ELSEVIER

Biochimica et Biophysica Acta 1371 (1998) 71–82

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Variations in the subunit content and catalytic activity of the cytochrome *c* oxidase complex from different tissues and different cardiac compartments

C. Vijayasarathy [a], Ida Biunno [a,1], Nibedita Lenka [a], Ming Yang [b], Aruna Basu [a,2], Ian P. Hall [a,3], Narayan G. Avadhani [a,*]

[a] *Laboratory of Biochemistry, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA*

[b] *Laboratory of Anatomy, Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA*

Received 15 August 1997; accepted 1 December 1997

Abstract

The composition and activity of cytochrome *c* oxidase (COX) was studied in mitochondria from rat liver, brain, kidney and heart and also in different compartments of the bovine heart to see whether any correlation exists between known oxidative capacity and COX activity. Immunoblot analysis showed that the levels of ubiquitously expressed subunits IV and Vb are about 8–12-fold lower in liver mitochondria as compared to the heart, kidney and brain. The heart enzyme with higher abundance of COX IV and Vb showed lower turnover number (495) while the liver enzyme with lower abundance of these subunits exhibited higher turnover number of 750. In support of the immunoblot results, immunohistochemical analysis of heart and kidney tissue sections showed an intense staining with the COX Vb antibody as compared to the liver sections. COX Vb antibody stained certain tubular regions of the kidney more intensely than the other regions suggesting region specific variation in the subunit level. Bovine heart compartments showed variation in subunit levels and also differed in the kinetic parameters of COX. The right atrium contained relatively more Vb protein, while the left ventricle contained higher level of subunit VIa. COX from both the ventricles showed high K_m for cytochrome *c* (23–37 μM) as compared to the atrial COX (K_m 8–15 μM). These results suggest a correlation between tissue specific oxidative capacity/work load and changes in subunit composition and associated changes in the activity of COX complex. More important, our results suggest variations based on the oxidative load of cell types within a tissue. © 1998 Elsevier Science B.V.

Keywords: Cytochrome *c* oxidase; Subunit content; Kinetic property; Kidney; Heart; Mitochondrion

Abbreviations: COX, cytochrome *c* oxidase; SMP, submitochondrial particles; TN, turnovernumber$_{max}$ [s^{-1}]

* Corresponding author. Fax: +1-215-898-9923; E-mail: narayan@vet.upenn.edu

[1] Present address: Consiglio Nazionale delle Ricerche, I.T.B.A., Via Ampere, 56, Milan (MI), Italy.

[2] Present address: Kimmel Cancer Center, Jefferson Medical College, Philadelphia, PA 19104, USA.

[3] Present address: Department of Medicine, Division of Therapeutics, University Hospital, Queen's Medical Centre, Nottingham, NG7 2UH, UK.

PII S0005-2736(97)00278-2

1. Introduction

Cytochrome *c* oxidase (COX, EC 1.9.3.1) is the terminal component of the respiratory chain that catalyzes the reduction of dioxygen to water from the electrons derived from ferrocytochrome *c*. COX is one of the three centers that create a proton gradient as an intermediate step in the conversion of redox energy to ATP. In mammals the enzyme is composed of 13 subunits and its biosynthesis involves a coordinated interplay between the nuclear and mitochondrial genomes. The largest subunits (I, II and III), which represent the catalytic core of the enzyme complex are encoded by the mitochondrial DNA and are synthesized within the mitochondrion [1,2]. The rest of the smaller subunits (IV, Va, Vb, VIa,b,c, VIIa, VIIb and VIII) are encoded on the nuclear DNA, synthesized in the cytosol, imported into mitochondria and assembled into the holoenzyme complex [3]. Genetic studies in yeast have elucidated the role of nuclear coded subunits in the assembly of the enzyme complex, as well as in the modulation of enzyme activity [4–6]. Nucleotide and reactive probe binding studies with the mammalian enzyme suggested a regulatory role for the nuclear coded subunits [7,8]. Selective removal of the nuclear encoded subunit VIb from the bovine heart enzyme complex increased the activity of the enzyme suggesting an inhibitory role for this subunit [9]. These and other studies support the essential role of the nuclear encoded subunits in the catalytic activity of the enzyme [10,11].

COX plays a major role in energy production and it is believed to be a major regulatory site that determines the mitochondrial respiration linked oxidative capacity [12]. Regulation of yeast COX by oxygen has been well documented [13,14]. Oxygen regulates the expression of subunit V isoforms in the yeast. The isoform Va is expressed under aerobic ($O_2 > 0.5\ \mu M$) conditions and the isoform Vb is expressed under anaerobic ($O_2 < 0.5\ \mu M$) conditions. The level of COX activity is determined by the number of holoenzyme molecules assembled in the presence or absence of Va or Vb. These isoforms have been shown to affect the TN of holoenzyme by altering the rates of intramolecular electron transfer between heme *a* and the binuclear reaction center [13,14]. However, currently very little is known about the effects of oxygen tension on the structure and activity of the mammalian enzymes.

In previous studies from our laboratory, we observed that though both COX IV and COX Vb are constitutively expressed in all tissues, Vb mRNA was found to be more abundant in the heart and kidney and low in the liver and brain [15]. Additionally COX Vb mRNA was found to be induced during the differentiation of myoblasts into myotubes [15]. Since heart is a tissue with higher mitochondrial abundance and high energy demand as compared to the liver, it would be interesting to see if the subunit composition of COX in these tissues show any difference and if there is any notable change in the COX activity. Previous studies aimed at elucidating the role of nuclear coded subunits in the activity of the mammalian COX enzyme arrived at some what different conclusions mainly because of the different methods of enzyme isolation and assay conditions used [16–18]. We have, therefore, used mitochondrial membrane fragments with a view to maintain the structural and functional integrity of the enzyme complex and investigated tissue specific differences in enzyme kinetics. Rat tissues which differ in energy demand such as, heart, brain, kidney and liver and different compartments of the bovine heart, which differ in the workload and hemodynamic properties were chosen as sources of the enzyme. Our results show marked differences in the catalytic activity and subunit composition of COX in different tissues as well as cardiac compartments.

2. Materials and methods

2.1. Preparation of mitochondria

Rat liver, heart, kidney and brain mitochondria were isolated as described earlier [19] following homogenization in H medium (70 mM sucrose, 220 mM mannitol, 2.5 mM HEPES, pH 7.4 and 2 mM EDTA) and differential centrifugation. The heart was first minced in a polytron before homogenizing in a motor driven Potter–Elvejham homogenizer. Brain mitochondria were isolated following a two step Percoll gradient [20]. Beef hearts were obtained from a slaughter house immediately after the animal was sacrificed and separated into right and left atria and right and left ventricles and the septal wall. The

mitochondria were prepared using a high speed blender in a medium containing 0.25 M sucrose, 0.01 M Tris–HCl (pH 7.8), 1 mM succinate and 0.2 mM EDTA [21]. Submitochondrial particles were prepared according to the method of Pedersen et al. [22]. All operations were performed at 4°C.

