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Bovine cytochrome *c* oxidases, purified from heart, skeletal muscle, liver and kidney, differ in the small subunits but show the same reaction kinetics with cytochrome *c*

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(1) Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate of purified cytochrome *c* oxidase preparations revealed that bovine kidney, skeletal muscle and heart contain different cytochrome *c* oxidase isoenzymes, which show differences in mobility of the subunits encoded by the nuclear genome. No differences in subunit pattern were observed between the oxidase preparations isolated from kidney and liver. (2) The kinetics of the steady-state reactions between bovine ferrocytochrome *c* and the four types of bovine cytochrome *c* oxidase preparation were compared under conditions of both high- and low-ionic strength. Also the pre-steady-state kinetics were studied. Only minor differences were observed in the electron-transfer activity of the isoenzymes. Thus, our experiments do not support the notion that the subunits encoded by the nuclear genome act as modulators conferring different activities to the isoenzymes of cytochrome *c* oxidase. (3) The cytochrome *c* oxidase preparation from bovine skeletal muscle was found to consist mainly of dimers, whereas the enzymes isolated from bovine kidney, liver and heart were monomeric.

Introduction

Cytochrome *c* oxidase (EC 1.9.3.1) is a multisubunit protein that catalyses the electron transfer from ferrocytochrome *c* to dioxygen and the concomitant translocation of protons across the mitochondrial inner membrane.

Mammalian cytochrome *c* oxidase consists of 13 different polypeptides [1], which are probably present in stoichiometric amounts [2–4].

The three largest subunits (I–III) are coded for by the mitochondrial genome and are generally believed to be involved in electron and proton transfer. Subunits I and II are supposed to contain all four prosthetic groups [5–7], whereas subunit III has been considered to play a role in proton translocation [8–10].

The ten smaller subunits are coded for by the nuclear genome. As yet, no specific function could be ascribed to these polypeptides. However, an important observation concerning these subunits was reported by Kadenbach and coworkers

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Abbreviations: TMPD, tetramethyl-*p*-phenylenediamine; HPLC, high-performance liquid chromatography; TN_{max}, maximal turnover numbers.

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[11–13], who found differences in apparent molecular mass of some of the small subunits of cytochrome *c* oxidase isolated from various organs of one mammalian species. On the basis of this finding they proposed that organ-specific isoenzyme forms of the oxidase exist, differing in one or more of the 10 small polypeptides. Considerable evidence now supports this hypothesis: tissue-specific isoenzymes of mammalian cytochrome *c* oxidase have been detected by N-terminal sequencing [14], immunological studies [1,15,16] and by investigation of the reactivity of cysteine and carboxylic groups of the small subunits [17,18]. An evaluation of these papers shows that at the moment organ specificity has been demonstrated for nearly all cytoplasmically synthesised subunits. Little is known about the number of isoforms per subunit. From immunological studies it appears that at least three different forms exist for most of the subunits investigated [16].

With the discovery of the natural occurrence of multiple types of most of the ten subunits coded for by the nuclear genome, a new tool has become available for the study of the function of these polypeptides. Kadenbach and coworkers [12] proposed that the small subunits act as organ-linked modulators of enzyme activity. A close comparison of the kinetic constants of the reactions between cytochrome *c* and various cytochrome *c* oxidase isoenzymes of one mammalian species might therefore reveal whether or not the small subunits are involved in the regulation of the electron-transfer reactions. However, the results published on this subject so far have been contradictory. Kadenbach et al. [12,13] compared the steady-state kinetics of cytochrome *c* oxidase from bovine liver and heart in a medium of low ionic strength, in which the enzyme is known to show biphasic kinetics [19]. Both for membrane bound and for partially or fully purified enzymes in the presence of deoxycholate, higher values were found for the high- and low-affinity TN_{max} (maximal turnover numbers) of the liver enzyme than for the heart isoenzyme. Differences were also observed in K_m values of the two isoenzymes. On the other hand, no differences were found in the steady-state kinetics of various porcine cytochrome *c* oxidase isoenzymes in the presence of laurylmaltoside [20]. Unfortunately, in all these experiments the reaction rates were measured polarographically. As pointed out by Ferguson et al. [21], in this system (in which ascorbic acid and tetramethyl-*p*-phenylenediamine (TMPD) are present), other electron-transfer reactions than the ones between cytochrome *c*, cytochrome *c* oxidase and oxygen may become rate-limiting.

