Chapter 13

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

Introduction

The enzyme cytochrome oxidase is the terminal sink of electrons in the electron transport chain of all aerobic organisms (1). 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, presence of one more Cu is also reported (2, 3). Of the thirteen polypeptides three high molecular weight peptides namely Viz. 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 (4).The enzyme exists as dimer deeply embedded in the inner membrane (4). The embedded enzyme is surrounded by core lipids: mainly phosphatidylcholine (PC), phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG) (5). The enzyme has an absolute requirement for DPG for its activity (6, 7). Since cytochrome oxidase is the terminal electron sink, the rates of respiration in mitochondria depend on 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 (8-15). However, the variations in the contents of the major phospholipids i.e. PC and PE, in mitochondria from different tissues are of lesser magnitude (8, 11-14).

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 cytochrome c site thus

substituting the function of the natural substrate, reduced cytochrome c (17, 18). 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. Attempts were also made to correlate the kinetic behavior with the lipid/phospholipid content of the mitochondria from these tissues. Results of the present study suggest that the kinetic properties of cytochrome oxidase complex indeed differ in a tissue-specific manner.

Materials and Methods

Details of chemicals used is given in chapter 7.

Adult male albino rats of Charles–Foster strain (200 - 225 g) were used. Isolation of liver, kidney and brain mitochondria according to the procedure given in chapter 2 and that of heart mitochondria according to method given in chapter 12.

Analytical methods

Contents of mitochondrial cytochromes were quantified as described previously (11, 16, 19). The extraction of mitochondrial lipids/phospholipids, estimation of cholesterol, and determination of phospholipid profile was by the methods described previously in Chapter 2 of the Thesis.

Assay of cytochrome oxidase activity is detailed in chapter 7.

For the substrate kinetics studies concentration of TMPD was varied in the range from 3 μM to 1000 μM.

Data analysis is as detailed in chapter 2 of the Thesis.

Results

The typical Eadie-Hofstee plots depicting effect of increasing concentrations of TMPD on the rate of oxidation in liver, kidney, brain and heart are shown in Figure 1 from which it is clear that mitochondria from liver, kidneys and heart mitochondria showed presence of a two kinetic components system for cytochrome oxidase. 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 (20-22)

The values of Km and Vmax for component I varied widely amongst the mitochondria from the four tissues (Table 1). The lowest value of Km (2.5 μM) was noted for heart mitochondria whereas this value was the highest for the kidney mitochondria (36 μM). The Vmax value was lowest for the liver mitochondria and the highest in the kidney mitochondria. For component II, the Km 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 Vmax matched with that for component I. In case of brain mitochondria a third kinetic component with Km of about 400 μM was evident (Table 1).

In view of this wide variability of Km and Vmax values in mitochondria from the four tissues as noted above, attempt was made to illustrate the relationship between the enzyme activity and Km values. These values expressed as a ratio of Kcat/Km, are

Table 1. Substrate kinetics characteristics of cytochrome oxidase from rat liver, kidney, brain and heart mitochondria

Tissue	Component I		Component II		Component III	
	Km	Vmax	Km	Vmax	Km	Vmax
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 Km (μM) and Vmax values were calculated based on the analysis by Lineweaver-Burk, Eadie-Hofstee and Eisenthal and Cornish-Bowdenmethods as described in the text. The values of Km and Vmax obtained by the three methods and 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

given in Table 2. As can be noted, the Kcat/Km values for component I were the highest for the brain and the heart mitochondria and lowest for the kidney mitochondria. For component II, the highest value is seen for the brain mitochondria whereas for the mitochondria from other three tissues, values of Kcat/Km are almost comparable. As it evident, the brain mitochondria were characterized by three values of Kcat/Km. It may also be pointed out that for a mitochondrial preparation from a given tissue the Kcat/Km values were the highest for component I and decreased in the subsequent component(s). The data in Table 3 show the contents 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 (16). As can be noted, consistent with previously reported values the content of cytochrome aa_3 was the highest in the heart mitochondria (9). The data on total phospholipid (TPL), cholesterol (CHL) and the molar ratio of TPL and CHL are given in the Table 4 while Table 5 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 4). Especially noteworthy is the wide variation in the DPG content (Table 5). 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 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 3 and 5).

Table 2. Kcat/ Km values of cytochrome oxidase kinetic components from rat liver, kidney, brain and heart mitochondria

Tissue	(Kcat/ Km) x 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 Kcat/ Km were computed as described in the text. The results are given as mean ± SEM of 6 independent observations.

Table 3. Cytochromes contents and cytochrome oxidase activity in mitochondria from rat liver, kidney, brain and heart

Tissue	Cytochromes content (pmol/mg protein)			Cytochrome oxidase activity, v
	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 the fixed concentration (100 μM) of TMPD. The cytochrome oxidase activity v, is expressed as nmol O_2 min^{-1} mg $protein^{-1}$.

Table 4. Total phospholipid, cholesterol and total phospholipid / cholesterol mol:mol 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.
TPL, total phospholipid; CHL, cholesterol.

Table 5. 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.

Lyso: Lysophospholipid; SPM: sphinghomyelin; PC: phosphatidylcholine; PI: phosphatidylinositol; PS: phosphatidylserine; PE: phosphatidylethanolamine; DPG: diphosphatidylglycerol.