2.2. Spectrophotometric measurements of COX activity in the steady state

COX was assayed by the method of Smith [23] wherein the rate of oxidation of ferrocytochrome *c* was measured by following the decrease in absorbency of its α band at 550 nm. Steady state activity assay conditions were similar to that described by Sinjorgo et al. [17]. The 1-ml reaction medium contained either 10 mM (low ionic strength) or 100 mM (high ionic strength) PO_4^{-2} (pH 7.0), 0.05% lauryl maltoside, 1 mM EDTA and 1–2 μg of protein in the form of submitochondrial particles. Ferrocytochrome *c* was added to a concentration of 0.12–80 μM. Reaction rates were measured using Cary-1E spectrophotometer. First order rate constants were calculated from mean values of four to six measurements at each ferrocytochrome *c* concentration. The cytochrome aa_3 content of enzyme preparations were calculated from the difference spectra (dithionate/ascorbate reduced minus ferricyanide oxidized) of mitochondria or submitochondrial particles solubilized in 2% deoxycholate using an absorption coefficient at (605–630 nm) of 16 mM^{-1} cm^{-1} [24]. All spectral measurements and enzyme assays were performed at ambient temperature in phosphate buffer. Protein concentrations were estimated by the method of Lowry et al. [25].

2.3. Electrophoresis of proteins and Western blot analysis

Proteins were subjected to electrophoresis on 12–18% SDS–acrylamide gels as described by Laemmli [26]. The conditions for Western blot analysis and immunodetection of proteins were as described earlier [27]. Specific antibody interactions were tested by probing with polyclonal or monoclonal antibodies and bound secondary antibody was detected and quantitated by incubating the membrane with chemifluorescent vista ECF substrate according to manufacturer's protocol (Amersham Life Sciences, Arlington Heights, IL). Polyclonal antibody against purified mouse COX was developed in rabbit using standard methods. Subunit specific monoclonal antibodies for COX I, Vb, VIa, and VIc were obtained from Molecular Probes, Eugene, OR, and the specificity of each antibody was verified by Western blot analysis of proteins from the rat liver and heart SMP as well as purified COX. Blots were quantified with an Image Scanner STORM (Molecular Dynamics, Sunnyvale, CA).

2.4. Northern blot analysis

Total RNA was extracted from rat tissues and bovine cardiac compartments by solubilization in guanidine thiocyanate followed by phenol extraction [28]. Denatured RNA samples were resolved by electrophoresis on formaldehyde containing agarose gels and transblotted to Nytran nylon membrane and hybridized using standard conditions described in the Schleicher and Schuell laboratory manual. COX subunit specific synthetic oligonucleotides (28–36 bases long) were 5′ end labelled using T4 Polynucleotide Kinase (New England Biolabs, Beverly, MA) and [γ ^{32}P] ATP (Amersham), and used as probes. The RNA loading was monitored by probing the blots with ^{32}P labelled 18S rDNA probe [29]. The northern blots were quantitated by scanning through a Phosphorimager 'Storm' system (Molecular Dynamics).

2.5. Oligonucleotides

The sequences for oligonucleotide probes were as follows.

COX I: 5′-AATGTGTGATATGGTGGAGGGCATCC-3′;
COX IV: 5′-CCTGTTCATCTCGGCGAAGCTCTC-3′;
COX Vb: 5′-ACCAGCTTGTAATGGGTTCCACAG-3′;
COX VIII (H): 5′-AGTGGGTGTTCTGGCTGGCTTGGCAGTGAT-3′.

2.6. Immunohistochemistry

Adult female mice were anesthetized with a mixture of ketamine and xylazine (10:7.5, 1.5 ml/kg, i.p.). All animals were perfused through the left

cardiac ventricle with 20 ml heparinized saline (1 u/ml of heparin in 0.15 M NaCl) followed by 60 ml cold Zambonis fixative (4% paraformaldehyde and 15% saturated picric acid in 0.1 M phosphate buffer). The heart, kidney, liver and brain were postfixed overnight in the same fixative containing 20% sucrose. All four organs were embedded on a single tissue stage with embedding medium (Tissue-TEK, Miles), cut into 8-μm thick sections in a cryostat at -18°C, and mounted on a single glass slide. The tissues were incubated in a drop (200 μl) of monoclonal antibody to COX I (1:50), COX Vb (1:200), COX VIa (1:100) or COX VIc (1:100) at 25°C for 20 h. The primary antibodies were diluted in 0.01 M phosphate buffered saline (PBS) containing 0.3% Triton X-100 and 2% normal donkey serum. Sections incubated in a drop of same buffer without any primary antibody served as controls. All sections were then incubated in 1:100 rhodamine–isothiocyanate conjugated donkey anti-mouse secondary antibody (Jackson Lab, NJ) at 25°C for 3 h. After each step, sections were rinsed with 0.01 M PBS (3×10 min). Finally, the sections were observed under a Leitz fluorescent microscope and photographed.

3. Results

3.1. Immunoblot analysis of COX subunits in different tissues

To minimize the loss of subunits that is inherent in various purification protocols, involving fractionation with different detergents, and/or high salt extraction, we have used submitochondrial particles for the Western immunoblot analysis. Since tissues differ in mitochondrial abundance and hence, in heme aa_3 content, proteins from different tissues were loaded on the gel based on equal heme content, rather than total protein content. Blots containing mitochondrial proteins from rat tissues that have been probed with polyclonal antibody to subunit IV, and monoclonal antibodies to subunits Vb and VIc are shown in Fig. 1A and B. On equal heme basis, the rat liver enzyme contained 8–10-fold lower levels of immunodetectable COX IV and Vb protein as compared to COX from heart, brain and kidney. The levels of these subunits in complexes from heart, brain and kidney