In order to investigate the involvement of the small subunits of cytochrome *c* oxidase in the electron-transfer reactions of the enzyme we compared the kinetic constants of the reactions between cytochrome *c* and various bovine isoenzymes using the more reliable spectrophotometric activity assay. The steady-state reactions were studied at low and at high ionic strength. Under the latter, more physiological conditions the enzyme shows simple Michaelis-Menten kinetics [22].

Furthermore, the pre-steady-state reaction of the isoenzymes was investigated. Using the stopped-flow method, the rate constants were calculated for the association reactions of ferrocytochrome *c* with the various oxidase isoenzymes.

Our studies revealed only minor differences in the electron-transfer activity of the isoenzymes, suggesting that the small subunits of cytochrome *c* oxidase do not function as modulators of the electron-transfer reactions of the enzyme.

Materials and Methods

Isolation of cytochrome c oxidase from various bovine organs. Cytochrome *c* oxidase was purified using either acid-precipitated submitochondrial particles [23] from heart or skeletal muscle, or mitochondria from liver or kidney as starting material.

Mitochondria were prepared by homogenising cleaned liver or kidney cortex in a Waring blender in 20 mM Tris-HCl (pH 7.3)/2 mM EDTA/0.25 M sucrose for about 20 s at speed set III (1 kg material in 5 l buffer). After centrifugation (3000 $\times g$ for 30 min) of the blended material the supernatant was further centrifuged at 30000 $\times g$ for 8 min. The supernatant and the fluffy top layer of the pellets were discarded and the firm pellets were collected and washed three times with isotonic buffer. Finally, the mitochondria were collected in 50 mM Tris-SO_4 (pH 8.0)/0.66 M sucrose and stored in liquid nitrogen.

Cytochrome *c* oxidase was isolated according

to the method of Fowler et al. [24] as modified in our laboratory [23]. Spectra taken of the purified enzymes showed the absence of other cytochrome contaminants. For determination of the concentration of cytochrome *c* oxidase a molar absorbance coefficient (reduced minus oxidised) of 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm was used [25].

Polyacrylamide gel electrophoresis. Gel electrophoresis was performed according to a method based on that of Kadenbach et al. [26] as described before [27].

HPLC size exclusion chromatography. Oxidase preparations were tested for the presence of dimers and larger aggregates of cytochrome *c* oxidase by HPLC chromatography using a method developed in our laboratory [28]. Samples (30 μl) equilibrated with laurylmaltoside were applied on to a Dupont GF-250 column and eluted at 1 $ml \cdot min^{-1}$ in 100 mM potassium phosphate (pH 7.4)/1 mM EDTA/0.1% laurylmaltoside. Protein and haem were detected at 280 and 405 nm, respectively.

Spectrophotometric measurement of cytochrome c oxidase activity in the steady state. Cytochrome *c* from bovine heart was prepared by the method of Margoliash and Walasek [29]. Ferrocytochrome *c* was obtained by incubating cytochrome *c* with ascorbate followed by gel filtration in 10 mM potassium phosphate (pH 7.4)/1 mM EDTA.

Concentrations were determined using an absorbance coefficient (reduced minus oxidised) at 550 nm of 21.1 $mM^{-1} \cdot cm^{-1}$ [30].

