Differences in Kinetic Properties of Cytochrome Oxidase in Mitochondria from Rat Tissues. A Comparative Study

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Substrate kinetic properties of cytochrome oxidase in rat liver, kidney, brain and heart mitochondria were examined using ascorbate + *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) as the electron donor system. Analysis of the substrate kinetics data revealed tissue-specific expression of kinetic components exhibiting differences with respect to *K*m, *V*max and *K*cat/*K*m values. Regression analysis data suggest that the enzyme activity may be regulated in a tissue-specific manner.

Key words: Cytochrome Oxidase, Cytochrome Content, Mitochondrial Phospholipids

Introduction

The enzyme cytochrome oxidase is the terminal sink of electrons in the electron transport chain of all aerobic organisms (Namslauer and Brzezinski, 2004). In the higher organisms the cytochrome oxidase complex (complex IV) comprises of 13 polypeptides, two hemes (heme a and heme a_3), two copper atoms (Cu A and Cu B), one Zn and one Mg atoms. Additionally, the presence of one more Cu atom is also reported (Capaldi, 1990; Carr and Winge, 2003). Of the thirteen polypeptides three high molecular weight peptides namely COX I, COX II and COX III are mitochondrial gene products and represent the minimum catalytic subunits. The remaining polypeptides are nuclear gene products and are regulatory polypeptides (Poyton and McEwen, 1996). The enzyme exists as dimer deeply embedded in the inner membrane (Poyton and McEwen, 1996). The embedded enzyme is surrounded by core lipids: mainly phosphatidylcholine (PC), phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG) (Daum, 1985). The enzyme has an absolute requirement for DPG for its activity (Fry and Green, 1980, 1981; Mc Millin and Dowhan, 2002). Since cytochrome oxidase is the terminal electron sink, the rates of respiration in mitochondria depend on the cytochrome oxidase content.

Interesting to note in this context is the fact that the rates of respiration in mitochondria from different tissues vary to a considerable extent, which is also true for the contents of cytochrome aa_3 and DPG (Katyare *et al.*, 1977, 1994; Katyare and Satav, 2005; Satav and Katyare, 2004; Swegert *et al.*, 1999; Bangur *et al.*, 1995; Billimoria *et al.*, 2005; Kunz, 2003). However, the variations in the contents of the major phospholipids, *i.e.* PC and PE, in mitochondria from different tissues are of lesser magnitude (Katyare *et al.*, 1977, 1994; Katyare and Satav, 2005; Satav and Katyare, 2004; Swegert *et al.*, 1999; Bangur *et al.*, 1995).

In view of these observations, it becomes interesting and important to find out if the kinetic properties of cytochrome oxidase are regulated in a tissue-specific manner. It has been reported that *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) transfers the electrons from ascorbate to cytochrome oxidase at the cytochrome c site thus substituting the function of the natural substrate, reduced cytochrome c (Jacobs, 1960; Jacobs and Sanadi, 1960). Hence employing the ascorbate + TMPD as the electron donor system we determined the rate of electron flux-dependent changes in the cytochrome oxidase activity in mitochondria from rat liver, kidney, brain and heart. We also attempted to correlate the kinetic behavior with the lipid/phospholipid content of the mitochondria from these tissues. Our results suggest that the kinetic properties of cytochrome oxidase complex indeed differ in a tissue-specific manner.

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Materials and Methods

Chemicals

Ascorbic acid, 4-morpholinopropanesulfonic acid (MOPS), ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and bovine serum albumin fraction V were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *N*,*N*,*N*′,*N*′-Tetramethyl-*p*-phenylenediamine (TMPD) was from British Drug Houses (Poole, Dorset, UK). All other chemicals were of analytical reagent grade and were purchased locally.

Isolation of mitochondria

Adult male albino rats of Charles–Foster strain (200–225 g) were used. Isolation of liver, kidney, brain and heart mitochondria was according to the procedures described previously (Katyare *et al.*, 1977, 1994; Katyare and Satav, 2005; Satav and Katyare, 2004; Swegert *et al.*, 1999; Bangur *et al.*, 1995; Billimoria *et al.*, 2005).

Analytical methods

The contents of mitochondrial cytochromes were quantified as described previously (Katyare *et al.*, 1977, 1994; Katewa and Katyare, 2004). The extraction of mitochondrial lipids/phospholipids, estimation of cholesterol, and determination of phospholipid profile were by the methods described previously (Swegert *et al.*, 1999; Pandya *et al.*, 2004).