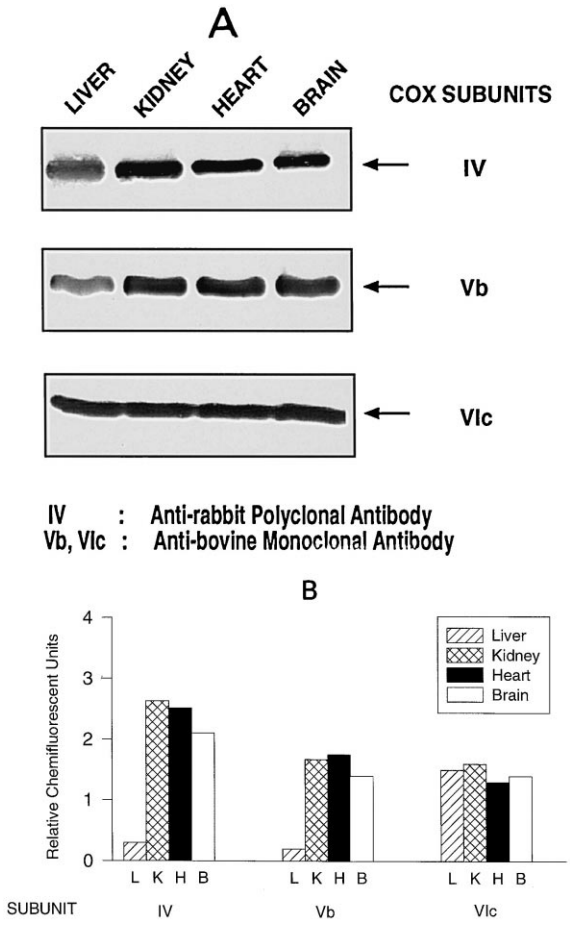

Fig. 1. The immunoblot analysis of mitochondrial proteins from different rat tissues. (A) The blots containing equal amount of COX (100 nmol of heme aa_3) from each tissue was probed with monoclonal anti-bovine antibodies (COX Vb and VIc) or polyclonal anti-rabbit antibody (COXIV) followed by the appropriate alkaline phosphatase conjugated secondary antibodies. The blots were then incubated with chemifluorescent substrate (vistaECF, Amersham) and the signals were detected and quantified with an Image scanner STORM (Molecular Dynamics). (B) Histogram showing the relative abundance of COX subunits by scanning the immunoblot in (A). The results represent an average of two separate experiments which showed less than 10% margin of error.

showed only marginal variations, and may account for a near normal (1:1) stoichiometry. A markedly lower levels of subunit IV and Vb in the liver may reflect substoichiometric levels of these subunits in the liver COX complex. The results also show no significant variation in the levels of the antibody cross-reactive subunit VIc in complexes from all the four tissues tested. Although not shown, the levels of

mitochondrial genome encoded subunits I and II did not vary significantly in all the four tissue samples loaded on the basis of equal heme content.

3.2. Steady state kinetics of COX from different tissues

The role of nuclear coded subunits in COX activity was probed by studying the reaction kinetics of the enzyme from different tissues. In most of the earlier studies, COX was assayed following partial or complete purification of the enzyme from a given source. These studies found that the buffer composition, pH, ionic strength, detergent and lipid composition influenced the COX activity [18]. To circumvent these problems we have used submitochondrial particles prepared by sonication for enzyme activity measurements. The membrane particles were solubilized in the presence of saturating amounts of laurylmaltoside, a non-ionic detergent that has been shown to prevent aggregation of COX [17].

The steady state kinetics of COX measured spectrophotometrically in submitochondrial particles from different tissues are presented in Table 1. In keeping with previous studies [17], spectrophotometric measurements of reaction kinetics in a medium of high ionic strength conditions (100 mM PO_4^{-2}) yielded a straight line Eadie–Hofstee plot (results not shown). In contrast, reaction kinetics in a low ionic strength medium (10 mM PO_4^{-2}) resulted in concave Eadie–Hofstee plots which represented the sum of two Michaelis–Menten rate equations [17,30]. Fig. 2 shows that steady state reactions of the COX from rat liver, heart, brain and kidney SMP when measured in a medium containing low ionic strength ($I = 27$ mM) buffer. Similar biphasic curves for COX activity at low ionic strength were reported previously with bovine heart SMP [30]. A comparison of values for submitochondrial particles from various tissues show differences in K_m and TN values both at low and high ionic strength conditions (Table 1). At high ionic strength (100 mM PO_4^{-2}), which mimics the physiological conditions, the heart enzyme exhibited higher K_m (26 μM) and lower TN (495) when compared to the liver enzyme with a low K_m (8 μM) and high TN (750). Under the same physiological conditions, both the brain and kidney enzymes showed K_m values similar to liver. However, their TN values were 40–50% lower than that of the liver enzyme. Both at low and high ionic conditions, the liver

Table 1
The kinetic parameters of rat cytochrome *c* oxidase isoenzymes

Isoenzyme	Steady state					
	$I = 27$ mM				$I = 226$ mM	
	High affinity		Low affinity			
	K_m (μM)	TN (s^{-1})	K_m (μM)	TN (s^{-1})	K_m (μM)	TN (s^{-1})
Brain	4	55	23	160	8	500
Heart	2	35	13	100	26	495
Kidney	3	80	37	200	9	340
Liver	3	120	22	200	8	750

The steady state reaction rates were determined by photometric assay in 1 ml medium containing either 10 mM PO_4^{2-} (low ionic) or 100 mM PO_4^{2-} (high ionic), 0.05% laurylmaltoside, 1 mM EDTA, 1–2 μg mitochondrial membranes and 0.16–80 μM ferrocytochrome *c*. First order rate constants were calculated from mean values of four to six readings at each ferrocytochrome *c* concentration. At high ionic strength, the K_m and TN values were determined directly from the straight line Eadie–Hofstee plots. The low ionic concave Eadie–Hofstee plot was resolved into two first degree functions and K_m and TN values of low and high affinity reactions were calculated from computer analysis as described earlier by Sinjorgo et al. [30].

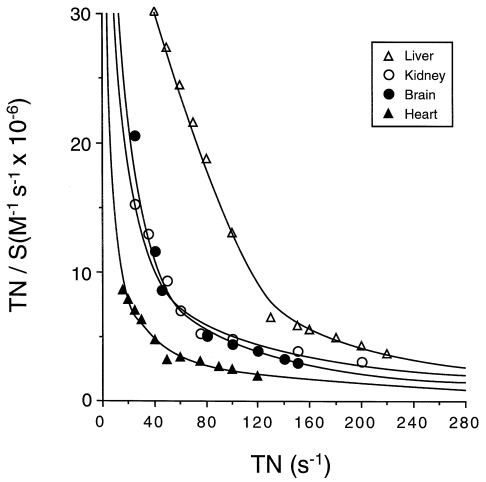

Fig. 2. The Eadie–Hofstee plots of steady state kinetics of COX at low ionic media. Spectrophotometric measurements were carried out in 10 mM PO_4^{2-} (pH 7.0), 0.05% laurylmaltoside, 1 mM EDTA and 0.12–80 μM ferrocytochrome *c*. SMP (1–2 μg) from different rat tissues was used as the source of enzyme. An average of four to six measurements at each concentration of cytochrome *c* was used to calculate the first order rate constants.

enzyme had generally higher TN values than enzyme from other tissues. In contrast, the high and low affinity phase K_m values (2 and 13 μM, respectively) for the heart enzyme at low ionic condition were significantly lower than those of other tissues (see Table 1). The difference in K_m and TN values between the tissues ranged 2–3-fold and probably reflects the tissue specific variation in the microenvironment of the enzyme.