The steady-state kinetics of the reaction between ferrocytochrome *c* and cytochrome *c* oxidase from various bovine organs were studied under conditions of both high- and low-ionic strength at 20 °C. In order to equilibrate the cytochrome *c* oxidase preparations with laurylmaltoside, 16 h prior to each experiment the enzyme was transferred to a medium containing 100 mM potassium phosphate (pH 7.4)/1 mM EDTA/0.5% laurylmaltoside and stored at 0 °C [22].

(A) Low ionic strength. Steady-state activity of the various cytochrome *c* oxidase preparations was determined in 10 mM potassium phosphate (pH 7.4)/1 mM EDTA/0.05% laurylmaltoside ($I = 27$ mM). Ferrocytochrome *c* was added to a concentration of 0.12–60 μM, cytochrome *c* oxidase to 1 nM. Reaction rates were measured using a modified Cary-14 spectrophotometer and a Durrum stopped-flow apparatus for the high and the low cytochrome *c* concentrations, respectively [22]. K_m and TN_{max} values of high- and low-affinity reactions were determined by computer analysis of the data [31].

(B) High ionic strength. The rate of oxidation of ferrocytochrome *c* was measured with our modified Cary-14 spectrophotometer [22] at 550 nm or 520 nm in 100 mM potassium phosphate (pH 7.4)/1 mM EDTA/0.05% laurylmaltoside ($I =$ 226 mM). Reduced cytochrome *c* was added to a final concentration of 5.1–51.3 μM; the concentration of cytochrome *c* oxidase was 1 nM. K_m and TN_{max} values were determined directly from the straight-line Eady-Hofstee plots obtained under these conditions [22].

Pre-steady-state kinetic measurements of cytochrome c oxidase activity. The pre-steady-state reaction of ferrocytochrome *c* with cytochrome *x* oxidase from various bovine organs was studied by following the initial reduction of the oxidase in 100 mM potassium phosphate (pH 7.4)/1 mM EDTA/0.05% laurylmaltoside (20 °C) using a Union-Giken RA-401 spectrophotometer. Cytochrome *c* oxidase was equilibrated with laurylmaltoside as described for the steady-state measurements. The final oxidase concentration was 1 μM, that of ferrocytochrome *c* 4–10 μM. Pseudo first-order rate constants (k_{obs}) were calculated after accumulation of 4–6 traces, using the program of the apparatus that fits the traces to an exponential function.

Results

The aggregation state of cytochrome c oxidase isolated from various bovine organs

As shown by HPLC size exclusion chromatography [28] and sucrose gradient centrifugation [27], our Fowler-type cytochrome *c* oxidase preparation from bovine heart is monomeric in the presence of the detergent laurylmaltoside. In order to assess the aggregation state of the cytochrome *c* oxidases we isolated from bovine kidney, liver and muscle, all preparations were subjected to chromatography on a HPLC size-exclusion column. Prior to these runs, the elution positions of monomeric and dimeric cytochrome *c* oxidase were determined using a bovine heart cytochrome *c*

oxidase preparation that had been partly dimerised through prolonged turnover of the enzyme [28] (not shown). Fig. 1 depicts the elution profile obtained for the cytochrome *c* oxidase preparations isolated from the four organs. The peak position found for kidney cytochrome *c* oxidase (A) is identical to that of the heart enzyme (B), demonstrating that also the kidney oxidase is fully monomeric after equilibration with laurylmaltoside. Some dimers are present in the liver preparation (C). Finally, the skeletal muscle enzyme (D) was found to be largely dimeric.

If larger aggregates of the enzyme would be present, they might not enter the column and would therefore not be detected. Therefore, we determine the recovery by injection of samples in the HPLC system from which the column had been removed. The detector peak areas thus obtained were identical to those of the samples injected on top of the column (not shown). We conclude that large aggregates did not occur in our oxidase preparations.

Since the absorptions both at 280 and at 405 nm were recorded, the HPLC chromatography also yielded information on the purity of the four oxidase preparations. The ratios of the absorbance at 280 and 405 nm in the monomer peaks of the heart and skeletal muscle preparation were the same (4.0). For the kidney and liver enzymes 10% higher values were obtained, which would indicate that these preparations were less pure.