Assay of cytochrome oxidase activity

The measurement of cytochrome oxidase activity was carried out polarographically using a Clarke-type oxygen electrode. The assay medium (final volume 1.6 ml) consisted of: 50 mM potassium phosphate buffer containing 0.4 mM each of $CaCl_2$ and $AlCl_3$ (Katyare *et al.*, 1970), and saturating amount of sodium ascorbate (10 mM). The concentration of TMPD varied from 3 to 1000 μM. In separate experiments, the cytochrome oxidase activity was also determined at fixed (100 μM) concentration of TMPD. The cytochrome oxidase activity, *v*, is expressed as nmol O_2 min^{-1} mg $protein^{-1}$.

Substrate kinetics data were computer analyzed using Sigma Plot version 6.1 by three methods, *i.e.* Lineweaver-Burk, Eadie-Hofstee and Eisenthal and Cornish-Bowden, from which *K*m and *V*max values were determined (Dixon and Webb, 1979; Dave *et al.*, 1999). The values of *K*m and *V*max obtained by the three methods were in close agreement and were averaged for the final presentation of the data. The values of *K*cat/*K*m were computed based on the respective values of *K*m and *V*max (Mathews and van Holde, 1996).

Regression analysis was carried out using Jandel Sigma Stat statistical software version 2.0.

Protein estimation was according to the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Results

The typical Eadie-Hofstee plots depicting effect of increasing concentrations of TMPD on the rate of oxidation in liver, kidney, brain and heart mitochondria are shown in Fig. 1, from which it is clear that mitochondria from liver, kidney and heart showed the presence of a two kinetic components system for cytochrome oxidase activity. As against this the brain mitochondria were characterized by the presence of three kinetic components. We have previously shown that the kinetic components represent the potential and the response of the enzyme to increasing concentrations of the substrate (Parmar *et al.*, 1995; Kaushal *et al.*, 1999; Katewa and Katyare, 2003).

The values of *K*m and *V*max for component I varied widely amongst the mitochondria from the four tissues (Table I). The lowest value of *K*m (2.5 μM) was noted for heart mitochondria whereas this value was the highest for the kidney mitochondria (36 μM). The *V*max value was lowest for the liver mitochondria and the highest in the kidney mitochondria. For component II, the *K*m value was comparable (120 μM for liver, brain and heart mitochondria) whereas this value for kidney mitochondria was 3.5 times higher. The pattern of *V*max matched with that for component I. In case of brain mitochondria a third kinetic component with *K*m of about 400 μM was evident (Table I).

In view of this wide variability of *K*m and *V*max values in mitochondria from the four tissues as noted above, we tried to illustrate the relationship between the enzyme activity and *K*m values. These values, expressed as a ratio of *K*cat/*K*m, are given in Table II. As can be noted, the *K*cat/*K*m values for component I were the highest for the brain and the heart mitochondria and lowest for the kidney

