex cannot be excluded, binding to isolated subunits does not prove their physiological location.

The following arguments, based on more recent data, suggest the location of the 2 copper atoms in subunit II and the 2 heme *a* in subunits I and II:

1. Subunit II shows amino acid sequence homology to the copper proteins azurin, plastocyanin and stellacyanin [59].
2. Subunit II is assumed to contain heme *a* since cytochrome *c* binds specifically to this subunit:
   (i) Dithiobis(succinimidylpropionate) crosslinked cytochrome *c* with subunit II when reacted with the cytochrome *c*–cytochrome *c* oxidase complex [74];
   (ii) Arylazido-derivatives of cytochrome *c* reacted with subunit II of beef heart [75,76] and yeast [77] cytochrome *c* oxidase.
3. Gel electrophoresis of beef heart cytochrome *c* oxidase at low temperatures and low ionic strength in the presence of a mixture of SDS and Triton X-100, which minimized redistribution of heme *a* and copper, showed all copper bound to subunit II and heme *a* distributed between subunits I and II in equal amounts [34].
4. Cytochrome *c* oxidase isolated from prokaryotes shows only 2 subunits with apparent M_r-values similar to subunits I and II of eukaryotic cells [68–70].

The translocation of protons through the enzyme complex is suggested to occur via subunit III. This is concluded from several observations:

1. Beef heart cytochrome *c* oxidase depleted of subunit III by alkaline treatment, has an unchanged or even stimulated respiratory activity [29] but fails to pump protons [78].
2. *N,N'*-Dicyclohexylcarbodiimide, a potent inhibitor of oxidative phosphorylation and mitochondrial ATPase [79], which reacts specifically with the proton-translocating proteolipid of mitochondrial, chloroplast and bacterial ATPases [80], was also found to inhibit proton translocation in reconstituted cytochrome *c* oxidase vesicles [8] and to bind specifically to subunit III of the enzyme [32].
3. The amino acid sequence of the *N,N'*-dicyclohexylcarbodiimide-binding region of subunit III resembles that of the ATPase proton-translocating proteolipid [81,82]. It contains a hydrophobic region interrupted by a glutamic acid [83].
4. A proton-translocating activity could not be shown with the isolated, in liposomes reconstituted 2-subunit cytochrome *c* oxidase from *P. denitrificans* [84]. Since in the intact bacterium a proton efflux coupled to electron transport via cytochrome *c* oxidase could be demonstrated [85], it was postulated by Ludwig [72] that during isolation a third protein subunit, involved in proton pumping activity, may have been lost.

7. Site of biosynthesis of subunits

In order to understand the function of the many individual subunits of cytochrome *c* oxidase, it is important to consider their dual site of synthesis in eukaryotic cells.

The 3 large subunits are coded for and synthesized on mitochondrial DNA and ribosomes, respectively. The various smaller subunits are coded for on nuclear DNA and synthesized on cytoplasmic ribosomes. This result, originally obtained for the enzyme from yeast and *N. crassa* [22], was corroborated for cytochrome *c* oxidase from oocytes of *Xenopus laevis* [86], embryonic bovine trachea cell cultures [87] and rat liver [88–90].

Mapping of the mitochondrial genome of yeast has unequivocally established the location of the structural genes for subunits I–III on mitochondrial DNA [91–93]. In addition, the mitochondrial genes for subunits I–III have been sequenced by Tzagoloff and his group [94–96], that for yeast subunit II also by Fox [97] and that for human placenta subunit II by Barrell et al. [98]. The predicted amino acid sequence of the yeast subunit II protein shows 49%, that of the human placenta subunit II protein 72% homology with the amino acid sequence of the bovine heart protein [59].

The synthesis and the location of structural genes of some protein components of respiratory enzyme complexes in mitochondria (cytochrome *b*, cytochrome *c* oxidase, ATPase), was taken as an argument for the endosymbiotic theory of the evolutionary origin of the eukaryotic cell [99–103]. Because of its large similarities to mitochondria, the bacterium *P. denitrificans* has been supposed to represent a descendant of an aerobic prokaryote which entered into an endocellular symbiosis with prokaryotes [103]. The evolutionary relationship between *P. denitrificans* and mitochondria is in particular evident from the amino acid sequence similarity of their cytochrome *c*, being much closer than those between *P. denitrificans* and many other bacteria [104].