SDS-polyacrylamide electrophoresis

Fig. 2 shows the subunit patterns obtained by gel electrophoresis of bovine kidney, liver, muscle and heart cytochrome *c* oxidase. Apart from the above-mentioned low A_{405}/A_{280} ratio on HPLC chromatography, the relatively lower purity of kidney and liver oxidase is also visible by the presence of additional bands in the high molecular mass region of the gel. In kidney cytochrome *c* oxidase also additional bands were observed between subunits IV and Va. Since subunit IV has been demonstrated to be extremely sensitive to proteolysis [32], these bands probably represent breakdown products of this polypeptide.

A comparison of the subunit pattern of our four bovine oxidase preparations confirms the observation [11–13] of organ-specific differences in the mobility of the small cytochrome *c* oxidase

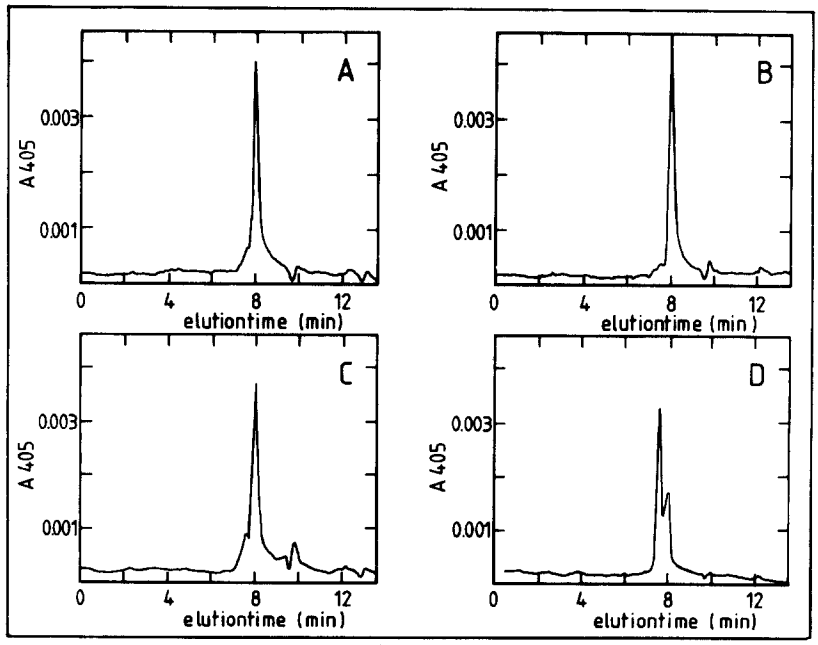

Fig. 1. Elution profile on a Dupont GF-250 column of cytochrome *c* oxidase purified from bovine kidney (A), heart (B), liver (C) and skeletal muscle (D).

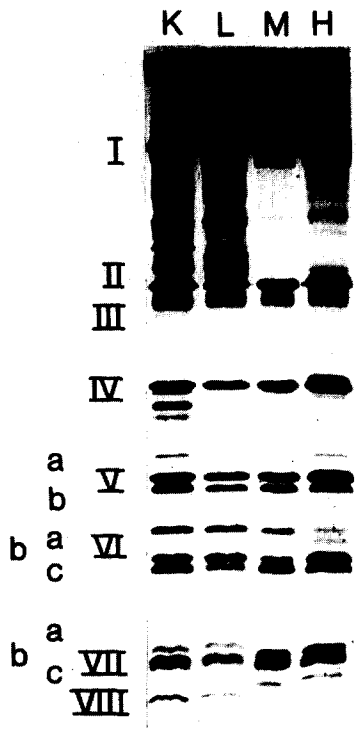

Fig. 2. Comparison of the subunit patterns of cytochrome *c* oxidase purified from bovine kidney (K), liver (L), skeletal muscle (M) and heart (H). Gel electrophoresis was performed by the method of Kadenbach et al. [26]. The subunit nomenclature of Kadenbach [14] is used.