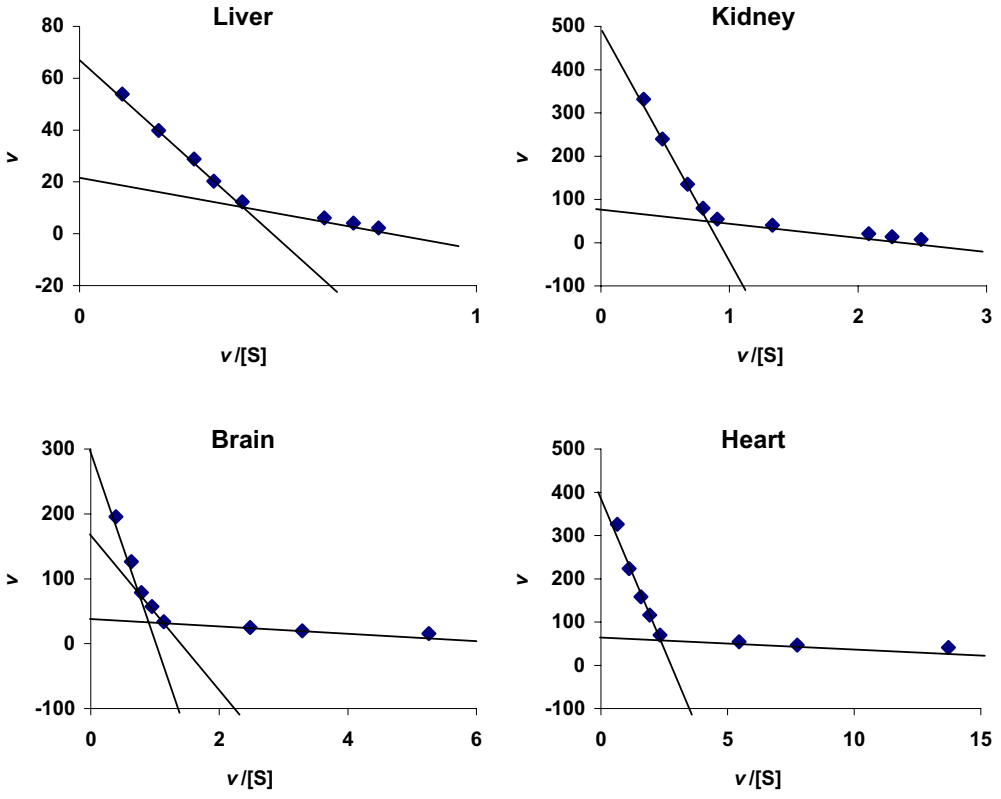

Fig. 1. Eadie-Hofstee plots for cytochrome oxidase from liver, kidney, brain and heart mitochondria. The enzyme activity *v*, is plotted versus *v*/[S] where *v* is the enzyme activity at the given TMPD concentration [S]. The plots are typical of 6 independent experiments. The variations in the individual data points from the mean value ranged from 4 to 6 SEM units.

mitochondria. For component II, the highest value is seen for the brain mitochondria whereas for the mitochondria from other three tissues the values of *K*cat/*K*m are almost comparable. As it is evident, the brain mitochondria were characterized by three values of *K*cat/*K*m. It may also be pointed out that for a mitochondrial preparation from a given tissue the *K*cat/*K*m values were the highest for component I and decreased in the subsequent component(s). The data in Table III show the con-

Table I. Substrate kinetic characteristics of cytochrome oxidase from rat liver, kidney, brain and heart mitochondria.

Tissue	Component I		Component II		Component III	
	*K*m	*V*max	*K*m	*V*max	*K*m	*V*max
Liver	17.3 ± 0.58	21.7 ± 1.38	120.2 ± 3.48	62.1 ± 0.75	–	–
Kidney	35.8 ± 2.69	82.4 ± 4.06	415.8 ± 11.47	467.8 ± 12.66	–	–
Brain	8.16 ± 0.83	38.9 ± 1.57	120.4 ± 9.10	165.4 ± 7.86	391.7 ± 20.10	369.1 ± 24.28
Heart	2.46 ± 0.12	73.2 ± 4.16	126.2 ± 7.26	349.6 ± 12.83	–	–

The *K*m (μM) and *V*max values were calculated based on the analysis by Lineweaver-Burk, Eadie-Hofstee and Eisenthal and Cornish-Bowden methods as described in the text. The values of *K*m and *V*max obtained by the three methods were averaged for the final presentation of the data. The results are given as mean ± SEM of 6 independent experiments. As indicated in the text, the kinetic components represent the potential and the response of the enzyme to increasing concentrations of the substrate.

Table II. *K*cat/*K*m values of cytochrome oxidase kinetic components from rat liver, kidney, brain and heart mitochondria.

Tissue	$(K\text{cat}/K\text{m}) \times 10^6$		
	Component I	Component II	Component III
Liver	0.29 ± 0.02	0.12 ± 0.01	–
Kidney	0.22 ± 0.01	0.11 ± 0.01	–
Brain	1.51 ± 0.16	0.43 ± 0.05	0.24 ± 0.02
Heart	1.62 ± 0.10	0.14 ± 0.01	–

The values of *K*cat/*K*m were computed as described in the text. The results are given as mean ± SEM of 6 independent observations.