3.3. Immunohistochemistry

The possibility of subunit Vb variation as observed in Fig. 1 was further investigated by immunohistochemical analysis of paraformaldehyde and picric acid fixed tissue sections. As shown in Fig. 3A, antibody to Vb gave a uniform, and relatively light staining of the liver sections. In support of the western blot data in Fig. 1, the heart sections were stained more intensely as compared to the liver (see Fig. 3B), suggesting differences in the abundance of the subunit in these tissues. It is also seen that the staining pattern of the heart sections were non-uniform, with some regions staining more intensely than the other suggesting intra-tissue heterogeneity in the level of COX Vb. Sections from mouse kidney cortex region (Fig. 3C) showed even more dramatic heterogeneous staining with COX Vb antibody. Some of the tubules with characteristic thick walls, were stained more intensely as compared to tubular structures with thin

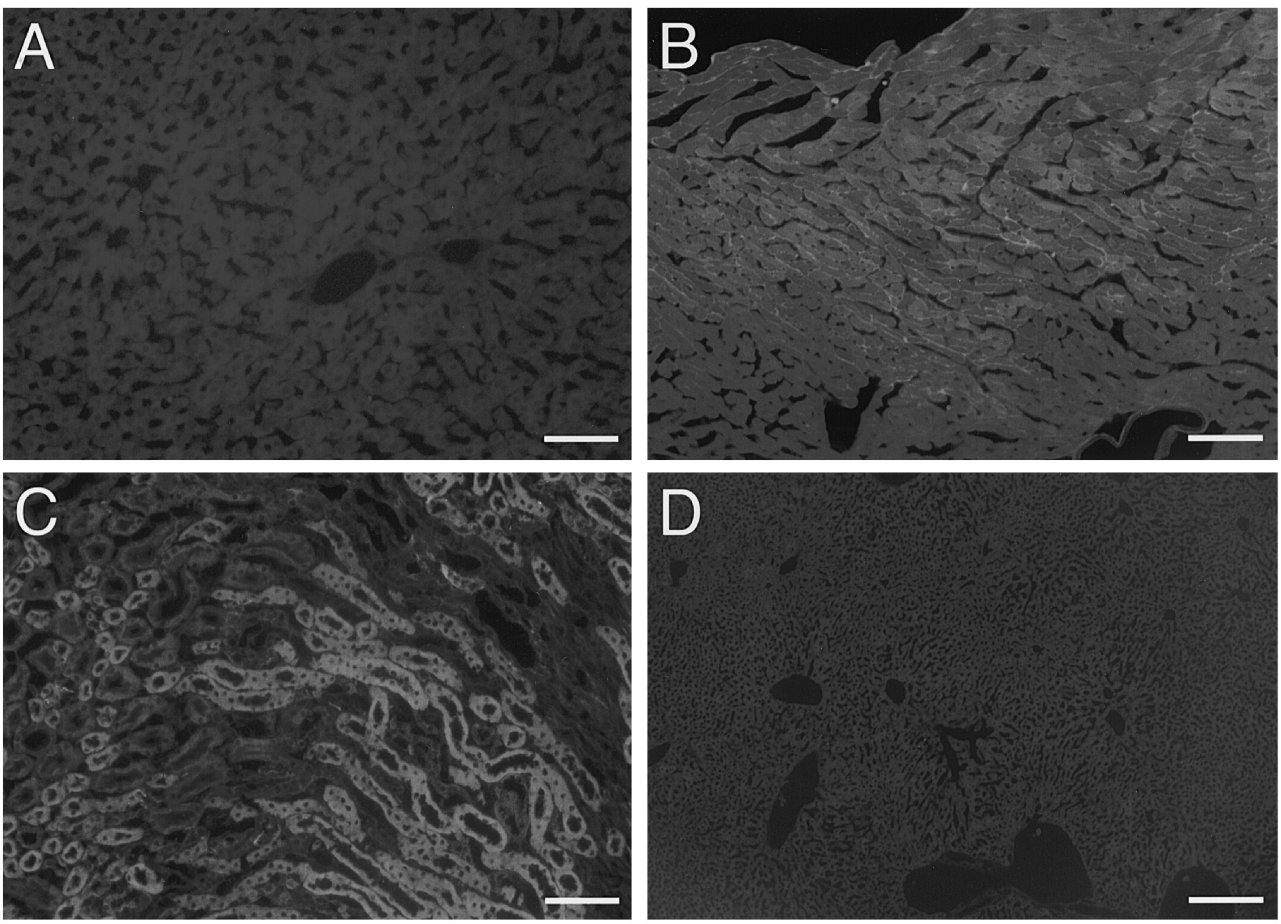

Fig. 3. The immunohistochemical localization of COX Vb in mouse liver, heart and kidney. (A) liver (scale bar = 100 μm), (B) heart (scale bar = 100 μm), (C) kidney (scale bar = 100 μm) and (D) liver, preimmune IgG control (scale bar = 50 μm). Sequential sections were incubated with anti-COX Vb monoclonal antibody and visualized by rhodamine isothiocyanate conjugated anti mouse IgG donkey antibody. Section in (D) was developed using pre-immune IgG.

walls. This intra-tissue heterogeneity in COX Vb level is consistent with the known heterogeneity in the level of oxidative metabolism in different compartments of kidney. Fig. 3D represents a control in which, a representative liver section was stained without added COX Vb antibody.

To investigate if the differential staining of the kidney regions represented the mitochondrial abundance in these cells, companion sections were probed with antibodies to mitochondrial encoded subunit I and a ubiquitous nuclear encoded subunit VIc. In contrast to a non-uniform discriminating pattern obtained with the Vb antibody (Fig. 4A), both subunit VIc (Fig. 4B) and subunit I (Fig. 4D) yielded more uniform staining patterns. Fig. 4C represents a higher magnification of the COX Vb antibody stained pattern shown in Fig. 4A. In Fig. 4E, a representative kidney section was stained without added primary antibody. These results suggest a possible intra-tissue heterogeneity in the abundance of COX Vb, possibly based on the oxidative, and/or metabolic load of the cell type.