subunits coded for the nuclear genome. An inspection of the subunit VI region reveals that, as mentioned previously [27], the heart enzyme shows four instead of three bands. In the other preparations the additional polypeptide was not observed. The extra band we observe may originate from proteolysis. However, it has been reported [33], that porcine liver and kidney subunits VIa consist of two different proteins. Therefore, we speculate that the additional subunit VI band observed in bovine heart oxidase is a true constituent of the enzyme, which is only visible at high electrophoretic resolution. The apparent absence of this polypeptide in other oxidase preparations suggests that, just as in porcine liver and kidney [33], it comigrates with one of the other subunit VI bands.

Between the kidney and liver enzymes on the one hand and those of heart and skeletal muscle on the other, clear differences were observed in the mobility of subunit VIII. Furthermore, minor differences in the positions of subunits VIa and VIIa were detectable. These results are in accordance with differences reported in electrophoretic mobility and N-terminal sequence of cytochrome *c* oxidase subunits VIa, VIIa and VIII from bovine heart and liver [12–14,26].

Summarising, we conclude that differences in polypeptide composition are observed between cytochrome *c* oxidase from bovine kidney, muscle and heart. Hence, these three proteins may be referred to as isoenzymes. Our results did not reveal differences in subunit pattern of the oxidase preparations isolated from kidney and liver. Since differences may exist that do not show up on SDS-polyacrylamide gel electrophoresis, it cannot yet be concluded that these two organs contain identical cytochrome *c* oxidase isoenzymes.

Steady-state kinetics

The steady-state kinetics of the electron-transfer reactions between bovine-heart ferrocytochrome *c* and the bovine cytochrome *c* oxidase

TABLE I

KINETIC PARAMETERS OF BOVINE CYTOCHROME *c* OXIDASE ISOENZYMES

Isoenzyme	Steady state						Pre-steady state
	I = 27 mM				I = 226 mM		I = 226 mM
	high affinity		low affinity				
	K_m (μM)	TN_{max} (s^{-1})	K_m (μM)	TN_{max} (s^{-1})	K_m (μM)	TN_{max} (s^{-1})	$k_1 \cdot 10^{-7}$ ($M^{-1} \cdot S^{-1}$)
Kidney	0.05	40	12	265	17	350	1.0
Liver	0.07	50	8	281	22	364	0.7
Heart	0.05	36	15	280	23	386	1.1
Muscle	0.05	39	11	258	25	328	0.7