Figure 1

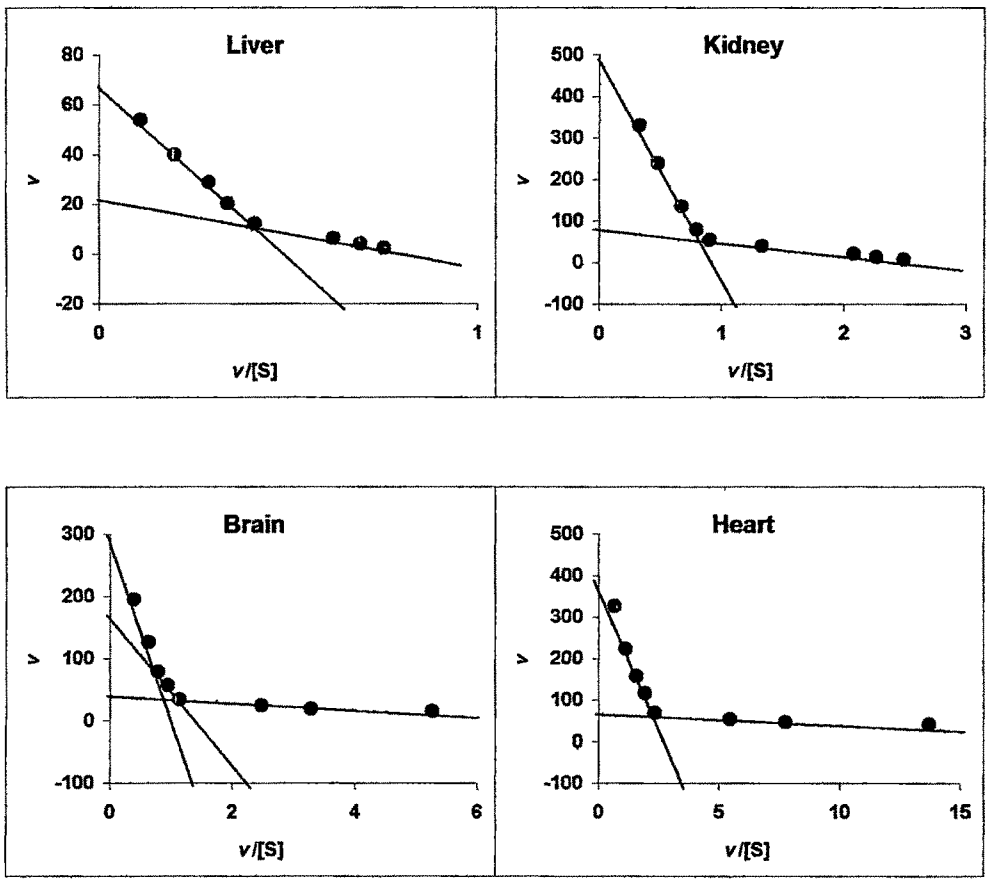
Liver
80
60
40
20
0
-20
v
0
1
v/[S]
Kidney
500
400
300
200
100
0
-100
v
0
1
2
3
v/[S]
Brain
300
200
100
0
-100
v
0
2
4
6
v/[S]
Heart
500
400
300
200
100
0
-100
v
0
5
10
15
v/[S]

Figure1. Eadie-Hofstee plots for cytochrome oxidase from liver, kidney, brain and heart mitochondria. The enzyme activity v on abscissa is plotted versus v /[S] on ordinate where v is the enzyme activity at the given TMPD concentration [S]. The plots are typical of 6 independent experiments in each group.

In view of these wide variations in the Km and Vmax values, cytochrome contents and lipid/phospholipid profiles, and cytochrome oxidase activity determined at the fixed concentration of TMPD (Tables 1-5, Fig. 1), it was of interest to see correlation existed between the enzyme activity and the above cited factors. This was achieved by regression analysis. These data are given in Table 6. 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 6).

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 cytochrome c site (17, 18). Secondly, when ascorbate is used in saturating concentration, the system follows normal substrate saturation kinetic pattern with respect to TMPD concentration. In 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 of reduced cytochrome c which far exceeds the intrinsic cytochrome c content (23).

Table 6. Correlation between activity and membrane lipid/phospholipid components

	Mitochondria			
	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.

The data in Fig. 1 and Tables 1 and 2 suggested 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 correlation ship between the DPG content and the activity determined at a fixed (100 μM) concentration of TMPD. The regression analysis data presented in Table 4 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 (5). Therefore the dependence of the enzyme activity on these factors only in brain but not other tissues was somewhat surprising. Also, what did seem surprising was that only in the brain mitochondria where DPG content is the lowest, DPG plays a regulatory role (Table 5). 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 (5, 6). 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 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 (6), the dogfish cytochrome

oxidase is a unique example where DPG is not present (24). 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 (24). DPG is known to be synthesized by the mitochondria themselves and the synthesis of DPG is regulated by thyroid hormones (25). 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 (11).

In more recent years it has been shown that the cytochrome oxidase activity in situ is regulated by NO and ATP (26, 27). NO inhibits the enzyme by dual mechanism whereas high concentration of ATP allosteric inhibitory action (26, 27). 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 investigating 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 (28).

Summary

Cytochrome oxidase activity was highest in heart and lowest in liver mitochondria. The activity was comparable in brain and kidney.

Content of cytochromes was highest in heart and lowest in brain mitochondria.

The typical Eadie-Hofstee plots for cytochrome oxidase from liver, kidneys and heart mitochondria showed presence of two kinetic components system. As against this the brain mitochondria were characterized by the presence of three kinetic components.

The values of Km and Vmax for component I varied widely amongst the mitochondria from the four tissues. In case of brain mitochondria a third kinetic component with Km of about 400 μM was evident

TPL and CHL content was lowest in liver whereas highest in the heart mitochondria.

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