3.4. COX subunit stoichiometry and activity in bovine cardiac compartments

To determine whether the level of mitochondrial and nuclear encoded mRNAs follow compartmental specific variations, equal amounts of total RNA from cardiac compartments were hybridized with subunit

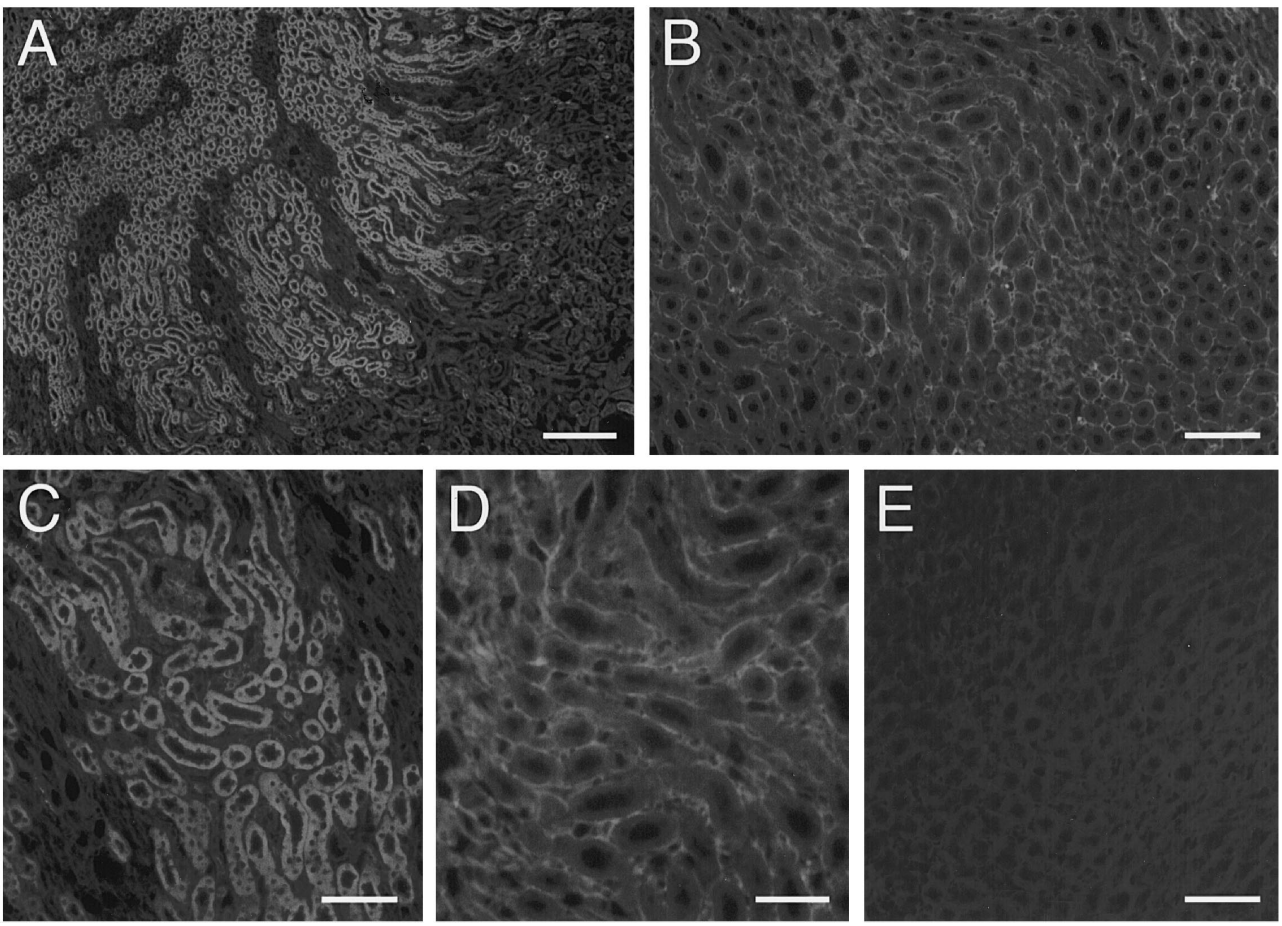

Fig. 4. The immunohistochemical localization of COX I, Vb and VIc in mouse kidney. (A) anti-COX Vb (scale bar = 250 μm), (B) anti-COX VIc (scale bar = 100 μm), (C) anti-COX Vb (scale bar = 100 μm), (D) anti-COX I (scale bar = 50 μm) and (E) preimmune IgG (scale bar = 100 μm). Sequential sections were incubated with anti-COX Vb monoclonal antibody and visualized by rhodamine isothiocyanate conjugated anti-mouse IgG donkey antibody.

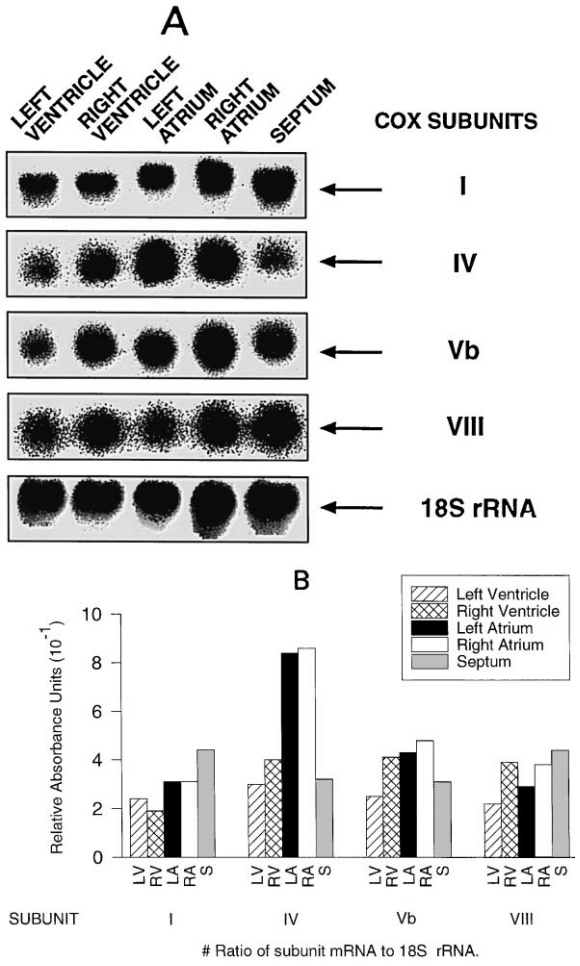

Fig. 5. The relative abundance of the COX mRNAs in different compartments of the bovine heart. (A) Northern blot analysis: total RNA (30 μg) from each cardiac compartment was separated on agarose–formaldehyde gel, transferred to nitrocellulose and hybridized with ^{32}P labelled subunit specific oligonucleotide probes. The blots were stripped and rehybridized with 18S rDNA probe to determine the loading level in each lane. (B) The autoradiograms in A were quantitated using an AGFA Arcus II scanner and the NIH image quantitation system. The values were normalized to the 18S rRNA level in each lane.

specific synthetic oligonucleotide from the conserved region of mRNAs. The relative abundance of mRNAs for different subunits in the bovine cardiac compartments is presented in Fig. 5A and B. Both the right and left atrial compartments showed higher levels of COX I, IV and Vb mRNAs. The right and left ventricles contained marginally varying levels of all the mRNAs studied. The left and right atrial compartments contained nearly 2–3-fold higher abundance of COX IV mRNA than the septal and ventricular compartments.