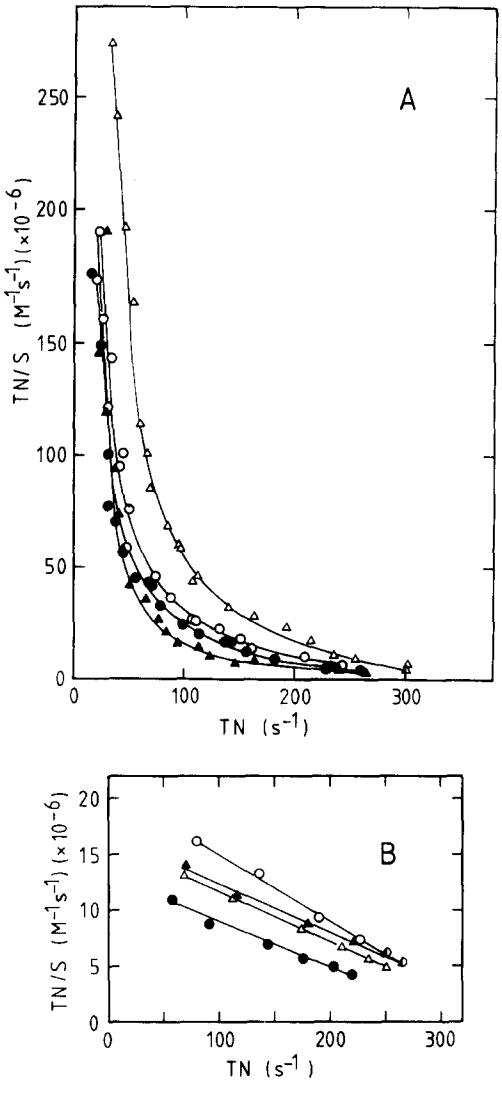

Fig. 3. Eadie-Hofstee plots of steady-state oxidation of bovine heart ferrocytochrome *c* by bovine cytochrome *c* oxidase isolated from: O——O, kidney; △——△, liver; ▲——▲, heart; ●——●, skeletal muscle. Temperature 20 °C. (A) Spectrophotometric measurement in 10 mM potassium phosphate (pH 7.4)/1 mM EDTA/0.05% laurylmaltoside ($I = 27$ mM). Final concentration of cytochrome *c* oxidase was 1 nM; that of ferrocytochrome *c* 0.12–60 μM. (B) Spectrophotometric measurement in 100 mM potassium phosphate (pH 7.4)/1 mM EDTA/0.05% laurylmaltoside ($I = 226$ mM). The final concentrations of cytochrome *c* oxidase and ferrocytochrome *c* were 1 nM and 5.1–51.3 μM, respectively.

isoenzymes were studied spectrophotometrically. Fig. 3A shows the steady-state reactions of the cytochrome *c* oxidase isoenzymes when studied in a medium of low ionic strength ($I = 27$ mM). Under these conditions biphasic Eadie-Hofstee plots are obtained, demonstrating that the reaction is not first-degree in substrate [34]. Table I shows the high- and low-affinity K_m and TN_{max} values calculated from the four curves. A comparison of the values obtained for the various oxidase preparations shows only minor differences in TN_{max} values, whereas the differences in K_m values do not exceed a factor of 2. Since the same variation in data was obtained when the kinetics were studied of various cytochrome *c* oxidase preparations from bovine heart (not shown), we feel the observed differences are not significant.

In media of high ionic strength, straight-line Eadie-Hofstee plots are obtained [22,34]. In previous studies we have demonstrated that the reaction observed under these conditions is related to the high-affinity reaction at low ionic strength [22]. Fig. 3B shows the steady-state kinetics of the bovine cytochrome *c* oxidase isoenzymes when studied at $I = 226$ mM. Again no significant differences were observed in the kinetic parameters calculated for the various enzyme preparations (Table I), which would indicate that the difference in polypeptide composition of the various isoenzymes does not affect the steady-state kinetics of cytochrome *c* oxidase.

Pre-steady-state kinetics

Pre-steady-state kinetic measurements were performed by monitoring the initial reduction of cytochrome *c* oxidase by ferrocytochrome *c* at 444 nm, using the stopped-flow technique. Cytochrome *c* was added in excess over cytochrome *c* oxidase, resulting in pseudo-first-order reaction traces, the pseudo-first-order rate constants (k_{obs}) of which were directly proportional to the cytochrome *c* concentration. The second-order rate constant (k_1) of the association of ferrocytochrome *c* to cytochrome *c* oxidase was calculated from the slope of the line obtained by plotting k_{obs} vs. the concentration of ferrocytochrome *c*.

The k_1 values thus determined for the four bovine cytochrome *c* oxidase preparations are summarised in Table I. Again, only minor differences were observed in the rate constants, just as was found for the steady-state kinetic parameters.

Discussion

We investigated the involvement of the ten small subunits of cytochrome *c* oxidase in the electron-transfer reaction catalysed by the enzyme, comparing the kinetics of the reactions of cytochrome *c* with cytochrome *c* oxidase from bovine kidney, liver, skeletal muscle and heart, respectively. The rationale for these studies lay in the observation by Kadenbach et al. [11–13] that organ-specific differences occur in these small subunits of cytochrome *c* oxidase. SDS-polyacrylamide gel electrophoresis of our four bovine oxidase preparations confirmed the existence of organ specific isoenzyme forms of cytochrome *c* oxidase.