tents of cytochromes in the mitochondria from the four tissues as well as the cytochrome oxidase activity determined at the fixed concentration of TMPD (100 μM) which is employed for all routine measurements (Katyare *et al.*, 1977). As can be noted, consistent with previously reported values, the content of cytochrome aa_3 was the highest in the heart mitochondria (Billimoria *et al.*, 2005). The data on total phospholipid (TPL), cholesterol (CHL) and the molar ratio of TPL and CHL are given in the Table IV while Table V summarizes the phospholipid profiles of the mitochondria from the four tissues. The wide variations in TPL, CHL and their molar ratios are self evident (Table IV). Especially noteworthy is the wide variation in the DPG content (Table V). Thus the DPG content of the brain mitochondria was the lowest and that in the heart mitochondria was the highest; the liver and the kidney mitochondria showed intermediate comparable values. As against the DPG content the variations in contents of PC and PE were of lesser magnitude. Interestingly, despite the lowest DPG content, the cytochrome oxidase activity in the brain mitochondria was almost comparable to that of the kidney mitochondria. Paradoxically, although the DPG contents of the liver and kidney mitochondria were almost comparable, the cytochrome oxidase activity in the liver mitochondria was almost 1/3 of that of the kidney mitochondria (Tables III and V).

In view of these wide variations in the *K*m and *V*max values, cytochrome contents and lipid/phospholipid profiles, and cytochrome oxidase activity determined at the fixed concentration of TMPD (Tables I–V, Fig. 1), it was of interest to see if correlation existed between the enzyme activity and the above cited factors. This was achieved by regression analysis. These data are given in Table VI. Thus, as can be noted the activity in the liver mitochondria correlated positively only with TPL content whereas in kidney mitochondria TPL and CHL but not their molar ratio were the major determinant. In case of brain mitochondria the activity correlated positively with DPG, PC, PE and molar ratio of PC and PE. For the heart mitochondria the positive correlation was obtained with CHL and molar ratio of TPL to CHL (Table VI).

Table IV. Total phospholipid (TPL), cholesterol (CHL) and molar phospholipid/cholesterol (TPL/CHL) ratio of mitochondria from rat liver, kidney, brain and heart.

Tissue	TPL [μg/mg protein]	CHL [μg/mg protein]	TPL/CHL [mol:mol]
Liver	175.3 ± 6.56	46.0 ± 1.04	1.91 ± 0.07
Kidney	284.3 ± 8.99	108.3 ± 3.38	1.32 ± 0.04
Brain	490.0 ± 18.87	482.5 ± 14.96	0.51 ± 0.02
Heart	283.5 ± 6.81	116.0 ± 6.95	1.25 ± 0.05

The results are given as mean ± SEM of 6 independent observations.

Table III. Cytochromes contents and cytochrome oxidase activity in mitochondria from rat liver, kidney, brain and heart.

Tissue	Cytochromes content [pmol mg protein^{-1}]			Cytochrome oxidase activity, *v* [nmol O_2 min^{-1} mg protein^{-1}]
	aa_3	b	$c+c_1$	
Liver	141.3 ± 2.89	226.3 ± 14.81	276.0 ± 11.4	28.1 ± 1.17
Kidney	347.9 ± 13.33	344.8 ± 15.20	596.9 ± 15.21	86.9 ± 3.91
Brain	130.0 ± 11.14	147.7 ± 8.97	225.7 ± 8.30	75.6 ± 1.70
Heart	632.4 ± 27.40	635.5 ± 41.52	707.8 ± 39.91	155.7 ± 9.60

The results are given as mean ± SEM of 6 independent observations. Cytochrome oxidase activity was determined at a fixed concentration (100 μM) of TMPD.

Table V. Phospholipids composition of mitochondria from rat liver, kidney, brain and heart.

Phospholipid class	Liver	Kidney	Brain	Heart
	(% of total)			
Lyso	1.52 ± 0.05	2.34 ± 0.18	3.54 ± 0.24	0.82 ± 0.05
SPM	2.78 ± 0.11	8.01 ± 0.22	7.66 ± 0.15	4.84 ± 0.17
PC	45.20 ± 0.46	36.41 ± 0.99	37.70 ± 0.63	37.41 ± 0.43
PI	1.52 ± 0.08	1.09 ± 0.18	3.68 ± 0.13	2.75 ± 0.13
PS	1.58 ± 0.24	0.96 ± 0.08	4.42 ± 0.16	1.62 ± 0.09
PE	36.20 ± 0.56	36.35 ± 0.51	39.31 ± 0.70	35.50 ± 0.40
DPG	11.22 ± 0.18	14.83 ± 0.34	3.70 ± 0.07	17.06 ± 0.31

The results are given as mean ± SEM of 6 independent observations.

Table VI. Correlation between activity and membrane lipid/phospholipid components.