Fig. 6 shows immunoblot analysis using submitochondrial particles from different bovine cardiac compartments. The COX from right atrium contained 1.6–2.2-fold higher levels of COX Vb protein as

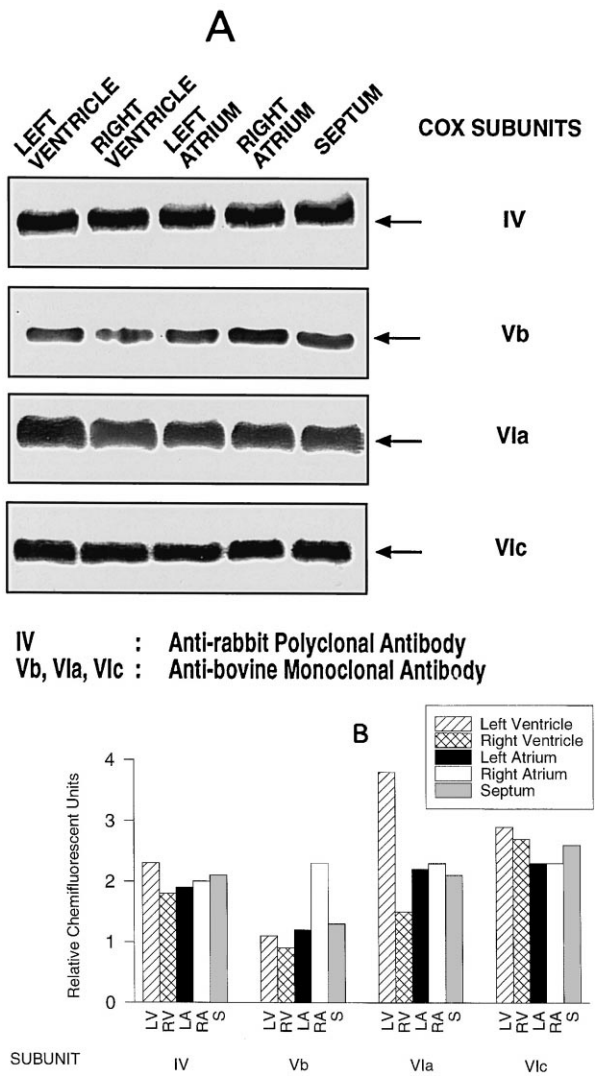

Fig. 6. The immunoblot analysis of mitochondrial proteins isolated from bovine cardiac compartments: In (A) the blots containing equal amount of COX (100 nmol of heme aa_3) from each cardiac compartment was probed with monoclonal anti-bovine antibodies (COX Vb, VIa and VIc) or polyclonal anti-rabbit antibody (COX IV). The immunoblot analysis was carried out as described in Fig. 1. (B) Histogram shows the relative abundance of COX subunits in mitochondria from different cardiac compartments.

Table 2
The kinetic parameters of bovine cardiac compartmental cytochrome *c* oxidase

Isoenzyme	Steady state					
	$I = 27$ mM				$I = 226$ mM	
	High affinity		Low affinity			
	K_m (μM)	TN (s^{-1})	K_m (μM)	TN (s^{-1})	K_m (μM)	TN (s^{-1})
Left ventricle	5	15	32	75	32	110
Right ventricle	4	20	48	80	28	105
Left atrium	2	20	11	85	8	105
Right atrium	1	10	26	60	19	95
Septum	2	15	27	75	22	110

The steady state reaction rates were determined by photometric assay in 1 ml medium containing either 10 mM PO_4^{2-} (low ionic) or 100 mM PO_4^{2-} (high ionic), 0.05% laurylmaltoside, 1 mM EDTA, 1–2 μg mitochondrial membranes and 0.16–80 μM ferrocytochrome *c*. The K_m and TN values were calculated as described in Table 1.

compared to the COX from other three compartments, i.e., the left atrium and both right and left ventricles. While subunit VIa content of COX was nearly similar in septum and atrial compartments, its level varied more than 2-fold between the left and right ventricles. The levels of COX IV and VIc subunits varied only marginally between the compartments.

Kinetic parameters for cardiac compartmental COX is presented in Table 2. There were minor differences in TN and K_m values between the right and left ventricular as well as septal compartments at both low and high ionic strength conditions. Right atrial enzyme exhibited lowest TN. The left atrial compartment showed lower K_m values and a TN value similar to that of left and right ventricles and septum. The K_m values between left and right atrium differed significantly with respect to high and low affinity phase binding sites for cytochrome *c*. The ventricular compartments differed significantly in their kinetic properties from the atrial compartments. COX from both the ventricles showed high K_m for cytochrome *c* (28–32 μM) as compared to the atrial COX which showed a lower K_m of 8–19 μM. These results provide insight on the inter-compartmental variations in the subunit composition and catalytic activity of COX in the cardiac tissue.

4. Discussion

Despite continued efforts in various laboratories, the functional roles of nuclear encoded ubiquitous and tissue specific isologs of COX in mammalian organisms remain unclear [1,31]. Subunit VIa has been extensively studied in this regard, and has been suggested to modulate enzyme activity possibly through nucleotide binding [8]. Studies in yeast have indicated that the nuclear coded smaller subunits influence the enzyme activity. The modulatory role of yeast COX subunit V isoforms, Va and Vb, in enzyme turnover as yeast switches from aerobic to anaerobic and vice versa is well documented [13]. Unlike the yeast, mammals depend entirely on oxygen for their survival. Under normal conditions when O_2 is not a limiting factor, the function of COX is solely determined by metabolic demands of the tissues. Based on the findings that some of the nuclear coded non-catalytic COX subunits are expressed as the liver (L) and muscle specific (H) isologs [32,33], it has been postulated that these subunits might have a modulatory role in enzyme function. In extension of these possibilities, our results suggest that ubiquitously expressed subunits IV and Vb may also have roles in the modulation of COX activity under certain physiological condition.