8. Postulated function of extramitochondrially synthesized subunits

From the above considerations it may be assumed that cytochrome *c* oxidase requires only 3 subunits (I–III) for its function. In addition regulation of respiratory activity and ATP-synthesis was shown to be exerted through cytochrome *c* oxidase by the same mechanism in eukaryotes and prokaryotes as well. Wilson and coworkers found that the respiratory chain between NADH and cytochrome *c* is at near equilibrium in eukaryotes [106–108] as well as in *P. denitrificans* [105] and suggested that regulation of the respiratory rate occurs through the third phosphorylation site, the cytochrome *c* oxidase–oxygen reaction. Application of a model, which contains only 3 variables, the ratios $[NAD^+]/[NADH]$ and $[ATP]/[ADP]\,[P_i]$ and the cytochrome *c* (or cytochrome *c* oxidase) turnover number [109], gave a good mathematical fit [110] with measured data from perfused heart, isolated liver cells, *Tetrahymena pyriformis*, cultured kidney cells and *P. denitrificans* as well.

The above described metabolic regulation does not require regulatory protein components of the oxidase. We therefore postulate that during evolution of eukaryotes further specific regulatory mechanisms have been introduced at the third coupling site of the respiratory chain by addition of 'regulatory' proteins to the 'catalytically' subunits I–III of cytochrome *c* oxidase. These additional regulatory mechanisms, assumed to be tissue-specific, could concern:

(i) The binding affinity of cytochrome *c* to cytochrome *c* oxidase. This is suggested from the variability of cytochrome *c*/*a* ratios in different tissues ([56] and table in [111]), the occurrence of 2 different binding sites for cytochrome *c* at the oxidase [112,113], the low affinity site being suggested to bypass electrons from sulfite via cytochrome *c* [114,115], and from extramitochondrial NADH via cytochrome b_5 and *c* [116] to the oxidase; and finally from the different kinetic data we have obtained for cytochrome *c* oxidases from beef liver and heart [56,117].

(ii) A variable stoichiometry of H^+/e^--ratios in cytochrome *c* oxidase. This is suggested from the easy 'uncoupling' of proton pumping activity from electron transport after alkaline treatment of the oxidase [57] which removes subunit III [29] and after DCCD treatment [32]; from the lack of proton pumping activity of the 2-subunit enzyme from *P. denitrificans* [84], and from the variable H^+/e^- stoichiometry measured with reconstituted cytochrome *c* oxidase [10]. The physiological basis of variable H^+/e^- ratios could be:

(i) Regulation of the various energy-dependent reactions occurring simultaneously at the inner

mitochondrial membrane: $\Delta\Psi$-dependent reactions (ATP/ADP-exchange [118,119], Ca^{2+}-uptake [120], electrogenic phosphate efflux [121,122]), ΔpH-dependent reactions (phosphate, pyruvate, glutamate, ornithine and tricarboxylate transport (review [123]) and $\Delta\mu H^+$-dependent reactions (ATP-synthase [124] and NADH/NADPH-transhydrogenase [125]);

(ii) Regulation of heat production without the involvement of the uncoupling protein [126].

Finally, it may be pointed out that also for other mitochondrial complexes like cytochrome bc_1 [127, 128] and ATP synthase [129], more protein components were found in higher evolved organisms than in lower eukaryotes or prokaryotes. Similar observations were made for enzyme complexes not involved in energy metabolism like RNA polymerase [130,131]. Thus it seems that evolution of higher animals was not restricted to neuronal cells and tissues, but included also basic reactions such as energy metabolism and translation. Its chemical basis seems connected with additional regulatory mechanisms brought about by additional proteins. Highly complex systems, characterized by a large number of regulatory proteins and tissue specificity, have in fact been demonstrated for glycogen synthesis and degradation [132] and for muscle contraction [133].

Acknowledgements

We thank Professor R. Thauer for fruitful discussions and Professors Wikström and Schatz for sending us samples of their cytochrome *c* oxidases. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 103, A2).

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