	Liver	Kidney	Brain	Heart
TPL	0.61	0.72	–	–
CHL	–	0.76	–	0.62
TPL/CHL	0.52	–	–	0.72
DPG	–	–	0.67	–
PC	–	0.51	0.91	–
PE	–	–	0.65	–
PC/PE	–	–	0.89	–

Regression coefficients were obtained by computer analysis using Jandel Sigma Stat Statistical software version 2.0 and are based on 6 independent experiments.

Discussion

The present studies were undertaken to examine if the cytochrome oxidase activity of mitochondria is regulated in a tissue-specific manner, and if so which are the regulatory factors. For evaluating these aspects we used ascorbate + TMPD as the electron donor system. There are two obvious advantages in employing ascorbate + TMPD as the electron donor system. First, it has been reported that the reduced TMPD directly donates electron at the cytochrome c site (Jacobs, 1960; Jacobs and Sanadi, 1960). Secondly, when ascorbate is used in saturating concentration, the system follows a normal substrate saturation kinetic pattern with respect to TMPD concentration. In the spectrophotometric assay using reduced cytochrome c, the reaction follows the first order kinetics thereby imposing complications in the interpretation of the data. Besides, the enzyme is constrained to interact with externally added reduced cytochrome c which far exceeds the intrinsic cytochrome c content (Smith, 1955).

The data in Fig. 1 and Tables I and II suggest that indeed tissue-specific regulatory mechanisms are operative as evinced in terms of characteristic substrate saturation kinetics and the differences with respect to kinetic components which were tissue-specific.

Also, we could not see any obvious correlationship between the DPG content and the activity determined at a fixed (100 μM) concentration of TMPD. The regression analysis data presented in Table VI are also in conformity of the point that the factors which influence the enzyme activity are tissue-specific.

PC and PE are the major phospholipids in the mitochondria and they form/constitute the peripheral core of the cytochrome oxidase (Daum, 1985). Therefore the dependence of the enzyme activity on these factors only in brain but not in other tissues was somewhat surprising. Also, what did seem surprising was that only in the brain mitochondria, where the DPG content is the lowest, DPG plays a regulatory role (Table V). It may be mentioned here that besides cytochrome oxidase, the electron transfer activity of mitochondrial complex I and complex III and the activity of FoF_1 ATPase are also dependent on DPG (Fry and Green, 1981; Daum 1985). In the light of this, it may be suggested that in our studies DPG emerged as a regulatory factor in the brain mitochondria only because its content could have been rate limiting. In other words, mitochondria from the other three tissues contained more than saturating amounts of DPG to fulfill requirement of all the enzyme systems and hence DPG was not rate limiting. Interesting to note here is the fact that although DPG is considered to be absolutely essential for the activity of cytochrome oxidase (Fry and Green, 1980), the dogfish cytochrome oxidase is a unique example where DPG is not present (Al-Tai *et al.*, 1984). In a comparative study of cytochrome oxidase from beef, dogfish and cod heart, it has been suggested that the enzyme system may not have any special lipid requirement other than membrane fluidity (Al-Tai *et al.*, 1984). DPG is known to be synthesized by

the mitochondria themselves and the synthesis of DPG is regulated by thyroid hormones (Hostetler, 1991). It may hence be suggested that possibly the brain mitochondria have only a limited capacity to synthesize DPG. Besides, it is traditionally believed that the mitochondria in adult brain are insensitive to thyroid hormone action (Katyare *et al.*, 1994).

In more recent years it has been shown that the cytochrome oxidase activity *in situ* is regulated by NO and ATP (Kadenbach, 2003; Brunori *et al.*, 2005). NO inhibits the enzyme by a dual mechanism whereas high concentration of ATP shows allosteric inhibitory action (Kadenbach, 2003; Brunori *et al.*, 2005). In the studies described in the present communication obviously NO and ATP are not involved. Nevertheless, it may be suggested that *in situ* the enzyme in mitochondria from different tissues may have differential accessibility to NO and ATP. In other words despite the similarity in the monomeric structure of the enzyme, subtle differences may exist at the level of the arrangement of regulatory subunits thereby restricting the accessibility of natural inhibitory ligands, *e.g.* NO and ATP. This possibility is worth to be investigated further.

In conclusion, results of our present studies have demonstrated that cytochrome oxidase activity is regulated in a tissue-specific manner for which a fine tuning mechanism may exist. Tissue-specific regulation of cytochrome c oxidase subunit expression by thyroid hormones is reported (Sheehan *et al.*, 2004).

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