Though the nuclear coded IV and Vb subunits are constitutively expressed in all the tissues the results presented in this study show that the COX from tissues with higher energy demand (O_2 consumption) contain higher levels of IV and Vb proteins. Liver with a lower energy demand and thus lower O_2 consumption contained lower levels of COX IV and Vb protein subunits. These observations are consistent with the previous studies showing 5–15-fold higher levels of COX Vb and IV mRNAs in the heart as compared to the liver [15,34]. A comparison of results in Figs. 5 and 6 show some degree of disparity in the steady state mRNA levels and subunit contents in different compartments of the heart. For example, both left and right atria contain nearly 2-fold higher levels of COX IV mRNAs as compared to other compartments although COX from all four compartments contain nearly similar subunit contents. This difference may largely be due to post-transcriptional or translational regulation, reported for different COX subunits under different physiological and develop-

mental conditions [35–39]. Results in Fig. 6 also show that COX from right atrium and left ventricle contain nearly 2-fold higher levels of subunits Vb and VIa (nearly 2-fold stoichiometry), respectively, as compared to near normal stoichiometric levels in COX from other compartments. Although reasons for the higher Vb and VIa contents of the right atrial and left ventricular complexes, respectively, remains unknown, it may be related to the biogenesis or activities of the respective COX complexes or reflects a yet unknown multifunctional properties of these two subunits.

Initial studies with bovine cytochrome *c* oxidase when assayed at low ionic condition (< 25 mM) showed that the steady state reaction rates do not follow a simple Michaelis–Menten rate kinetics [40]. Since then, a number of studies have shown that at low ionic strength the reaction yields a concave Eadie–Hofstee plot which represents two first degree functions, suggesting two separate rate kinetics corresponding to a high affinity (low K_m) and low affinity (high K_m) reactions [30,41]. The high affinity reaction is believed to be due to cytochrome *c* binding to subunit II of COX, which is also described as the catalytic site [42]. The low affinity or non-catalytic site was shown to be due to cytochrome *c* binding to cardiolipin through electrostatic interaction [42,43]. The straight line Eadie–Hofstee plots obtained under high ionic conditions is thought to be analogous to high affinity reaction at low ionic strength [44]. It is suggested that high ionic conditions essentially reduce the low affinity binding of ferrocytochrome *c* by interfering with electrostatic interaction. Although reactions under high ionic condition is believed to be physiologically important, the differences in kinetic rates under low ionic conditions are important parameters for comparing COX from different tissues under different microenvironments, including different subunit compositions. We have therefore, carried out activity measurements under both of these conditions to gain insight on tissue specific differences in COX complex.

Kinetic characterization of the isoenzymes reveal sharp differences in enzyme activity between the tissues. We postulate that these kinetic parameters reflect observed differences in subunit stoichiometry of the COX enzyme. While the heart isoenzyme with a higher abundance of COX IV and Vb protein showed lower TN, the liver enzyme with a lower abundance of COX IV and Vb exhibited higher TN. Our results, therefore, suggest an inverse relationship between COX IV and Vb abundance and corresponding changes in the kinetic properties of the enzyme.

In initial studies using spectrophotometric assays under high ionic strength conditions, Kadenbach et al. [16] and Sinjorgo et al. [17] reported similar reaction kinetics for bovine COX purified from heart, skeletal muscle, liver and kidney with their known differences in the tissue specific isolog contents. These results therefore suggested that the nuclear encoded subunits might not influence the catalytic activity of the enzyme COX. In a series of subsequent studies, Kadenbach et al. [16] demonstrated that the bovine liver and heart COX reconstituted in phospholipid vesicles exhibited different reaction kinetics. These and subsequent studies suggested that the organ linked variation in nuclear coded subunits is an adaptation to differences in phospholipid composition of the mitochondrial membranes [44,45]. It was also shown that reconstituted bovine heart COX, but not the liver COX, responds to intra-liposomal ADP. This response was stimulated by monoclonal antibody to COX VIa, probably by inducing a conformational change in the enzyme complex [8]. These studies strongly support the possibility that the modulation of COX activity by lipids is mediated by nuclear coded subunit VIa. The stimulatory role of cardiolipin and free fatty acids on COX activity have also been documented [18,46]. Stimulation of COX activity in hyperthyroid rat was solely attributed to the increase in membrane cardiolipin content [47].

In support of the above listed studies, under near native conditions the liver isoenzyme exhibits low K_m and high TN, in tune with its low O_2 consumption rate. On the other hand, heart, with high oxidative capacity, shows low TN and a higher K_m, possibly a mechanism to prevent oxidative injury. The isoenzymes of brain and kidney whose O_2 consumption rates are lower than that of the heart, but higher than that of liver exhibit intermediate kinetic properties. The kidney isoenzyme presented a unique picture. While its TN at high ionic condition reflected it to be a heart like isoenzyme (high oxidative capacity), at low ionic state it resembled the COX from a tissue with low oxidative capacity, such as liver, possibly because of mixed population. This interpretation is

consistent with the known variations in the levels of oxidative metabolism between the cortical and medullary regions of the kidney.

Within the heart tissue, different compartments with varying workloads exhibited heterogeneity in subunit stoichiometry. While Vb was more abundant in right atrium, left ventricle contained a higher level of VIa. Though a direct link between subunit abundance and kinetic properties of the cardiac compartmental isoenzymes is not apparent, the ventricular compartments differed significantly from atrial compartments in their kinetic properties. Though the left ventricular output is known to be slightly greater than that of right ventricle, both the ventricular compartments exhibited similar kinetic properties for the COX enzyme. Atrial compartments with a lower workload (low O_2 consumption) differed significantly from ventricular compartments. Under high ionic conditions, all the heart compartments showed essentially similar TN. The higher abundance of Vb in right atrium is consistent with its lower TN. It is quite likely that the heart specific COX VIa or COX VIII may also be involved in fine tuning of the cardiac compartmental COX enzymes. This is consistent with our observation that COX VIa is differentially distributed within the cardiac compartments, with a higher abundance in the left ventricle.

5. Conclusion

Our results using enzyme complex with structural and functional integrity close to the native mitochondrial membrane associated COX define a link between oxygen dependency of the tissue, the abundance of nuclear coded COX IV and Vb subunits and the kinetic properties of the isoenzymes. Through the immunoblot and immunohistochemical analyses, we demonstrate for the first time, an inter and intra tissue specific differences in the COX subunit levels. Although the precise mechanisms of altered kinetic parameters remain unknown, our results open up the possibility of using tissue regions or cells containing high and low Vb subunit contents to address these questions. The higher expression of COX Vb/IV might be an adaptive mechanism employed by the highly oxidative tissues like heart, brain and proximal convoluted tubules of the kidney to prevent oxygen mediated injury.