Our steady-state kinetic measurements were performed spectrophotometrically, since this system yields unambiguous information about the kinetic parameters of the enzyme-catalysed reaction, in contrast to the polarographic method where the presence of ascorbic acid and TMPD induces artificial side reactions that may become rate-limiting [21,35].

We found a good correspondence in high- and low affinity K_m and TN_{max} values determined for the four bovine cytochrome *c* oxidase preparations in a medium of low ionic strength containing laurylmaltoside (Table I). These results contrast with the reports of the group of Kadenbach [12,13], who found clear differences in steady-state kinetic parameters determined for cytochrome *c* oxidase from bovine liver an heart, respectively. However, not only were their measurements performed polarographically, but they also used 0.04–0.08% (w/v) of the detergent deoxycholate to keep the enzymes in solution during measurements. Such reaction conditions are far from favourable, since deoxycholate is present in a concentration below the critical micelle concentration and, as found in recent experiments [41], cytochrome *c* oxidase then self-associates into aggregates. In experiments performed in the presence of saturating amounts of laurylmaltoside (a condition under which in our experiments no aggregation of the oxidase occurred), Kadenbach et al. [20] found no differences in the kinetics of various porcine isoenzymes. Finally, bovine heart and liver cytochrome *c* oxidase reconstituted in phospholipid vesicles were reported to show kinetic differences [37]. The significance of this observation is, however, not clear, since in photometric measurements performed at low ionic strength no biphasic kinetics was observed. Biphasic kinetics was found in the polarographic assay but only when TMPD was omitted. Under these conditions, however, the rate of reduction of cytochrome *c* by ascorbic acid may become rate-limiting [21].

Since conditions of high ionic strength give a closer approximation of the situation in vivo, we also compared the steady-state kinetics of our cytochrome *c* oxidase isoenzymes at high ionic strength. Again, hardly any differences were observed in kinetic parameters calculated for the four cytochrome *c* oxidation preparations (Table I). Finally, also in our pre-steady-state kinetic determination of the association rate constant of ferrocytochrome *c* and cytochrome *c* oxidase, no significant differences were found between the isoenzymes.

Summarising, we conclude that in our studies differences in the small subunits of cytochrome *c* oxidase did not cause differences in electron-transfer activity. Consequently, a function of the small subunits as direct modulators of the electron-transfer activity of the oxidase seems unlikely. However, several conceivable possibilities remain. The small subunits (coded for by the nuclear genome) may be involved in proton translocation, in binding of ligands that affect the catalytic activity of the enzyme, or in coupling of electron and proton transfer. Furthermore, Buse and Steffens [38] suggested that these small subunits might have a structural role, perhaps by 'anchoring' the enzyme to the membrane. Organ-linked variation in the small subunits could then be an adaptation to differences in phospholipid composition, which were reported for mitochondria isolated from several mammalian tissues [13].

In contrast to our kinetic data we did observe differences in aggregation state between the bovine isoenzymes (Fig. 1). Our cytochrome *c* oxidase from skeletal muscle was found to be largely dimeric, whereas the other three oxidase preparations contained almost exclusively the monomeric enzyme.

The variation in tendency of cytochrome *c*

oxidase to form dimers in the presence of laurylmaltoside is known. Under identical conditions both monomers [28] and dimers [39,40] of the enzyme from bovine heart have been found. We speculate that this discrepancy is caused by differences in the amount of phospholipid in the preparations resulting from the fact that different isolation procedures were used. Although our four bovine oxidase preparations were all isolated by the same Fowler procedure [23,24], differences in phospholipid composition of the mitochondrial inner membranes of the various organs might result in differences in the composition or amount of phospholipids in the final preparations.

Acknowledgements

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