Acknowledgements

We thank Mrs. Alka Agarwal and Dr. Ravi Pidikiti for their help in different aspects of this study. We also thank Dr. Leon Weiss for his advice on the immunohistochemistry. This research was supported in part by NIH grant GM-49683.

References

[1] R.A. Capaldi, Ann. Rev. Biochem. 59 (1990) 569–596.
[2] B. Kadenbach, J. Barth, R. Akgun, D. Linder, S. Possekel, Biochim. Biophys. Acta 1271 (1995) 103–109.
[3] R.A. Capaldi, Arch. Biochem. Biophys. 280 (1990) 252–262.
[4] R.O. Poyton, C.E. Trueblood, R.M. Wright, L.E. Farrell, Ann. NY Acad. Sci. 550 (1988) 289–307.
[5] R. Lightowlers, Z. Chrzanowska-Lightowlers, M. Marusich, R.A. Capaldi, J. Biol. Chem. 266 (1991) 7688–7693.
[6] R. Aggeler, R.A. Capaldi, J. Biol. Chem. 265 (1990) 16389–16393.
[7] R. Bisson, G. Schiavo, C. Montecucco, J. Biol. Chem. 262 (1987) 5993–5998.
[8] G. Anthony, A. Reimann, B. Kadenbach, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 1652–1656.
[9] A. Weishaupt, B. Kadenbach, Biochemistry 31 (1992) 11477–11481.
[10] J.W. Taanman, R.A. Capaldi, J. Biol. Chem. 268 (1993) 18754–18761.
[11] B. Kadenbach, A. Stroh, F.J. Huther, A. Reimann, D. Steverding, J. Bioenerg. Biomembr. 23 (1991) 321–334.
[12] C. Ostermeier, S. Iwata, H. Michel, Curr. Opin. Struct. Biol. 6 (1996) 460–466.
[13] R.O. Poyton, J.E. Mc Ewen, Ann. Rev. Biochem. 65 (1996) 563–607.
[14] L.A. Allen, X.-J. Zhao, W. Caughey, R.O. Poyton, J. Biol. Chem. 270 (1995) 110–118.
[15] A. Basu, N. Lenka, J. Mullick, N.G. Avadhani, J. Biol. Chem. 272 (1997) 5899–5908.
[16] B. Kadenbach, H. Stroh, M. Ungibauer, L. Kuhn-Nentwig, U. Buge, J. Jarausch, Methods Enzymol. 126 (1986) 32–45.
[17] K.M.C. Sinjorgo, I. Durak, H.L. Dekker, C.M. Edel, T.B.M. Hakvoort, B.F. van Gelder, A.O. Muijsers, Biochim. Biophys. Acta 893 (1987) 251–258.
[18] U. Buge, B. Kadenbach, Eur. J. Biochem. 161 (1986) 383–390.
[19] N.K. Bhat, B.G. Niranjan, N.G. Avadhani, Biochemistry 21 (1982) 2452–2460.
[20] N.R. Sims, J. Neurochem. 55 (1990) 698–707.
[21] G.F. Azzone, R. Colonna, B. Ziche, Methods Enzymol. LV (1979) 46–50.

[22] P.L. Pedersen, J.W. Greenawalt, B. Reynafarje, J. Hullihen, G.L. Decker, J.W. Soper, E. Bustamente, Methods Cell. Biol. 20 (1978) 411–481.
[23] L. Smith, in: D. Glick (Ed.), Methods in Biochemical Analysis, Vol. 2, 1955, p. 427.
[24] G. Von Jagow, M. Klingenberg, FEBS Lett. 24 (1972) 278–282.
[25] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.
[26] U.K. Laemmli, Nature 227 (1970) 680–685.
[27] H. Towbin, T. Staehelin, J. Gordon, Proc. Natl. Acad. Sci. U.S.A. 76 (1979) 4350–4354.
[28] P. Chomczynski, N. Sacchi, Anal. Biochem. 162 (1987) 156–159.
[29] P.K. Wellauer, I.B. David, J. Mol. Biol. 128 (1979) 289–303.
[30] K.M.C. Sinjorgo, J.H. Meijling, A.O. Muijsers, Biochim. Biophys. Acta 767 (1984) 48–56.
[31] B. Kadenbach, L. Kuhn-Nentwig, U. Buge, Curr. Top. Bioenerg. 15 (1987) 113–161.
[32] B. Kadenbach, A. Stroh, A. Becker, C. Eckerskorn, F. Lottspeich, Biochim. Biophys. Acta 1015 (1990) 368–372.
[33] J.-E. Tannman, R.E. Hall, C. Tang, M.F. Marusich, N.C. Kennaway, R.A. Capaldi, Biochim. Biophys. Acta 1225 (1993) 95–100.
[34] J. Virbasius, R.C. Scarpulla, Nucleic Acid Res. 18 (1990) 6581–6586.
[35] E. Lefai, A. Vincent, O. Boespflug-Tanguy, A. Tanguy, S. Alziari, Biochim. Biophys. Acta 1318 (1997) 191–201.
[36] K. Luciakova, R. Li, B.D. Nelson, Eur. J. Biochem. 207 (1992) 253–257.
[37] D.M. Medeiros, L. Shiry, T. Samelman, Comp. Biochem. Physiol. 117 (1997) 77–87.
[38] T. Preiss, R.N. Lightowlers, J. Biol. Chem. 268 (1993) 10659–10667.
[39] R. Sewards, B. Wiseman, H.T. Jacobs, Mol. Gen. Genet. 245 (1994) 760–768.
[40] B. Errede, M.D. Kamen, Biochemistry 17 (1978) 1015–1027.
[41] S. Ferguson-Miller, D.L. Brautigan, E. Margolish, J. Biol. Chem. 251 (1976) 1104–1115.
[42] R. Bisson, B. Jacobs, R.A. Capaldi, Biochemistry 19 (1980) 4173–4178.
[43] S.B. Vik, G. Georgevich, R.A. Capaldi, Proc. Natl. Acad. Sci. U.S.A. 78 (1981) 1456–1460.
[44] K.M.C. Sinjorgo, O.M. Steinebach, H.L. Dekker, A.O. Muijsers, Biochim. Biophys. Acta 850 (1986) 108–115.
[45] P. Merle, B. Kadenbach, Eur. J. Biochem. 125 (1982) 239–244.
[46] M. Sharpe, I. Perin, P. Nicholls, FEBS Lett. 391 (1996) 134–138.
[47] G. Paradies, F.M. Ruggiero, G. Petrosillo, E. Quagliariello, Biochim Biophys. Acta 1225 (1994) 165–170.